CoA is a dimeric CO-sensing heme protein from *Rhodospirillum rubrum*. The heme iron in reduced CoA is six-coordinate; the axial ligands are His-77 and Pro-2. CO displaces Pro-2 and induces a conformation change that allows CoA to bind DNA and activate transcription of coo genes. Equilibrium CO binding is cooperative, with a Hill coefficient of \( n = 1.4 \), \( P_{50} = 2.2 \mu M \), and estimated Adair constants \( K_1 = 0.16 \) and \( K_2 = 1.3 \mu M^{-1} \). The rates of CO binding and release are both strongly biphasic, with roughly equal amplitudes for the fast and slow phases. The association rates show a hyperbolic dependence on [CO], consistent with Pro-2 dissociation being rate-limiting. The kinetic characteristics of the transiently formed five-coordinate heme are probed via flash photolysis. These observations are integrated into a kinetic model, in which CO binding to one subunit decreases the rate of Pro-2 rebinding in the second, leading to a net increase in affinity for the second CO. The CO adduct exists in slowly interconverting “open” and “closed” forms. This interconversion probably involves the large-scale motions required to bring the DNA-binding domains into proper orientation. The combination of low CO affinity, slow CO binding, and slow conformational transitions ensures that activation of CoA only occurs at high (micromolar) and sustained (≥1 min) levels of CO. When micromolar levels do occur, positive cooperativity allows efficient activation over a narrow range of CO concentrations.

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\( K_n \thickspace/\thickspace \mu M, \quad n = 1.4 \) 

\( K_1 = 0.16 \) and \( K_2 = 1.3 \mu M^{-1} \)
of CooA variants, and a model for the protein conformational change was recently proposed (15).

In the present work the equilibria and kinetics of CO binding to CooA have been measured and used to develop a kinetic model that can explain cooperative CO binding in terms of two successive Pro-2 displacement reactions. Rates of CO binding to and release from CooA were measured in stopped-flow, rapid mixing experiments. Studies were performed with both wild-type protein and a truncated CooA variant, ΔP3R4, in which the N terminus of each subunit is shortened by about 6 Å, causing strain on the Pro-2–Fe(II) bond and facilitating its dissociation. Direct determination of the rate constants for Pro-2 coordination and CO binding to five-coordinate heme intermediates was achieved by analyzing nanosecond and microsecond laser photolysis experiments and monitoring rebinding by absorbance and resonance Raman changes. Our results indicate that Pro-2 coordination serves to inhibit CO binding to CooA.

Determination of Equilibrium Constants for CO Binding—

Sample Preparation—CooA was purified as previously described (16), stored in 25 mM MOPS/0.1 M NaCl (pH 7.4), and then diluted into an appropriate buffer solution to give concentrations of ~2–5 μM for the equilibrium binding titrations, ~5–25 μM for the rapid mixing experiments, and ~50 μM for time-resolved resonance Raman and microsecond laser photolysis experiments. CooA concentrations are expressed in terms of heme. CooA and its CO adduct were prepared by adding the protein to a syringe or cuvette that contained buffer equilibrated with 1 atm of N2 or CO/N2 mixtures, respectively. If not stated otherwise, solid CO was purged with argon, and the other with CO, both at 1 atm; the concentration of dissolved CO was assumed to be 1000 μM. The CooA sample was titrated by making mixtures of the two stock solutions in a Hamilton syringe and then adding 20 μl of the appropriate mixture to each cuvette to obtain the concentration of CO needed. A separate CO/CooA mixture was prepared for each spectrum shown below in Fig. 2A. The cuvette headspace was minimized to ~30 μl, approximately equal to the volume of the added solution, to avoid degassing of CO. Complete saturation of CooA was achieved by addition of 1 atm of CO gas.

The concentration of CO was corrected for the bound fraction: [CO] = [CO]_{total} \times \frac{[\text{CooA}]_{total}}{[\text{CooA}][\text{CO}]} because CO binds tightly to CooA. The fractional saturation, Y, was determined experimentally by the fractional change between the absorbance of the CooA/CO mixture and that of the unliganded protein normalized to the maximum change, at 1 atm CO: \[ Y = \frac{[\text{CO}]^n}{K_{n1} + [\text{CO}]^2} \] (Eq. 1)

where \( K_{n1} \) is defined as the Hill equilibrium constant for the dissociation of \( n \) ligand molecules from the protein. [CO] was initially set to [CO]_{total} and then iteratively readjusted by the calculated value of Y until convergence occurred. The final parameters obtained by the above procedure were applied to Reaction 1 and Equation 2 to extract Adair constants,

\[
\begin{align*}
K_1 &= k_1/k_{-1} \\
(k\text{CooA})_2 + \text{CO} &\rightleftharpoons (k\text{CooA})_2\text{CO} \\
K_2 &= k_2/k_{-2} \\
(k\text{CooA})_2\text{CO} &\rightleftharpoons (k\text{CooA})_2\text{CO}_2
\end{align*}
\]

REACTION 1

\[
Y = \frac{k_1[\text{CO}] + 2k_2[\text{CO}]^2}{2k_1 + K_1[\text{CO}] + K_2[\text{CO}]^2}
\] (Eq. 2)

where \( K_1 \) and \( K_2 \) are the association equilibrium constants for the binding of the first and second CO molecules to the CooA dimer.

Determination of CO Association Rate Constants by Rapid Mixing—

Reduced CooA was mixed with CO at various concentrations, and binding was followed by measuring absorbance changes in the visible or Soret wavelength regions. In one set of experiments, ~25 μM CooA was mixed with CO and visible spectra from 335 to 650 nm were recorded.

**Fig. 1.** Ribbon diagram of the structure of CooA in its inactive Fe(II) state (a) and of CAP in its active, cAMP-bound state (b). The black structures in the center of each effector domain represent the heme (for CooA) and cAMP (for CAP). The homology of the two structures suggests that CO binding to CooA induces a conformational change of the protein that brings the DNA-binding domains (αp, colored yellow) into positions similar to that of active CAP. The figure also indicates the B and C α-helices. (Adapted from Ref. 11.)
Dynamics of Carbon Monoxide Binding to CooA

**Determination of CO Dissociation Rate Constants**—Two types of experiments were used to examine CO dissociation from CooA-CO. In the first, a solution of the CO adduct (5 μM) was rapidly mixed with 1 mM NO, and the displacement of CO was measured by the absorbance change at 422 nm. In the second experiment, the CO adduct was rapidly mixed with H64L mesoheme deoxyMb, which acts as an efficient scavenger of CO ($k_{\text{CO}} = 30 \mu \text{M}^{-1} \text{s}^{-1}$, $K_{\text{d}} = 11,000 \mu \text{M}^{-1}$) and has a Soret peak for MbCO at ~416 nm. The decrease in absorbance due to the loss of CO from CooA-CO was again monitored at 422 nm. In both cases the solution contained little or no free CO, and conditions were adjusted so that the rate-limiting step was CO dissociation.

**Flash Photolysis of CooA-CO; Absorbance Measurements**—Two types of experiments were conducted. In the first, time-resolved UV-visible spectra were recorded to identify and characterize the intermediates in CO rebinding after short, ~7-ns, YAG laser excitation pulses (Spectra Physics Quanta Ray GCR-170 Nd:YAG laser/MOPO-710 operated at 10 Hz with 20-mJ pump pulses). The configuration of photolysis laser and UV-visible probe laser has been described (17). A solution of the CO adduct was transferred to an airtight 2-mm path length quartz cuvette that had been flushed with 1 atm of CO. The protein was excited at 535 nm, in the Soret absorption band of the heme group. UV-visible absorption changes were extracted by singular value decomposition of time/wavelength/intensity matrices as described by Hofrichter et al. (18).

In the second set of experiments, CO rebinding was measured after excitation with an intense 0.3-μJ pulse from a Phaser-2100B dye laser using rhodamine 577 in ethanol. In these experiments, 30–50 μM CooA-CO was placed in a 1-mm path length cuvette containing a small amount of sodium dithionite and an appropriate concentration of CO. Because complete photolysis could be achieved, the dye laser system was used to determine the kinetic parameters that define the competition between CO rebinding and Pro-2 coordination to five-coordinate intermediates. Time courses were recorded at multiple [CO], fitted to exponential expressions, and analyzed in terms of the dependences of the fast and slow phase rates and amplitudes on CO concentration as described by Hargrove (19) for similar six-coordinate plant HbCO systems.

The rapid phase was followed at 420 nm, the Soret peak for CooA-CO, and represents CO recombination to five-coordinate heme on 0.1- to 50-μs time scales. The slow phase was followed at ~429 nm, close to the Soret absorbance maximum of reduced hexacoordinate CooA, and represents CO displacement of recombined Pro-2 on 0.1- to 10-s time scales. The dye laser was also used to determine the overall quantum yield for complete photolysis of CooA-CO, using MbCO as a reference (20). The value obtained, $\Phi_{\text{photolysis}} = 0.02$ for CooA-CO, is in agreement with the more direct picosecond rebinding studies of Aono and coworkers (12–14).

**Time-resolved Resonance Raman Spectroscopy**—The experimental setup for the time-resolved resonance Raman measurements has been described elsewhere (21, 22). The second harmonic of a Q-switched Nd:YLF laser (Photonics International GM-30-527) was used to pump a Ti:sapphire laser (Photonics International TU-UV), which gave a narrowed laser frequency output (<0.1 cm⁻¹) tunable between 810 and 920 nm. The Ti:S laser output (~25 ns at 1 kHz) was frequency doubled using a non-linear lithium triborate crystal to get a 419-nm pulse. A...
parallel laser system was used to obtain 426-nm probe pulses. The optimum pump laser power to achieve maximum photolysis was 140 milliwatts. Photolysis due to the probe laser itself was minimized by keeping the power at ~1 milliwatt. A DG535 delay generator controlled the time delay between the two laser pulses (200 ns to 300 μs). The beams were overlapped and then focused with a pair of cylindrical lenses onto the sample. The sample solution was contained in a NMR tube and spun around a stationary stirring wire for vertical mixing of the sample. Above the sample surface a continuous stream of CO was delivered through a stainless-steel tube to maintain the CO concentration at 1 mM. The scattered light was collected and focused onto a single spectrograph (SPEX 1269, 3600 grooves/mm) equipped with a gated intensified photo diode array detector. Six 30-s acquisition scans were averaged. Spectra were calibrated with acetone, cyclohexane, and methylcyclohexane.

The spectra were deconvoluted using the GRAMS/32 version 5.10 software. Parameters for the curve fitting included use of a mixed Gaussian/Lorentzian band shape and the incorporation of fixed band-widths. The fractional composition after photolysis was calculated from the corrected intensities, e.g. \( I_{\text{CooA}}/I_{\text{CooA} + I_{\text{CooA}} + I_{\text{CooA-CO}}} \). Multiplying \( I_{\text{CooA}} \) and \( I_{\text{CooA-CO}} \) by the total protein concentration provides the concentration of each of the species. The concentrations measured from probe-only spectra were subtracted to calculate the change in the concentration. The relative change was then obtained by dividing these numbers by the total protein concentration.

RESULTS

Equilibrium Binding of CO Is Cooperative—CooA was carefully titrated with CO at pH 7.4 while monitoring the visible absorption spectrum (Fig. 2). The spectral changes (Fig. 2A) were used to calculate fractional saturation changes as a function of the concentration of free CO in the sample (Fig. 2B) as described under “Experimental Procedures.” When the binding data were analyzed in terms of the Hill equation (Equation 1), the fitted value of \( P_{50} \) was 2.2 μM, with \( n = 1.4 \), indicating substantial positive cooperativity; theoretical binding curves for a non-cooperative dimer (\( n = 1 \)), and one showing maximum cooperativity (\( n = 2 \)), are shown for comparison. The data were also used to extract Adair association constants, with fitted values of 0.17 and 1.25 μM\(^{-1}\) for \( K_1 \) and \( K_2 \), respectively (Equation 2).

CO Binding Kinetics Are Biphasic and Limited by Pro-2 Dissociation—In stopped-flow mixing experiments, CO binding to reduced CooA occurs on time scales of minutes and is biphasic (Fig. 3; [CO] = 500 μM). The data, followed over 1000 s, were fit to a two exponential expression, yielding fast and slow pseudo-first-order rate constants, \( k_f \) and \( k_s \). When the experiments were repeated at 250 and 100 μM CO, both rates diminished, but the changes were not proportional to [CO]. This result suggested rate limitation by a step prior to CO binding, presumably dissociation of the coordinated internal Pro-2 ligand present in reduced CooA dimers (Fig. 4).

This interpretation is supported by the time course for CO binding to the CooA variant, ΔP3R4, in which residues Pro-3 and Arg-4 are deleted (Fig. 3B). This variant is fully active in the presence of CO, and the Fe(II) form is predominantly six-coordinate and low spin, suggesting that Pro-2 remains bound to the heme. However, the ligand field is weaker (15), indicating that shortening the N terminus strains the Pro-2–Fe bond. Consistent with this finding, the pseudo first order rate constant for the fast phase at 500 μM [CO] is much larger than that for wild-type CooA, \( k_f = 100 \text{ s}^{-1} \) and 0.37 s\(^{-1}\) for ΔP3R4 and WT CooA, respectively. A slow phase is also seen for the ΔP3R4 variant, and its rate (\( k_s = 0.11 \text{ s}^{-1} \)) is much closer to that for the slow phase of WT CooA (\( k_s = 0.07 \text{ s}^{-1} \)) (Fig. 3).

To define the mechanism of binding more accurately, stopped-flow experiments were conducted at lower CooA concentrations (2.5 μM), following absorbance changes at 420 nm, the Soret band maximum of the CO adduct. The [CO] was varied from 10 to 500 μM. Biphasic kinetics were still observed (Fig. 5), and the fitted values of \( k_f \) and \( k_s \) show a clear hyperbolic dependence on [CO] (Fig. 6).

The observed fast and slow pseudo first order rate constants shown in Fig. 6 were analyzed in terms of the mechanism
shown in Fig. 4, which involves the pre-dissociation of Pro-2 followed by CO binding to a transient pentacoordinate (5c) intermediate. This intermediate is spectrally undetectable in the stopped-flow experiments, because, as will be shown in the next section, the rates of bimolecular CO binding, $k_{CO}[CO]$, and Pro rebinding, $k_{Pro}$, to the 5c intermediate are very rapid compared with the rate constant for Pro-2 dissociation, $k_{-Pro}$. The latter rate constant limits the overall rate of CO binding in the rapid mixing experiments. Consequently, a steady-state assumption can be made for the rate of change of [CooA(5c)], and the observed pseudo first order rate constant for overall CO binding to CooA containing bound Pro-2 (19, 24, 25) is given by Equation 3.

$$k_{obs} = \frac{k_{pro}[CO] + k_{CO}[CO] + k_{pro}k_{CO}}{k_{pro} + k_{CO} + k_{CO}[CO]} \quad \text{(Eq. 3)}$$

Equation 3 reduces to a simple hyperbolic expression when the rates of proline and CO dissociation are very small (i.e. $k_{-pro} \ll k_{pro} + k_{CO}[CO]$ and $k_{-CO} \ll k_{CO}[CO]$).

$$k_{obs} = \frac{k_{pro}[CO]}{k_{pro} + k_{CO}[CO]} = \frac{k_{pro}[CO]}{k_{pro}k_{CO}} \quad \text{(Eq. 4)}$$

Equation 4 applies to both the fast and slow observed rate constants, $k_{obs,f}$ and $k_{obs,s}$, and holds rigorously at CO concentrations where complete binding occurs. The limiting rates for both phases are the rate constants for Pro-2 dissociation, $k_{-Pro,f}$ and $k_{-Pro,s}$. The solid lines in Fig. 6 represent fits to Equation 4, with $k_{-Pro,f} = 0.4 \text{ s}^{-1}$, $k_{pro}/k_{CO}$ (fast phase) = 50 $\mu$M and $k_{-Pro,s} = 0.07 \text{ s}^{-1}$, $k_{pro}/k_{CO}$ (slow phase) = 60 $\mu$M.

**CO Rebinding to the 5-Coordinate Intermediate**—The kinetics of CO rebinding to the five-coordinate intermediate, CooA(5c), were determined via flash-photolysis techniques. Initial experiments employed short (7–25 ns) flashes and detection of early transients, using absorption or resonance Raman spectroscopy. The experiments in the next section employed longer (300 ns) flashes to achieve complete photolysis and monitor more directly the competition between CO and Pro rebinding on longer time scales.

Fig. 7 shows transient absorption difference spectra, collected at 10 ns and 100 $\mu$s after CooA-CO photolysis with a 7-ns, 535-nm YAG laser pulse (Fig. 7B). These spectra have the same features (438-nm maximum and 421-nm minimum) as the transient (10 ns) difference spectrum of the CO adduct of myoglobin, MbCO (Fig. 7C) and not those of the equilibrium difference spectrum of CooA and its CO adduct (Fig. 7A). This result established that the intermediate contains a five-coordinate heme, analogous to that in deoxyMb. Moreover, singular value decomposition of time/wavelength/intensity matrices (18) showed that the transient absorbance changes at pH 7.4 exhibit only one basis spectrum, the one seen in Fig. 7B. However, data obtained at pH 11 (not shown) contained an additional, low amplitude basis spectrum, corresponding to the CooA/CooA-CO absorption difference spectrum. Hence, Pro-2 rebinding begins to compete with CO rebinding at high pH, even at 1000 $\mu$M CO. The absorbance changes indicated low photolysis yields at 7 ns, consistent with the fast geminate rebinding found by Aono and coworkers (12). The estimated yield was $-10\%$ at pH 7.4 but increased to $-30\%$ at pH 11.
suggesting that more of the photogenerated CO escapes into solution at high pH.

These inferences are supported by time-resolved resonance Raman spectra (Fig. 8). The intense $\nu_4$ porphyrin band (26) can be resolved (Fig. 9, inset) into well separated contributions from CooA (1360 cm$^{-1}$), CooA-CO (1372 cm$^{-1}$), and the five-coordinate (5c) photoproduct (1356 cm$^{-1}$), allowing all three to be monitored and quantitated independently. Fig. 9 shows the time courses of the species concentrations, obtained from deconvolved peak areas after correction for different enhancement factors (similar for CooA and CooA-CO but 3-fold lower for the five-coordinate intermediate). In agreement with the transient absorption data, the fraction of CooA reformed after photolysis was negligible at pH 7, but a small fraction (10%) was detected at pH 11.

The recombination of CO and the disappearance of the five-coordinate intermediate (Fig. 9) are well described by a simple two exponential expression ($A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t)$) with pseudo first order rate constants equal to 32,000 s$^{-1}$ (60% amplitude) and 2,000 s$^{-1}$ (40% amplitude). Analysis of the absorption transients with 7-ns pulse excitation yielded similar observed rate constants, 67,000 and 2,000 s$^{-1}$, respectively. Because [CO] was 1000 $\mu$M in these experiments, the observed rate constants correspond to second-order $k_{CO}$ values of 32 and 2 $\mu$M$^{-1}$ s$^{-1}$ for the 25-ns experiments and 67 and 2 $\mu$M$^{-1}$ s$^{-1}$ for the 7-ns experiments, respectively. The larger bimolecular rate constants are in agreement with that reported by Aono and coworkers: 35 $\mu$M$^{-1}$ s$^{-1}$ (12). The slower rate constants lie between the two smaller values that Aono and coworkers extracted from their flash photolysis data: 6.8 and 1.2 $\mu$M$^{-1}$ s$^{-1}$ (12). A more rigorous analysis of the ligand concentration dependence of CO and Pro-2 binding to the 5c intermediate is described below, using a longer laser excitation pulse.

**Rebinding Competition between CO and Pro-2**—Using an intense 300 ns dye laser pulse permitted complete photolysis of CooA-CO and monitoring of subsequent rebinding events over a longer time interval. In initial experiments, the quantum yield for CO photodissociation was determined to be $\approx 0.02$ by analyzing the dependence of the rebinding absorbance change on laser power and comparing the results with those obtained using sperm whale MbCO as control with a quantum yield of $\approx 1$ (20). These data showed that complete photolysis of CooA-CO could be achieved at maximum dye laser power.

By fully photolyzing the sample and lowering [CO] to 100 $\mu$M, we were able to monitor both the fast rebinding to the five-coordinate intermediate and the slow displacement of re-coordinated Pro-2 (Fig. 10A). The fast phase has wavelength maxima and minima corresponding to CO binding to five-coordinate heme, whereas the slow phase (Fig. 10A, inset) could be monitored at 429 nm, which is the wavelength maximum of the CooA/CooA-CO difference spectrum. The amplitude of the slow phase is determined by the competition between internal Pro-2 and bimolecular CO rebinding and is approximately equal to $k_{Pro}/(k_{Pro} + k_{CO}[CO])$ (see Fig. 4). The fractional amount of Pro-2 rebinding diminishes to $\approx 10\%$ as [CO] is raised to 1000 $\mu$M (Fig. 10B), consistent with the short flash data (Figs. 7–9). As in the stopped-flow experiments, the slow phase is biphasic, and both rates show a hyperbolic dependence on [CO] (see diamonds in Fig. 6).
A rigorous analysis of the fast and slow phases together involves complex quadratic expressions of the rate parameters shown in Fig. 4, as has been described by Hargrove (19) for the analysis of CO rebinding to non-symbiotic, hexacoordinate plant hemoglobins. These equations reduce to simple expressions when \( k_{\text{Pro}} / H_{11002} \) is much less than both \( k_{\text{Pro}} \) and \( k_{\text{CO}} / H_{11032} \), as is the case for CooA (see Table I). Under these conditions, the pseudo-first-order rate constant for the slower, proline displacement phase is given by Equation 4, above, and the rate constant for the rapid bimolecular phase is given by Equation 5.

\[ k_{\text{rapid}} = k_{\text{Pro}} + k_{\text{CO}}[\text{CO}] \]  

(Eq. 5)

The fast phases in Fig. 10 were fitted to a single exponential expression, and a plot of \( k_{\text{rapid}} \) versus [CO] gave a slope of \(-16 \mu \text{M}^{-1} \text{s}^{-1}\) and an intercept of \(-1000 \text{ s}^{-1}\), which correspond to \( k_{\text{CO}} \) and \( k_{\text{Pro}} \), respectively (dotted line in Fig. 11B). However, just as in the short pulse experiments, the rapid rebinding phase is best represented as a two exponential process (see comparison in Fig. 11A), and plots of \( k_{\text{rapid}} \) versus [CO] (Fig. 11B) yield two sets of rate parameters. The intercepts of the linear plots yield the first order rate constants for proline rebinding to the five-coordinate heme, \( k_{\text{Pro}} = 2000 \) and \( 500 \text{ s}^{-1}\) (Fig. 11B). The slopes give \( k_{\text{CO}} = 32 \) and \( 9 \mu \text{M}^{-1} \text{s}^{-1}\). The larger rate constant is in agreement with the short pulse resonance Raman and transient absorbance measurements and that of Aono and coworkers (12). The other bimolecular rate constant, \( 9 \mu \text{M}^{-1} \text{s}^{-1}\), is larger than the values obtained using 7- and 25-ns YAG laser pulses. It is possible that the short-pulse time courses contain a small slow phase contribution from the displacement of re-coordinated Pro-2, which was not accounted for in the fitting analyses.

The 300-ns dye laser was also used to examine CO rebinding at different laser intensities, to compare time courses after
100% photolysis, when the photoproduct is CooA(5c)2, and after 10% photolysis when the photoproduct contains only one bound ligand, i.e. CooA2(CO)(5c). At 1000 μM CO, the time courses for rebinding are the same (Fig. 12), showing that bimolecular CO binding to a pentacoordinate CooA subunit is not affected by the presence or absence of CO bound to the other subunit. However, at 100 μM CO, the rate of the rapid phase is smaller at 10% than at 100% photolysis. Under these conditions, the rate of Pro-2 binding is affecting the observed rate (Equation 5), and the smaller rate constant observed at 10% photolysis implies that Pro-2 rebinding is slowed when one CO is bound to a CooA dimer.

Kinetics of CO Dissociation from CooA-CO—Time courses for CO dissociation from CooA-CO are shown in Fig. 13. Two independent techniques were used to monitor CO release. In the first, solutions with high concentrations of NO were mixed with the CO complex of CooA at moderately low [CO]. Under these conditions the rate-limiting step for NO displacement is the dissociation of CO. Immediately after CO dissociates, NO rapidly and tightly binds to the heme, preventing CO rebinding. However, NO binding is known to release the proximal histidine ligand in CooA (27), and thus some of the observed spectral changes could be due to the proximal histidine displacement reaction.

In the second type of experiment, H64L deoxyMb containing mesoheme was mixed with the CO complex of CooA, and the time course for the transfer of CO from CooA-CO to the scavenging reagent was followed at 420 nm. The distal histidine in the Mb mutant is replaced by leucine, which dramatically increases both the rate (kCO ~ 30 μM⁻¹s⁻¹) and affinity (KCO ~ 1,100 μM⁻¹) of CO binding (28). Protoheme was replaced by mesoheme to shift the Soret peak of the MbCO complex to 416 nm, which facilitates absorbance measurements at 420 nm, the CooA-CO Soret peak. Disappearance of the CooA-CO peak indicates the dissociation of the bound ligand, which is rapidly and almost irreversibly bound by the reagent.

Both the NO displacement and H64L deoxyMb scavenging time courses are biphasic (Fig. 13). Fits to two exponential expressions give rate constants of 0.02–0.04 s⁻¹ and 0.002 s⁻¹ for the fast and slow phases, which have roughly equal amplitudes.
DISCUSSION

Cooperativity and Biphasic Kinetics—The central issue for understanding the dynamics of CO binding to CooA is reconciling positive cooperativity in the equilibrium curve with biphasic kinetics. The observation of fast and slow phases, of roughly equal amplitudes, for CO association would seem to suggest a decrease in reactivity toward ligands after one CO molecule has been bound. The observation of fast and slow phases for CO dissociation suggests that stabilization of bound CO is enhanced after one ligand molecule has been released. Both kinetic observations seem in apparent contradiction to cooperative equilibrium CO binding.

This contradiction cannot be reconciled for CO dissociation. The fast and slow dissociation phases shown in Fig. 13 must arise from roughly equal populations of very slowly interconverting protein conformers. However, because the equilibrium constant for the conformation transition is roughly one, it will have little effect on the overall CO equilibrium binding curve. The faster rate of CO dissociation, ~0.02 s⁻¹, is assigned to an “open” conformation, because k_{-CO} value is essentially the same as for MbCO. The slower rate constant (k_{-CO} ~ 0.002 s⁻¹) is assigned to a “closed” conformation, in which the binding pocket obstructs egress of the CO. Thus the slower dissociation rate could represent either the rate of interconversion of the conformers or the rate of CO escape from the closed form, whichever is faster. Open and closed forms of the CO-bound form of CooA also provide a plausible explanation for two bimolecular rates of CO recombination after photolysis of the saturated CO complex. Functionally similar open and closed conformations have been reported for hexacoordinate plant and bacterial hemoglobins (19). In the case of CooA, the transition from open to closed is probably related to the large conformational changes required for high affinity binding to DNA.

In contrast, the decelerating association time courses shown in Figs. 3 and 5 can be reconciled with positive equilibrium cooperativity. Each CO association reaction is itself a two-step process: Pro-2 dissociation is followed by competition between CO binding and Pro-2 re-association. The overall deceleration in CO binding to hexacoordinate CooA observed in mixing experiments implies that Pro-2 dissociation slows down after one CO is bound. However, there appears to be an even greater slowing of Pro-2 re-association, so that the equilibrium constant for Pro-2 coordination decreases significantly. In this interpretation, the decrease in K_{Pro} (Fig. 4) after one CO has bound will facilitate the binding of a second CO and account for the observed positive cooperativity shown in Fig. 2.

Kinetic Model—The interpretations described above were used to assign equilibrium and rate constants for a model of CO
binding to CooA, as diagrammed in Fig. 14; the parameter values are given in Table I. These parameters were selected as follows: 1) The equilibrium constant between the open and closed form of CooA₂(CO)₂ is unity based on the equal amplitudes of the fast and slow CO dissociation phases (Fig. 11), and the interconversion rate (sum of the forward and backward rate constants) is 0.002 s⁻¹, based on the observed rate of the slow CO dissociation phase. 2) CO dissociation and association rate constants for binding to pentacoordinate CooA subunits are the measured values for the open form of CooA₂(CO)₂, 0.02 s⁻¹ and 32 μM⁻¹ s⁻¹, and are assumed not to depend on the ligation state of the second subunit in the dimer. The latter assumption is based on the independence of the rapid, bimolecular CO recombination rate on the degree of photolysis at high [CO] (Fig. 12A). These parameters are in the range, 0.02–0.05 s⁻¹ and −25 μM⁻¹ s⁻¹, determined for Mb variants in which the distal histidine is replaced by an apolar residue, producing a hydrophobic binding pocket, similar to that in CooA-CO (28). The assigned association constant for the closed form of CooA₂(CO)₂ is 9 μM⁻¹ s⁻¹ but is not relevant to CO binding to CooA in mixing experiments. 3) The Pro-2 dissociation and association rate constants do depend strongly on the ligation status of the adjacent subunit, consistent with the observed slowing of Pro-2 re-association after a 10% photolysis flash at low [CO] (Fig. 12B). Pro-2 dissociation limits the rates of the CO association in mixing experiments with CooA at high [CO] (Equation 4 and Fig. 6). For the first binding step there is a statistical factor of 2, because binding is equally probable for the two equivalent subunits. Thus, the intrinsic values of $k_{1,CO}$ are 0.2 and 0.07 s⁻¹ for the first and second steps of CO binding to CooA₂(Pro₂), which predict $k_{1}$ and $k_{2}$ values of 0.4 and 0.07 s⁻¹, respectively, for the limiting values in Fig. 6. The Pro-2 association rates were fixed by requiring consistency with the measured equilibrium association constants for the two CO binding steps (Equation 2). Expressions for the Adair constants were derived in Equations 6 and 7.

$$K_1 = \frac{K_{CO}(K_{Pro,2} + 1)}{(2 + K_{Pro,1}K_{Pro,2})} = 2 \frac{K_{CO}}{K_{Pro,1}} = 0.16 \mu M^{-1}$$  
(6)

$$K_2 = \frac{1}{2} \left( \frac{1}{K_{Pro,1}} + \frac{1}{K_{Pro,2}} \right) = \frac{1}{2} \left( \frac{K_{CO}}{K_{Pro,1}} \right) = 1.3 \mu M^{-1}$$  
(7)

where $K_{Pro,1}$ and $K_{Pro,2}$ are the rate constants for the first and second Pro binding steps, respectively. $K_{Pro,1}$ is based on the assigned $K_{CO}$ and $K_{Pro,2}$ values, then $K_{Pro,1}$ and $K_{Pro,2}$ must equal 20,000 and 1,200, and the required values for $k_{Pro,1}$ and $k_{Pro,2}$ are 4,000 and 84 s⁻¹, respectively. The values for $k_{Pro}$ estimated for the photoproduct CooA₂(5c)₂ are 2,000 and 500 s⁻¹ for the open and closed forms (γ intercepts in Fig. 11B), are in this range.

The model in Fig. 14 and the parameters in Table I provide a reasonable, quantitative interpretation of the equilibrium binding curve and the biphasic time courses for CO association.
to and dissociation from CooA dimers. They also can describe microsecond time courses for rebinding to the transient five-coordinate intermediates generated by laser photolysis. The key feature of the model is that the increase in CO affinity, which occurs after one ligand is bound, is caused by a decrease in the affinity and rate constants for Pro coordination to the remaining unliganded subunit.

The biphasic association kinetics could alternatively be explained by two populations of slowly interconverting CooA conformers, one with large CO and Pro binding rate constants, and one with significantly smaller parameters. Consecutive binding and conformational heterogeneity cannot readily be distinguished via kinetics alone. The equation to describe consecutive binding under pseudo first order conditions is,

\[ (1 - Y_t) = \frac{2k_2 - k_1}{2k_2 - k_3} \exp(-k_1 t) + \frac{k_1}{2k_2 - k_3} \exp(-k_2 t) \]  

(Eq. 8)

where \((1 - Y_t)\) is the fraction of unreacted CooA remaining at time, \(t\), and \(k_1\) and \(k_2\) are the rate constants for the two successive CO binding steps. If the absorption change is assumed to be the same for each binding step, Equation 8 predicts that the amplitudes of the fast and slow phases should be \(-40\%\) and \(-60\%\), respectively, which is consistent with what is observed by simple two-exponential analysis (55–60% slow phase, Fig. 5). The dependences of \(k_1\) and \(k_2\) on [CO] are still hyperbolic and represented by Equation 4, because each CO binding step requires the initial dissociation of Pro-2, regardless of mechanism. The dashed lines in Fig. 6 calculated using the model parameters in Table I provide satisfactory fits to the observed data, except at the lowest [CO] values for the slower phase, where the fitted rate parameters are the least reliable.

Thus, the two-step, consecutive binding and conformational heterogeneity models are both consistent with the observed kinetic data. However, an interpretation based entirely on conformational heterogeneity cannot explain the cooperativity observed for equilibrium CO binding. Thus we feel that the model presented in Fig. 14 and Table I provides the best description of the functional behavior of CooA dimers.

Structural Interpretation—We have previously proposed that the transition from CooA\(_2\)(Pro)\(_2\) to CooA\(_2\)(CO)\(_2\) involves displacement of the heme into an adjacent cavity and a shift of the C-helix toward the opposite heme (Fig. 15), based on the crystal structure of CooA, resonance Raman data, and mutational analyses (15). The key feature of this proposal is that the Pro-2 ligand in CooA\(_2\)(CO)\(_2\) is provided by the N terminus of the opposite subunit.

The Pro-2–Heme Bond—An obvious role for Pro-2 is to inhibit CO binding. The equilibrium affinity of CooA (\(0.5 \mu M^{-1}\)) is three orders of magnitude lower than the calculated value of \(K_{CO} (1600 \mu M^{-1})\) for CO binding to the five-coordinate inter-

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**Fig. 15.** Detail of the two heme-binding domains of CooA, showing the proposed displacement (dashed arrows) of the hemes and C-helices, upon CO replacement of the Pro-2 ligands (from Ref. 15).
mediate, reflecting the energetic cost of displacing the endogenous Pro-2 ligand.

In addition, the interlocking arrangement of the two Pro-2 ligands provides a natural explanation of cooperativity, which results from a lowering of the Pro-2 binding affinity after one CO molecule is bound. The equilibrium constant for Pro-2 binding drops from 20,000 in CooA2(Pro)(CO)2 to 1,200 in CooA2(Pro)(CO). It is reasonable to assume that the heme and C-helix displacement that is initiated by dissociation of the first Pro-2 ligand makes it easier to dissociate the second one, because the second Pro-2 is attached to the subunit containing the displaced, heme-bound CO (Figs. 1 and 15). The lowered Pro-2 affinity in CooA2(Pro)(CO) results from a large reduction in the Pro-2 association rate constant, from 4,000 to 84 s⁻¹, which overcomes a smaller decrease in the Pro-2 dissociation rate constant, from 0.2 to 0.07 s⁻¹. This lowering of k_pro suggests that CO binding to the first subunit causes the Pro-2 N terminus to move farther from its heme target in the second unliganded subunit, thereby slowing its reassociation. This displacement would be a natural result of protein rearrangement induced by the inward movement of the first heme group (Fig. 15).

The rate of Pro-2 dissociation is a measure of the strength of its coordination to the heme iron. Therefore, a decrease in k_pro implies strengthening of the Fe–Pro bond after the first CO is bound, despite the lowered affinity, which results from the greater reduction in k_pro. The Fe–Pro bond in CooA2(Pro)2 may be strained because of the interlocking subunits, and this strain would be released when the first Pro-2 is displaced. This interpretation is supported by the kinetic data for the ΔPro84 CooA mutant. Although the first CO binding step is accelerated by shortening the N-terminal tail of the protein, the second step is nearly as slow as in the wild-type protein. This result indicates that the truncated N terminus can form a strong Fe–Pro bond in the CooA2(Pro)(CO)2 intermediate, after the first Pro-2 ligand is displaced.

Open and Closed Forms—The heme and C-helix displacements that occur upon CO binding must initiate the repositioning of the DNA-binding domains (Fig. 1). The presence of open and closed forms of CooA2(CO)2, defined by the kinetics of CO dissociation, implies the presence of slowly interconverting conformations when both subunits are fully bound by CO. The open form may represent protein molecules in which the conformational changes required for DNA binding are not complete, despite the proper CO-induced heme displacement. Although resonance Raman spectroscopy reveals a minority population of CooA2(CO)2 with characteristics suggesting undisplaced hemes, this population appears to be quite small (15).

In addition preliminary experiments with mutational variants of CooA reveal no correlation between the undisplaced heme-CO population and the kinetically determined open/closed fraction. Thus closed and open populations cannot be distinguished by the resonance Raman spectrum of the heme group or by its absorption spectrum.

We propose that the activation of CooA by CO occurs in two stages: 1) concerted heme displacements as diagrammed in Fig. 15 and 2) longer range movements of the C-helices that bring the DNA-binding domains into proper alignment for transcriptional regulation. The second stage is suggested to correspond to the open-closed conversion. The protein motions in the second stage are far enough from the CO binding site so that they are not sensed by changes in heme spectral properties. However, this conformational change is proposed to decrease the rate of CO dissociation, presumably by causing tighter packing around the distal heme pocket.

This interpretation implies that only the closed form of CooA2(CO)2 can bind its target DNA. Because the closed and open populations appear to be equal, it would appear that only half the CooA2(CO)2 molecules are competent to bind to DNA rapidly and activate the coo genes, but would still be sufficient to saturate the two coo promoters.

Physiological Implications—Because the sensitivity of CooA to CO is lowered ~1000-fold by Pro-2 ligation, CooA activation will not occur until the CO concentration reaches micromolar levels. This desensitization may be physiologically important, because CooA activation induces synthesis of a suite of CO-metabolizing proteins, at significant energy cost to the organism. Responding to trace levels of CO in the environment would be wasteful. At the same time, positive equilibration cooperativity ensures an efficient response over a narrow range of CO concentrations once micromolar levels have been reached.

The slow rate of CO binding and the slow interconversion between the open and closed forms of CooA2(CO)2 may also be beneficial for dampening the effects of transient fluctuations in CO levels. Premature CooA activation is prevented by the slow rate of Pro dissociation, and, at the same time, deactivation of DNA-bound CooA2(CO)2 also occurs slowly, on the time scale of minutes (τ diss ~ 500 s), due to the low rate of CO dissociation from the closed form. Thus, the slow rates of Pro dissociation, and of the closed-open interconversion, act as noise filters to avoid wasteful biochemical responses to rapid fluctuations in the ambient CO levels.

Acknowledgments—We thank Dr. Alex Pevsner for carrying out a principal component analysis on the stopped-flow data and Jose Serate for excellent technical assistance.

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J. Biol. Chem. 2004, 279:21096-21108.
doi: 10.1074/jbc.M400613200 originally published online February 27, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400613200

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