In crystallo thermodynamic analysis of conformational change of the topaquinone cofactor in bacterial copper amine oxidase

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In the catalytic reaction of copper amine oxidase, the protein-derived redox cofactor topaquinone (TPQ) is reduced by an amine substrate to an aminoresorcinol form (TPQ$_{amr}$), which is in equilibrium with a semiquinone radical form (TPQ$_{sq}$). The transition from TPQ$_{amr}$ to TPQ$_{sq}$ is an endothermic process, accompanied by a significant conformational change of the cofactor. We employed the humid air and glue-coating (HAG) method to capture the equilibrium mixture of TPQ$_{amr}$ and TPQ$_{sq}$ in noncryocooled crystals of the enzyme from Arthrobacter globiformis and found that the equilibrium shifts more toward TPQ$_{sq}$ in crystals than in solution. Thermodynamic analyses of the temperature- and pH-dependent equilibrium also revealed that the transition to TPQ$_{sq}$ is entropy-driven both in crystals and in solution, giving the thermodynamic parameters that led to experimental determination of the crystal packing effect. Furthermore, we demonstrate that the binding of product aldehyde to the hydrophobic pocket in the active site produces various equilibrium states in two forms of the product Schiff-base, TPQ$_{amr}$ and TPQ$_{sq}$, in a pH-dependent manner. The temperature-controlled HAG method provides a technique for thermodynamic analysis of conformational changes occurring in protein crystals that are hard to scrutinize by conventional cryogenic X-ray crystallography.

Copper amine oxidases (EC 1.4.3.6, CuAOs) catalyze the oxidative deamination of various primary amines to produce the corresponding aldehydes, hydrogen peroxide, and ammonia through a ping-pong mechanism comprising two half-reactions (Fig. 1A) (1, 2). All CuAOs so far characterized structurally are the homodimers of a 70–95-kDa subunit, each containing a Cu$^{2+}$ ion and a protein-derived quinone cofactor, topaquinone (TPQ) (3). We have determined X-ray crystal structures of the enzyme from Arthrobacter globiformis (AGAO) that had been reduced with substrate under anaerobic conditions (4–8) and demonstrated that the reduced cofactor formed in the initial reductive half-reaction has two distinct conformations: a semiquinone radical form (TPQ$_{sq}$), in which the 4-hydroxyl group of the cofactor is ligated axially to the copper center, and an aminoresorcinol form (TPQ$_{amr}$), in which the cofactor has no direct contact with the copper (designated “on-copper” and “off-copper” conformations, respectively; Fig. 1B) (8). Previous spectrophotometric studies revealed that the equilibrium between TPQ$_{sq}$ and TPQ$_{amr}$ is affected significantly by temperature and pH (8, 9); at higher pH values and temperatures, the equilibrium shifts toward TPQ$_{amr}$, which suggests that the conformational change of TPQ is accompanied by a change in enthalpy. However, the structural basis for the pH- and temperature-dependent equilibrium remains unresolved.

In this study, we applied the humid air and glue-coating (HAG) method to thermodynamically analyze the conformational change of TPQ in noncryocooled crystals of AGAO. The HAG method was originally developed as a protein crystal-mounting method applicable to noncryogenic X-ray crystallography, in which a protein crystal is coated by water-soluble polymer and held in an air stream with controlled humidity (10). Recently, the HAG method was further improved by using N$_2$ gas (enabling the maintenance of anaerobic conditions) with a system for rigorously controlling the temperature and humidity around the mounted crystal and applied to the time-resolved structural study of bovine heart cytochrome c oxidase at 4°C (11); the temperature control of the crystal is expected to be more accurate and direct than for the crystal enclosed in the capillary generally used in noncryogenic crystallography. By using this method throughout the procedure from the anaerobic soaking of crystals with substrate to X-ray diffraction measurement, we could visualize the equilibrium mixture of TPQ$_{amr}$ and TPQ$_{sq}$ in AGAO crystals under noncryogenic conditions. Importantly, rigorous temperature control of the whole process led to the development of in crystallo thermodynamics of conformational changes that are

Significance

This study elucidated conformational changes of the redox quinone cofactor in bacterial copper amine oxidase during the catalytic reaction. The reaction intermediates were kept in noncryocooled crystals by coating with water-soluble polymer and placing in a humid N$_2$ gas stream. By changing the temperature, we could collect X-ray diffraction data of the crystals that led to the structural determination of an equilibrium mixture of different states of the cofactor. Such thermodynamic analyses of conformational changes occurring in protein crystals have been very difficult, if even possible, for cryogenic X-ray crystallography. Thermodynamic parameters obtained by the in crystallo thermodynamic analysis are more directly linked to structural changes than those obtained by ordinary methods in solution.


The authors declare no conflict of interest.

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Data deposition: Atomic coordinates and structure factors for 35 structures have been deposited in the Protein Data Bank (accession nos. 5ZP5–5ZP9, 5ZPA–5ZPF, 5ZOU, 5ZOW–5ZPI, 5ZPT–5ZP4, and 5ZPJ–5ZPP).

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of AGAO crystals were included in the calculation of the averaged occupancies of TPO intermediates (TPOamr and TPOsq). As summarized in SI Appendix, Table S2, the observed variance of occupancies of the assigned intermediates between the two monomers, and also among the ethylamine-reduced crystals obtained under the same conditions, is insignificant. Moreover, similar levels of B-factors for TPOamr and TPOsq, which are sufficiently low and comparable to those of the averaged protein B-factors shown in SI Appendix, Table S1, support the conclusion that the models are correctly accommodated in the electron density with reliable occupancies. It is also noteworthy that the similarity of B factors is consistent with their similar levels of the degree of disorder/mobility (thermal fluctuation). In marked contrast to the cryogenic crystallography that showed electron densities corresponding to a single conformer of TPO (see, e.g., figs. 7 and 8 in ref. 8), those determined at four different non-cryogenic temperatures clearly showed a mixture of the two conformers (off-copper TPOamr and on-copper TPOsq) in the Fo − Fc omit maps for residue 382 (TPO; Fig. 2A). Relative occupancies of each conformer (SI Appendix, Table S2) clearly indicate that the off-copper TPOamr shifts to the on-copper TPOsq as the temperature increases (Fig. 2B). The occupancies of on-copper TPOsq were also consistent with the single-crystal ultraviolet-visible absorption spectra observed by the temperature-controlled HAG method, which showed that the TPOsq-specific absorption bands around 350 (shoulder), 440, and 468 nm decreased in intensity as the temperature dropped from 20 °C to 5 °C (Fig. 2C).

For comparison, the temperature dependence of the TPOsq/TPOamr equilibrium in the ethylamine-reduced AGAO was also studied in solution at pH 6.0. Again, absorption bands of TPOsq gradually decreased at decreasing temperatures (SI Appendix, Fig. S1A), as in crystals. The spectral change was found to be fully reversible at least in solution; reversibility of the spectral change in the crystal remains unsettled because of crystal cracking when the temperature of the blowing nitrogen gas was slowly returned from 5 °C to 20 °C after the measurement at successively decreasing temperatures in the experiment shown in Fig. 2C. The TPOsq content was calculated from the absorbance at 468 nm, using the extinction coefficient of TPOsq (~5,000 M⁻¹ cm⁻¹ at 450 nm) (12). Thermodynamic parameters (Table 1) obtained from van't Hoff plots (Fig. 2D) of Keq values for both crystalline and solution states indicated that the significant consumption of heat (ΔHβ > 0) in the TPOsq–TPOamr transition is mostly or excessively compensated for by a large gain of entropy (ΔS° > 0), resulting in a minuscule change of the free energy (ΔG°). Thus, we conclude that the TPOsq–TPOamr equilibrium is an energetically well-balanced facile process occurring both in crystals and in solution. However, we noted that the magnitude of Keq (the ratio of TPOsq/TPOamr) was considerably larger in crystals than in solution at all temperatures examined (e.g., 3.6 ± 0.98 vs. 0.44 ± 0.011, respectively, at 20 °C; Fig. 2D).

On the basis of the modeled structure, the TPQ ring in the off-copper conformation of TPOamr is restricted in its motion, being sandwiched between the side chains of Asn381 and Tyr384/Val282 in a narrow wedge-shaped space (4) (Fig. 1B and SI Appendix, Fig. S2A), and tethered by a short (strong) hydrogen bond (~2.2 Å) with the side-chain hydroxyl group of Tyr284 (SI Appendix, Fig. S2B). Meanwhile, the TPQ ring of the on-copper TPOsq has sufficient freedom to rotate around the C8–Cy bond (SI Appendix, Fig. S2A), even though it is tethered by a weak hydrogen bond (~3.0 Å) to the S6 atom of Met602 (SI Appendix, Fig. S2B) (8). Thus, with regard to the rotatory movement of the TPQ ring, TPOsq has an increased mobility compared with TPOamr. Moreover, the side chains of Asn381 and Tyr384/Val282 would gain an increased mobility in the absence of the sandwiched TPOamr. Altogether, the TPOsq state should have higher entropy than the TPOamr state. These structural differences

Fig. 1. Presumed catalytic mechanism and active-site structure of AGAO. (A) Presumed catalytic mechanism. TPQox, oxidative form of TPQ; TPQsub, substrate Schiff-base of TPQ; TPQprod, product Schiff-base of TPQ; TPQamr, aminoresorcinol form of TPQ; TPQsq, semiquinone radical form of TPQ; TPQimq, iminoquinone form of TPQ. (B) Stick model of the active site. Off-copper conformer (TPQamr) and on-copper conformer (TPQsq) are drawn in magenta and green, respectively.
strongly suggest that the heat consumed during the transition to on-copper TPQsq is mainly used for breaking the strong hydrogen bond to the 4-OH group of Tyr284 in TPQamr, whereas the significant gain of entropy is a consequence of the increase in the mobility of the TPQ ring and other residues restricting the movement of TPQamr. Hence, the TPQamr–TPQsq conformational change is entropy-driven. Furthermore, the magnitudes of \( \Delta H^\circ \) and \( \Delta S^\circ \) are both \( \sim 1.5 \)-fold larger in crystals than in solution (Table 1). It is likely that the crystal packing leads to more energetically favorable noncovalent interactions in the crystal than in solution; this is more significant in TPQamr than in TPQsq. The ligation of TPQ to copper anchors it in place in both crystal and solution. In contrast, TPQamr is more flexible and is more easily accommodated by the surrounding residues, leading to optimization of interactions and decrease in enthalpy and entropy of TPQamr compared to TPQsq. The packing exerts its effect on these interactions through the protein side chains and, therefore, has a more significant effect on the thermodynamic terms of TPQamr. Here, the packing leads to increased optimization of interactions and further decreases the enthalpy and entropy of TPQamr. Collectively, the crystal packing effect was experimentally and accurately evaluated from the differences in thermodynamic parameters; \( \Delta H^\circ (\text{crystal}) - \Delta H^\circ (\text{solution}) = 12 \text{ kJ/mol and } \Delta S^\circ (\text{crystal}) - \Delta S^\circ (\text{solution}) = 56 \text{ (J/mol)/K.} \)

**pH Dependence of TPQsq/TPQamr Equilibrium in Crystal.** We next investigated the effect of pH on the TPQsq/TPQamr equilibrium in the ethylamine-reduced AGAO crystals by the HAG method conducted at a constant temperature (15 °C) (SI Appendix, Table S3). Surprisingly, the occupancies of TPQsq and TPQamr were almost constant in the crystals determined at pH 6–10, showing that the equilibrium is pH-independent with nearly constant \( K_{eq} \) values of \( \sim 3.0 \) (Fig. 3A and SI Appendix, Table S4). The magnitude of the TPQsq-derived absorption peak at 468 nm also did not significantly change in solution at pH 6–10 (SI Appendix, Fig. S3), giving an approximate \( K_{eq} \) value of 0.25 at 25 °C. These results demonstrate that the TPQsq/TPQamr equilibrium is intrinsically independent of pH both in crystals and in solution. This conclusion markedly contrasts with the previous observations that the TPQsq/TPQamr equilibrium formed in the 2-PEA-reduced enzyme is dependent on pH (in solution), involving two ionizable groups with \( pK_a \) values of 5.96 and 7.74 (SI Appendix, Fig. S3) (8). Thus, the pH-dependent equilibrium shift appeared to be a substrate-specific phenomenon. To identify the structural basis for the distinct pH dependences, noncryogenic structures were also determined for the 2-PEA-reduced crystals at different pH values (SI Appendix, Table S5). Actually, the \( F_o - F_c \) omit maps for the TPQ moiety were considerably different, depending on the measured pH (Fig. 3B and SI Appendix, Table S6). In addition to the off-copper TPQamr observed at pH 8.0 and on-copper TPQsq observed at pH 8.0–10.0, two forms of a product Schiff-base of TPQ (TPQpsb), which differed in configuration with regard to the Schiff-base double bond (cis or trans) (SI Appendix, Fig. S4), were assigned for the \( F_o - F_c \) omit maps. cis-TPQpsb assigned with \( \sim 55 \% \) occupancy and another TPQpsb in trans configuration with \( \sim 45 \% \) occupancy, which is similar, but slightly different in conformation, to the phenyl ring moiety from trans-TPQpsb formed during the reductive half-reaction (Fig. 1A) (4–8). This trans-TPQpsb was also probably formed from the bound PAA, suggesting that the aldehyde group of the bound PAA has a relatively unrestricted conformation at a noncryogenic temperature, as described here, so that the nucleophilic attack by the 5-NH2 group of TPQamr is allowed from either the

### Table 1. Thermodynamic parameters of equilibrium between TPQamr and TPQsq

<table>
<thead>
<tr>
<th>State</th>
<th>( K_{eq}^{\circ} ) in solution at pH 6.0</th>
<th>( K_{eq}^{\circ} ) in crystal at pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta G^\circ )</td>
<td>2.0 ± 0.26</td>
<td>( \Delta G^\circ )</td>
</tr>
<tr>
<td>( \Delta H^\circ )</td>
<td>26 ± 1.8</td>
<td>( \Delta H^\circ )</td>
</tr>
<tr>
<td>( \Delta S^\circ )</td>
<td>83 ± 6.2</td>
<td>( \Delta S^\circ )</td>
</tr>
<tr>
<td>( \Delta S^\circ /K^\circ )</td>
<td>24 ± 0.18</td>
<td>( \Delta S^\circ /K^\circ )</td>
</tr>
</tbody>
</table>

*Estimated from \( \varepsilon_{100} \) of 5,000 M\(^{-1}\)

\( \text{cm}^{-1} \) for TPQsq (12) or average occupancies in the crystal structures for TPQamr using the equation: \( K_{eq} = (\Delta G^\circ /R T) + \Delta S^\circ /R T \), where \( \Delta H^\circ \) and \( \Delta S^\circ \) represent the standard enthalpy and entropy changes, respectively, and \( R \) and \( T \) are gas constant and absolute temperature, respectively. The values are at 20 °C. 

+ Calculated using the equation: \( \Delta G^\circ = \Delta H^\circ - T \Delta S^\circ \) (in solution) or from \( K_{eq} \) (in crystal). The values are at 20 °C.

+ Calculated from the van’t Hoff plot (Fig. 2D).
Si or the Re face of the carbonyl carbon of PAA with different conformations to form cis- or trans-TPQpsb, respectively (SI Appendix, Fig. S4 A and B; see following for details). At pH 8.0–10.0, an isolated electron density assignable to PAA was observed. At pH 8.0, the occupancy of TPQsq was 76%, and at pH 9.0 and 10.0, TPQsq was detected exclusively (Fig. 3B). In contrast, in the ethylamine-reduced structures having the empty hydrophobic pocket, both TPQsq and TPQamr were constantly detected with occupancies of 79–81% and 19–21%, respectively, at pH 8.0–10.0 (Fig. 3A). This difference suggests that the binding of PAA to the hydrophobic pocket facilitates the transition to TPQsq at pH 9.0 and 10.0. Altogether, these results confirm that the TPQsq/TPQamr equilibrium is dependent on pH in the 2-PEA-reduced crystals and demonstrate that the pH dependence is derived from PAA bound in the hydrophobic pocket.

It is conceivable that the hydrophobicity introduced by the aromatic ring of the bound PAA could be a primary driving force for the TPQamr–TPQsq transition in the 2-PEA-reduced crystals at pH > 8. The short hydrogen bond between Tyr284 and the product aldehyde contoured at 3.5σ. Electron density maps and modeled structures at pH 6.0, 7.0, 8.0, 9.0, and 10.0 in A were generated from datasets AGAOETA-p2 (subunit B of PDB entry 5ZOW), AGAOETA-p4 (subunit B of 5ZOY), AGAOETA-p6 (subunit B of 5ZP0), AGAOETA-p8 (subunit A of 5ZPR), and AGAOETA-p10 (subunit B of SZPH), respectively (SI Appendix, Table S3). Electron density maps and modeled structures at pH 6.0, 7.0, 8.0, 9.0, and 10.0 in B were generated from datasets AGAOPEA2 (subunit A of 5ZPK), AGAOPEA4 (subunit A of 5ZPM), AGAOPEA5 (subunit A of 5ZPN), AGAOPEA9 (subunit B of 5ZPT), and AGAOPEA11 (subunit A of SZPT), respectively (SI Appendix, Table S5). Residue 382 is represented by a stick model with the same color as in the bar graph. Water molecules and the Cu centers are represented by cyan and orange spheres, respectively. Average occupancies of the intermediates are shown by bars with SE (n ≥ 4; cyan, cis-; purple, trans-; green, TPQsq).

A possible mechanism for the pH-dependent formation of cis-TPQpsb and trans-TPQpsb at pH ~7 and below is schematically shown in Fig. 4, in which the reductive half-reaction from the initial oxidized form of TPQ (TPQox) to TPQsq is depicted at the center and the formations of trans-TPQpsb and cis-TPQpsb are on the left and right sides, respectively. Both trans-TPQpsb and cis-TPQpsb are considered to be protonated and have one more proton than TPQamr. Thus, the two forms of TPQpsb become more stable relative to TPQamr with decreasing pH. We assume involvement of the active-site base, Asp298, having an unusual pKₐ (7.5 at 30 °C) (4), in the pH dependence. Our previous study demonstrated that Asp298 participates in the α-H abstraction from substrate amine, as well as in the hydrolysis of TPQpsb in the reductive half-reaction (4). In the noncryogenic structure determined at pH 6.0 and 7.0, the carboxyl group of Asp298 was located close (2.8–3.4 Å away) to the imine group of TPQpsb. At an alkaline pH (>8), the deprotonated carboxyl group of Asp298 would promote the attack of a water molecule on the imine carbon atom of trans-TPQpsb and cis-TPQpsb, and hence cause the hydrolysis of TPQpsb (Fig. 4) (4). These factors account for the fact that the TPQpsb structures are observed only at pH ≤ 7 (Fig. 3B). In addition, the protonated carboxyl group of Asp298 (major species at pH < 7.5) can form a hydrogen bond with the carbonyl O atom of PAA, with Asp298 being the hydrogen donor. This hydrogen bond fixes the aldehyde group of PAA so that its Si face is attacked by the amino group of TPQpsb, resulting in the formation of cis-TPQpsb (Fig. 4, Right and SI Appendix, Fig. S4B). In contrast, the hydrogen bond cannot be formed between the deprotonated carboxyl group of Asp298 and the carbonyl O...
atom of PAA. In this case, PAA could adopt a conformation in which the Re face is attacked by the amino group of TPQamr (SI Appendix, Fig. S4A), resulting in the formation of trans-TPQpsb (Fig. 4, Left). Presumably, the aldehyde group of the bound PAA has a relatively unrestricted conformation at a noncryogenic temperature compared with the aromatic ring tightly bound at the hydrophobic pocket. In fact, the $F_{o}-F_{c}$ omit map observed at an elevated contoured level ($8\sigma$) indicates that the aldehyde group of PAA shows considerably lower electron density than the aromatic ring and the surrounding residues such as Asp298 (SI Appendix, Fig. S4C). With Asp298 far from the carbonyl group (SI Appendix, Fig. S4D), it is most likely that a water molecule would participate in the condensation as a proton donor (Fig. 4). Considering the $pK_a$ value of Asp298, its carboxyl group is mostly protonated at pH lower than 6, which favors the formation of cis-TPQpsb. Only at pH 7 in the series of experiments shown in Fig. 3B, the deprotonated form of Asp298 could occupy a significant fraction that brings about a detectable amount of trans-TPQpsb. Altogether, our findings suggest that the protonation/deprotonation state of Asp298 affects the reactivity of PAA with TPQamr or the lability of TPQpsb to hydrolysis, generating the deprotonation state of Asp298 affects the reactivity of PAA with TPQamr or the lability of TPQpsb to hydrolysis, generating the de

Fig. 4. Possible mechanism of pH-dependent equilibrium changes in the 2-PEA-reduced AGAO crystal.

Conclusions

We demonstrated in this study that the temperature-controlled HAG method is useful for determining the equilibrium mixture of the two catalytic intermediates, TPQamr and TPQsq, in AGAO crystals under noncryogenic conditions. Moreover, the HAG method is applicable to thermodynamic analyses of conformational changes occurring in protein crystals that are difficult to study by conventional cryogenic X-ray crystallography (13, 14). Importantly, thermodynamic parameters obtained by the in crystallo thermodynamic analysis are more directly linked to the structural changes of interest than those obtained by the ordinary methods in solution, such as spectroscopic and calorimetric analyses. Concerning the applicability of the HAG method, it should be noted that the method is limited to the conformational changes that do not lead to crystal cracking, as has been often observed in several protein crystals when soaked with ligands (15, 16). In the present study, no crack was formed on anaerobic soaking of AGAO crystals with substrate. The determined structures revealed that the conformational change is limited to the active-site region without significant global conformational changes in the main chain (4, 8).

In crystallo thermodynamic analysis in the present study showed that $\Delta H^\circ$ and $\Delta S^\circ$ of the TPQamr–TPQsq transition in crystal are enlarged by ~1.5-fold compared with those in solution (Table 1). Interestingly, the increase of $\Delta H^\circ$ and $\Delta S^\circ$ ascribed to the packing effect is comparable to the effect of polyethylene glycol or dextran-generating circumstances mimicking a physiological macromolecular crowding state of the cell interior (17). The environments of proteins in noncryocooled crystals might resemble those within the cell interior, such as the cytosol. This similarity suggests that the conformational changes and thermodynamic parameters determined by the temperature-controlled HAG method in the noncryocooled crystals reflect those under physiological conditions.

Various techniques of time-resolved crystallography, including the serial femtosecond crystallography with X-ray free electron laser under noncryogenic conditions, are now available to visualize motions in the enzyme active site (11, 18). The extreme brightness and ultrashort duration of X-ray free electron laser pulses enable the collection of diffraction data of protein crystals on the femtosecond timescale after initiation of the reaction. Although the serial femtosecond crystallography is an excellent method to monitor the conformational changes in crystal at ambient temperature, the temperature-controlled HAG method is useful to observe the structural equilibrium among states having distinct conformations under specified measurement conditions with a conventional X-ray beamline. The definition of conditions such as temperature and pH is essential for thermodynamic analyses. We expect that the temperature-controlled HAG method will provide an important addition to these techniques currently being developed.

Materials and Methods

Materials. Recombinant AGAO was purified as its inactive precursor form and converted to the Cu/TPQ-containing active form, as reported previously (19, 20). Protein and TPQ concentrations were determined spectrophotometrically, using molar extinction coefficients of $c_{\text{TPQ}} = 93,200 \text{ M}^{-1}\text{cm}^{-1}$ (19) and $c_{\text{TPQ}} = 5,000 \text{ M}^{-1}\text{cm}^{-1}$ (12), respectfully. Amine substrates, 2-PEA and ethylamine, were neutralized with 1 M H$_2$SO$_4$.

Spectrophotometric Measurements. To achieve fully anaerobic conditions, the enzyme and substrate solutions were kept in a vacuum-type glove box.
cryoloop (LithoLoops; Protein Wave) and coated with aqueous polymer glue at constant temperature (4 °C) performed on-site in an anaerobic chamber that was maintained at a constant ionic strength (I = 0.35 ± 0.03 M) that was adjusted with 100 mM Na2SO4. The mixture was transferred to a quartz cuvette with a gas-tight screw-cap, and temperature of the cell folder was changed from 25 °C to 20, 15, 10, and 5 °C, sequentially. Absorption spectra were measured using an Agilent 8453 photodiode-array spectrophotometer after 5 min preincubation at each temperature. After the measurement at 5 °C, temperature was returned to 25 °C to examine the reversibility of the spectral change.

Preparation of AGAO Crystals. AGAO was crystallized by microdialysis essentially according to the method described previously (4). Briefly, a 15 mg/mL protein solution was dialyzed in a 50–L dialysis button at 16 °C against 1.05 M potassium-sodium tartrate in 25 mM Hepes buffer (pH 7.4). Single crystals with approximate dimensions of 0.5 × 1.0 × 0.2 mm grew in about 2 wk.


For the data collection at BL38B1, the wavelength of the synchrotron radiation and oscillation range were 1.0 Å (12.40 keV) and 1.0°, respectively. Typically, the beam was elliptical shape with 200.0 × 100.0 μm (height × width) dimensions. A CCD detector (MX225HE; Rayonix) was used for detection. To minimize the radiation damage on the data collection under noncryogenic conditions, the X-ray beam was attenuated with a 700-μm-thick aluminum attenuator, and the beam radiation points were moved on the crystal gradually by 0.05 μm during the data collection. A total of 360 images were collected. The X-ray dose of the crystal was evaluated to be 4.8 kGy by RADDose-3D (22) (www.raddo.se), which is well below the level that causes significant damage to the crystal during data collection.

Single-Crystal Microspectrophotometry in the HAG Method. For the ethylamine-reduced single crystal mounted in the HAG method, temperature was gradually decreased from 20 °C to 5 °C under anaerobic conditions, and absorption spectra were measured at 20, 15, 10, and 5 °C, using the microspectrophotometer system as reported previously (4), which was settled in the hall close to the experimental hatch of the beamline.

Data Process and Refinements. The collected datasets were processed and scaled using HKL2000 (23). The initial phase was determined by molecular replacement with Phaser (24). The search model was based on the coordinates of the AGAO monomer (Protein Data Bank code 1U7U) after removing all water molecules and metal ions. Refinements, electron density map calculations, and assignment of solvent molecules were performed using Phenix (25). Manual building was performed using Coot (26), and water molecules and other ligands, such as metal ions, were added step-by-step to the model during the refinement process. PyMOL version 1.8 (Schrödinger, LLC) was used for figure drawings. Details and statistics pertaining to the data collection and refinement are summarized in SI Appendix, Tables S1, S3, and S5. Atomic coordinates and structure factors have been deposited in the Protein Data Bank with the accession codes as shown in SI Appendix, Tables S1, S3, and S5.
