Dissociation and Recombination between Ligands and Heme in a CO-sensing Transcriptional Activator CooA

A FLASH PHOTOLYSIS STUDY*

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Shigeichi Kumazaki‡, Hiroshi Nakajima, Takahisa Sakaguchi, Emi Nakagawa, Hidenori Shinohara, Keitaro Yoshihara, and Shigetoshi Aono

From the School of Materials Science, Japan Advanced Institute of Science and Technology, Tatsunokuchi, Ishikawa 923-1292, Japan

CooA from Rhodospirillum rubrum is a transcriptional activator in which a heme prosthetic group acts as a CO sensor and regulates the activity of the protein. In this study, the electronic relaxation of the heme, and the concurrent recombination between ligands and the heme at ~280 K were examined in an effort to understand the environment around the heme and the dynamics of the ligands. Upon photodissociation of the reduced CooA at 400 nm, electronic relaxation of the heme occurred with time constants of 0.8 and 1.7 ps. The ligand rebinding was substantially completed with a time constant of 6.5 ps, followed by a slow relaxation process with a time constant of 173 ps. In the case of CO-bound CooA, relaxation of the excited heme occurred with two time constants, 1.1 and 2.4 ps, which were largely similar to those with reduced CooA. The subsequent CO recombination process was remarkably fast compared with that of other CO-bound heme proteins. It was well described as a biphasic geminate recombination process with time constants of 78 ps (60%) and 386 ps (30%). About 10% of the excited heme remained unligated at 1.9 ns. The dynamics of rebinding of CO thus will help us to understand how the physiologically relevant diatomic molecule approaches the heme binding site in CooA with picosecond resolution.

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through the sample. The pump pulses were chopped at 5 Hz, and the spectra were synchronously recorded at 10 Hz.

In the second scheme (single wavelength method), the dual photodiode array was replaced by two photodiode detectors. A mechanical chopper at half the repetition rate of the pulses (500 Hz) modulated the pump beam with a fixed phase to the pulse train. One photodiode detector monitored the energy of the white continuum pulse at 440 (±5) nm after transmission through the sample irradiated by the pump beam, and the other monitored the energy fluctuation of the same portion of the white continuum pulse without transmission through the sample. The probe and reference signals were carefully balanced and integrated by gated integrators for each laser shot. The difference signal between the two outputs of the gated integrators was sent to a digital lock-in amplifier. The amplitude of the modulation, which is synchronous to the pump pulse train was used to calculate the transient absorbance changes. The sample was continuously irradiated by the laser beams for at most 2 s to avoid build-up of photoproducts. A dark period of at least 1 s was introduced before measuring the transient absorbance change at each time delay.

The instrument-response function and the time zero at each probe wavelength were estimated by optical Kerr effect (OKE) cross correlation between pump and white continuum pulses. Neat o-dichlorobenzene in the sample cell was used as the OKE medium. The full width at half-maximum of the instrument-response function was 0.55–0.65 ps in the 420–510-nm region. The delay time where the OKE signal shows maximal intensity is defined to be ~0.3 ps at each wavelength. With this definition, more than 90% of the instrumental-response limited transient absorption signal is developed at 0.0 ps. Curve-fitting analysis was applied to the data in the time region later than 0.0 ps. The angle between the pump and probe polarizations was set at the magic angle (54.7°) in all transient absorption measurements.

The sample was introduced under nitrogen gas atmosphere into a gas-tight cylindrical cuvette with a path length of 2.5 mm. The CooA concentration was typically ~25 μM on the basis of heme content. This cuvette was cooled (~280 K) and rotated at ~3 Hz during the measurements to ensure that each pair of pump-probe pulses would irradiate a new portion of the sample. The absorption spectrum before and after each measurement did not show any change. The energy of the pump pulse was 1.5 μJ in the transient spectrum method and 1 μJ in the single wavelength method. There was no essential change in the kinetics at 440 nm when the pump power was decreased to 0.4 μJ (data not shown). The probe pulse energy was <80 nJ. The diameters of the pump and probe beams at the sample positions were 0.3 and 0.2 mm, respectively. We estimated that at most 40% of the heme was excited by a 1 μJ pump pulse.

RESULTS

The electronic absorption spectra of the reduced and CO-bound CooA, together with the probe wavelength range for flash photolysis, are shown in Fig. 1. Transient absorption spectra at representative delay times are shown in Fig. 2, a and b. Similar changes in the transient spectral shapes occurred in the case of both the reduced and CO-bound CooA within 5 ps upon excitation. After 5 ps, the spectral shape remained almost constant but the amplitude decayed. The decay seemed to be much faster in the case of reduced CooA than that in the case of CO-bound CooA. Transient absorption kinetics at representative wavelengths are shown in Fig. 3. The signal at 427 nm was dominated by photobleaching of the absorption of the six-coordinate heme in the ground state. Between ~430 and ~510 nm, the five-coordinate heme showed an absorption band (12). The appearance of a positive absorbance change above 480 nm was instrument-response limited (e.g. at 488 nm in Fig. 3, a and b; Ref. 9). There were two phases in the transient absorbance changes from 0.2–5.6 ps. The first phase was the growth of the positive signal at around 440 nm and the negative signal at around 425 nm (Fig. 2, a and b). Evidence of this phase was the delayed peak of the transient signals at 427 and 443 nm compared with that at 488 nm (Fig. 3, a and b). The second phase was observed from 1.2–5.6 ps in which the wavelength that gave the maximum positive absorbance change shifted from 441 nm to ~438 nm (Fig. 2, a and b). The difference between the reduced and CO-bound CooA became clear at 23 ps and later times, where relatively larger transient absorbance changes remained in the case of CO-bound CooA.

These complicated spectral changes were analyzed with the
help of global curve fitting analysis. The kinetics over the whole wavelength region within 45 ps were simultaneously fit by the sum of triple exponential functions with common time constants. This yielded time constants of 0.8, 1.7, and 6.5 ps for the reduced CooA, and 1.1, 2.4, and 78 ps for the CO-bound CooA. For each time constant, the decay-associated spectrum (DAS), which is a plot of the exponential amplitude against wavelength, is given in Fig. 4.

The single wavelength method was employed to study the long-lived transients in a more extended time region up to 1.9 ns (Fig. 5). The kinetic trace of the reduced CooA was fit by a sum of two exponential functions with time constants of 6.5 ps, 173 ps, and an offset (a very long time constant) (Fig. 5a). The kinetic trace of the CO-bound CooA was fit by a sum of three exponential functions with time constants of 4, 78, and 386 ps and an offset (Fig. 5b). The spectral shapes of the transient absorption spectra of the CO-bound CooA within 45 ps were compared in Fig. 2c. All of the above parameters obtained by the curve fit are summarized in Table I.

**DISCUSSION**

**Distinction between Relaxation of Heme and Ligand Rebinding**—It has been reported that the dissociation of diatomic ligands from heme upon photoexcitation of cytochrome c oxidase (13), hemoglobin, or myoglobin (14) occurs on a ~0.1-ps time scale with a quantum yield close to unity. It is assumed that dissociation of ligands in CooA occurs within our instrumental response time with a quantum yield of unity. Rubtsov et al. have studied the transient absorption changes in the mid-IR region after photolysis, and their study revealed an instrument-response limited bleaching of the vibrational band of the bound CO. This also supports our assumption. All of the time-resolved processes in this work are thus associated with relaxation of heme and/or rebinding of ligands.

For both reduced and CO-bound CooA, we have resolved three exponentially decaying components within 45 ps (Table I). The fastest two of the three processes seem to be common to both the reduced and CO-bound CooA. This is supported by the following key points of the data. Firstly, the shortest two time constants of the reduced CooA, 0.8 and 1.7 ps, are similar to those of the CO-bound CooA, 1.1 and 2.4 ps, respectively. Secondly, the shapes of the DASs of the 0.8 and 1.7 ps components are also similar to those of the 1.1 and 2.4 ps components, respectively (Fig. 4).

The dynamics of photodissociation and recombination between diatomic ligands and heme proteins have been extensively investigated for many heme compounds, such as myoglobin, hemoglobin, and cytochrome c oxidase (9–12, 16, 17). It

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1 The abbreviation used is: DAS, decay-associated spectrum.

2 I. V. Rubtsov, T. Zhang, H. Nakajima, S. Aono, G. I. Rubtsov, S. Kumazaki, and K. Yoshihara, submitted for publication.
absorption around 480 nm (12). It has been recently shown that a proximal ligand (His-77) is common to the reduced and CO-bound CooA (3, 25). It is thus possible that the energy dissipation of the excited heme through CO dissociation in the CO-bound CooA is more effective than that of the distal ligand in the reduced CooA.

The slowest-decaying components in Fig. 4 are very different in terms of time constants comparing the reduced CooA (6.5 ps) and CO-bound CooA (78 ps). It should also be noted that the DASs of the 6.5 and 78 ps components are similar to the difference spectrum between five- and six-coordinate heme (12). These components are thus attributable to recombination of the ligand. Given the common proximal ligand for reduced and CO-bound CooA (3, 25), the great difference in the time constant probably results from the different natures of the distal ligands. It should be noted that heme itself undergoes a relatively slow relaxation process, like vibrational population relaxation, which affects the absorption spectrum in the Soret band region. The exponential decay time of vibrationally hot states of the electronically relaxed heme could be as slow as 6–16 ps (18, 19). The DASs for the 6.5-ps time constant may be affected by vibrational cooling processes.

There is a minor component with a time constant of 173 ps in the kinetics of the reduced CooA (Fig. 5a and Table I). In carboxyhemoglobin, there is similarly a slow relaxation process upon photodissociation of CO (20, 21). The ligation state of the proximal histidine changes with a time constant of 100 ps (probed by α(Fe-His) Raman band shift, Ref. 21), and the restructuring of the asymmetric protein environment requires about 300 ps (probed by circular dichroism at 355 nm) (20). An even slower relaxation on the time scale of nano- to micro-seconds has been detected by probing weak iron-porphyrin charge transfer transitions (22), though the major change is complete on the subnanosecond time scale. These are intrinsic to myoglobin, because such slow processes are not seen in the case of isolated heme in protein-free solution (21). It is thus likely that the 173-ps component observed here is indicative of a relaxation of the CooA polypeptide upon photoexcitation of the heme, which affects the heme absorption spectrum.

**Ligand Rebinding Dynamics**—The rebinding of the ligand to the reduced CooA is very fast (6.5 ps, Figs. 4a and 5a). This recombination time seems to be comparable with that (7–20 ps) of the cytochrome c center in reduced cytochrome c oxidase (13, 17), where the heme is six-coordinate with the axial ligands being histidine residues (23). The 6.5-ps rebinding indicates that both the proximal and distal ligands of the ferrous heme in the reduced CooA are endogenous ligands of high inertia, not exogenous small molecules or ions, for which recombination times of at least tens of picoseconds are expected. These results are consistent with the proposed coordination structure of the heme in CooA (25).

The fastest component of 4 ps in the 440-nm kinetics of CO-bound CooA (Fig. 5b) seems to be associated with the relaxation process of excited heme (Fig. 4 and Table I). There seems to be no great change in the transient spectral shape of CO-bound CooA from 23–630 ps (Fig. 2c). These findings lead us to conclude that the recombination dynamics of CO in CooA is multiexponential: 78 ps (60%) and 386 ps (30%). About 10% of the dissociated CO remains free at 1.9 ns. This overall rebinding dynamics is largely consistent with that obtained by mid-IR probing of the CO vibrational band (CO-bound CooA (0.5 mM) in D$_2$O) or by resonance Raman probing (25). A small but clear difference between our findings and the results re-
CO Geminate Recombination in a CO-sensing Heme Protein, CooA

The reactivity between CO and the heme protein in CooA is crucial for its function as a CO sensor. The rate and yield of the geminate recombination of CO in CooA (Table I) are strikingly faster and higher than those of CO-bound CooA in the sample cell. Changes in the apparent decay dynamics of the five-coordinate CooA provide a much smaller free volume than that of myoglobin (26). The axial ligand to His-77 in the trans pocket and/or the time dependence of the reactivity of CO with amino acid residues that interact directly with the CO bound to the heme (8). Thus, there seem to be no steric restrictions for the fast recombination of CO in CooA. The geminate recombination between a diatomic ligand and heme on a picosecond time scale (10–500 ps) has been best studied in the case of NO rebinding in myoglobin (10, 15). Rapid NO recombination can be achieved by specific mutations in two ways: (i) removing steric restrictions directly adjacent to the iron atom and (ii) inhibiting ligand movement away from the iron atom by placing diffusional barriers. Recent results obtained by resonance Raman spectroscopic study and EXAFS analysis by this group have suggested that there are no distort amino acid residues that interact directly with the CO bound to the heme (8). Thus, there seem to be no steric restrictions affecting the binding of CO to heme at its binding site. In addition, we propose that the distal side of the heme pocket in CooA provides a much smaller free volume than that of myoglobin, which should cause a decrease of the CO recombination time by three orders of magnitude as compared with that in the case of myoglobin (26). The axial ligand trans to His-77 in the reduced CooA is replaced by CO upon CO binding (3, 25). It is thus probable that the released ligand acts as a diffusional barrier against the dissociated CO.

The rebinding dynamics of CO in CooA is clearly nonexponential. There are at least three models by which one can explain the nonexponential rebinding kinetics (24, 15). (i) The multiple-site model invokes several intermediate sites for CO after the photodissociation, which differ in terms of the potential barriers to recombination with heme. The microscopic origin probably arises from the complicated structure of the protein environment into which CO is ejected by photolysis. (ii) The relaxation model incorporates a potential barrier that evolves as the heme and protein structure relax following photodissociation, which gives a time-dependent rate constant of the CO-recombination process in CO-bound CooA in D2O by probing CO vibrational bands, which also supports this model. It should be noted that the real rebinding dynamics could be caused by a mechanism consistent with the multiple-site and relaxation models combined. Conformers of CooA may show different recombination rates (as in the inhomogeneous model). However, there has been no report on CooA conformers with a population ratio comparable with the amplitude ratio of the 78 and 386 ps components of the CO recombination dynamics (Table I).

In summary, this study has shown that CO geminate recombination proceeds with a high yield (90%) on a picosecond time scale. These features may be relevant to the physiological function of CooA which should hold CO tightly while it is activated to regulate transcription of the target DNA. The fast CO re-binding is possibly because of the crowded heme pocket in which the ligand released from the ferrous heme acts as a barrier against CO diffusion. The CO re-binding dynamics is multieponential. This is explained by the presence of multiple intermediate states of the dissociated CO in the distal heme pocket and/or the time dependence of the reactivity of CO with the heme.

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