Electrochemical Evidence of the Redox-controlled Ligand Exchange of the Heme in the CO-sensing Transcriptional Activator CooA

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The CO-sensing transcriptional activator CooA contains the six-coordinate, low-spin protoheme as a CO sensor. In this work, the reduction and oxidation midpoint potentials of CooA were determined by electrochemical redox titrations, which were -320 mV and -260 mV (vs. NHE), respectively. The different midpoint potentials provide evidence for exchange of the heme axial ligand during the change in the redox state of the heme.

Rhodospirillum rubrum CooA, a transcriptional activator that regulates the expression of the *coo* operons,[1-3] contains a protoheme as a prosthetic group.[4-6] The heme in CooA plays a central role in sensing carbon monoxide (CO) that is a physiological effector of CooA, and in regulating the transcriptional activator activity of CooA.[4-8] CooA is the first example of a hemeprotein in which CO plays a physiological role, though CO is widely used as a probe to study the biochemical and biophysical properties of hemeproteins.

The transcriptional activator activity of CooA is controlled by CO.[7-9] Only CO-bound CooA, in which CO is bound to the ferrous heme as an axial ligand, can bind the target DNA and thereby activate the transcription of a family of genes concerned with CO metabolism in *R. rubrum*, indicating that CO activates CooA as a transcriptional activator.[2,3,7-9] The heme in CooA is in the six-coordinate in the ferric, ferrous, and CO-bound forms.[6,7,10] Therefore, the exchange of the axial ligand takes place when the heme binds the effector. Furthermore, the possibility has been reported that the exchange of the axial ligand takes place during the change in the redox state of the heme in CooA.[7,9]

In this study, we elucidate the redox properties of the heme in CooA by means of electrochemical redox titration and indicate the experimental evidence supporting the proposition that the redox-controlled ligand exchange of the heme takes place in CooA.

The expression and purification of CooA were carried out as reported previously.[7] A Sephacryl S-100 (Amersham Pharmacia Biotech.) gel filtration column equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl was used at the final step of the purification. The purified CooA was concentrated to about 30 μM by ultrafiltration with a YM-10 membrane (Amicon, Inc.).

The electrochemical experiments were made in an electrochemical cell equipped with a quartz optical cell (40 (height) x 10 (width) x 1 mm (thickness)). The optical path length of the optical cell is 1 mm. A working electrode, a gold mesh electrode (40 x 9 x 0.7 mm), was immersed in the optical cell. A platinum wire and Ag/AgCl (3M KCl) electrodes (RE-1B, BSA) were used as auxiliary and a reference electrodes, respectively. The potential was controlled by a potentiostat (HA-301, Hokuto Denko Co.). The electronic absorption spectra were measured on a U-3300 Hitachi UV/Vis spectrophotometer.

For electrochemical redox titrations, the following redox mediator dyes (2 μM each) were added in the sample solution (the value of $E_{1/2}$ (vs. NHE) of each mediator dye is shown in the parentheses): phenazine methosulfate (+80 mV),[11] gallocyanine (+20 mV),[11] indigo trisulfonate (-80 mV),[11] 2-hydroxy 1,4-naphthoquinone (-120 mV),[12] anthraquinone 2-sulfonate (-230 mV),[11] benzyl viologen (-350 mV),[11] and methyl viologen (-440 mV).[13] CooA solution containing the mediator dyes was repeatedly degassed and flushed with Ar prior to the measurement, then approximately 2.5 ml of the sample solution was transferred into the electrochemical cell by using a gas-tight syringe under Ar atmosphere. The electrochemical cell was put in a thermoelectric cell holder of a U-3300 Hitachi UV/Vis spectrophotometer. The cell holder was kept at 15 °C during the electrochemical titration.

The redox reaction of CooA was pursued by recording the absorbance change at the regions of the Soret and Q bands. The electronic absorption spectra at the desired potential were recorded when the redox equilibrium of CooA was accomplished.

In the presence of redox mediator dyes, electron transfer between a gold mesh electrode and CooA proceeded and the redox equilibrium of CooA could be achieved at the desired potential. After changing the potential applied to the working electrode, it took about 5 minutes to accomplish the redox equilibrium of CooA. The electronic absorption spectra of CooA were measured at each potential when the redox equilibrium of CooA had been achieved. Figure 1 shows a typical spectral change of CooA during reductive titration; i.e., the reduction of the ferric CooA, when the potential applied to the working electrode was varied from -100 to -650 mV with a step width of 25 or 50 mV. The electronic absorption spectrum at the potential of -100 mV was identical to that of the ferric CooA as isolated, showing the Soret peak at 423.5 nm. As the potential applied to the working electrode was shifted negatively, the Soret peak at 423.5 nm decreased and the new Soret peak at 424.5 nm increased with the appearance of the α and β bands at 558.5 nm and 528.0 nm, respectively. No spectral change was observed below the potential of -600 mV. The electronic absorption spectrum observed below the potential of -600 mV was identical to that of ferrous CooA chemically reduced by sodium dithionite.

After measuring the electronic absorption spectrum at the potential of -600 mV in the reductive titration, the potential was started to shift positively to re-oxidize CooA for the electrochemical oxidative titration. In the oxidative titration, changes in the electronic absorption spectrum of CooA were the reverse of those observed in the reductive titration, during which the potential applied to the working electrode was shifted positively. Finally, an electronic absorption spectrum almost identical to that before the redox titration was observed. Isosbestic points were observed at 411.0, 434.5, 510.0, and 569.5 nm on the spectral change during the redox titrations.

These results show that, in the electrochemical redox titration system described herein, the redox reaction of CooA proceeds quantitatively and with no side reaction.

Nernst plots to determine the reduction and oxidation midpoint potentials of CooA are shown in the inset of Figure 1. The reduction and oxidation midpoint potentials of CooA were determined to be -320 mV and -260 mV, respectively. In the case of CooA, the reduction and oxidation midpoint potentials were not identical; i.e., the oxidation midpoint potential was

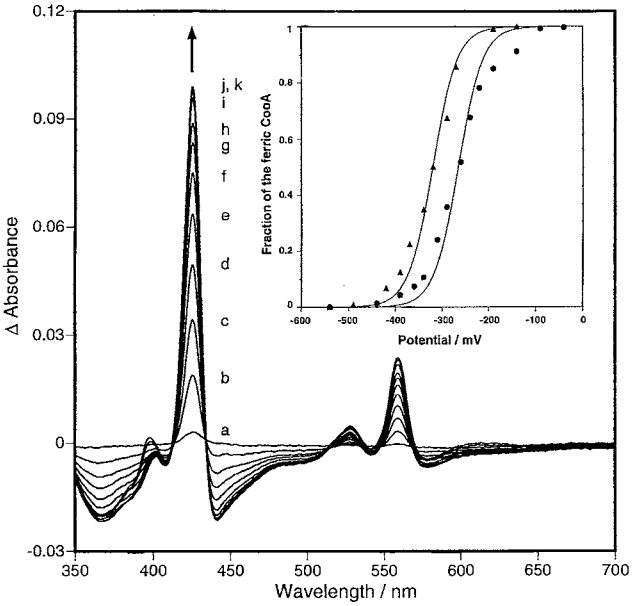

Figure 1. Typical spectral change of CooA during reductive titration. CooA (15 μM in dimer) was dissolved in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl. The difference spectra, the electronic absorption spectra at appropriate potential minus the spectrum of oxidized CooA, are shown. The spectra were measured at potentials of (a) -200, (b) -250, (c) -275, (d) -300, (e) -325, (f) -350, (g) -375, (h) -400, (i) -450, (j) -500, and (k) -550 mV. No spectral change was observed at potential of -100 mV. The spectra measured at potentials at -600 and -650 mV were identical to the spectrum (k). **Inset.** Reductive (triangles) and oxidative (circles) titrations of CooA. The fraction of the ferric CooA was plotted as a function of E_h. The individual data points are an average of at least three independent measurements. The solid lines are theoretical Nernst curves for one-electron reduction (triangles) and oxidation (circles) with midpoint potentials of -320 and -260 mV, respectively.

shifted positively by 60 mV compared with the reduction midpoint potential. The reduction and oxidation midpoint potentials would be identical if a simple redox reaction of the heme in CooA had taken place during the electrochemical redox titrations. On the other hand, the reduction and oxidation potentials will differ if the redox reaction of a heme is accompanied by heme ligand switching.[16] Mutational and spectroscopic experiments suggest that Cys75 is an axial ligand in the ferric heme and that His77 replaces Cys75 upon reduction of the heme in CooA.[7,9] The experimental results reported herein, namely, that the reduction and oxidation midpoint potentials are different for CooA, provide direct evidence of the redox-driven ligand exchange of the heme in CooA. The reactions (1) and (2) are thought to proceed in the reductive and oxidative titrations, respectively. In the following equations, Fe^{3+}, Fe^{2+}, and L represent the ferric heme, the ferrous heme, and an unknown axial ligand, respectively. Although [Cys-Fe^{2+}-L] and [His-Fe^{3+}-L] seem to be formed transiently in the redox titrations of CooA, their electronic absorption spectra are not observed.

$$\text{Cys-Fe}^{3+}\text{-L} \rightarrow [\text{Cys-Fe}^{2+}\text{-L}] \rightarrow \text{His-Fe}^{2+}\text{-L} \quad (1)$$
$$\text{His-Fe}^{2+}\text{-L} \rightarrow [\text{His-Fe}^{3+}\text{-L}] \rightarrow \text{Cys-Fe}^{3+}\text{-L} \quad (2)$$

Recently, a similar redox-controlled ligand exchange of the heme has been observed during catalysis in cytchrome cd1 nitrite reductase from *Paracoccus denitrificans* (formerly *Thiosphaera pantotropha*).[17,18] In the case of *Paracoccus denitrificans* cytochrome cd1, the exchange of the axial ligands is thought to be responsible for the adjustment of the redox potential of the hemes in order to regulate the internal electron transfer and for the release of the reaction product (NO) from the d1 heme.[17,18]

In the case of CooA, the change in the redox state of the heme is thought to be concerned with the activation and deactivation of CooA as a transcriptional activator. If this is the case, the redox-controlled ligand exchange would be responsible for the regulation of the redox potential at which the reduction and oxidation of the heme in CooA proceeds efficiently *in vivo* for activation and deactivation, respectively.

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