Cystathionine β-synthase (CBS) 1 is a key enzyme in the regulation of homocysteine in humans (1,2). Mutations in CBS are the most common cause of hereditary homocystinuria, in which elevated plasma homocysteine levels are associated with a range of pathologies, including mental retardation and cardiovascular disease (3). CBS is a pyridoxal phosphate (PLP)-dependent enzyme, which catalyzes the condensation of homocysteine with serine to give cystathionine. In addition to the PLP cofactor, human CBS contains a heme group. The heme is not required for enzyme activity and is absent in CBS produced by yeast and protozoa (4). A fragment of the human enzyme in which the 69-residue N-terminal region is deleted, no longer binds heme, but retains ~40 % activity (4). However, the heme can play a regulatory role; the Fe(II) form of the enzyme is inhibited upon binding CO or NO (5,6), while activity is doubled when the Fe(II) is oxidized to Fe(III) (7). The redox state of the heme is pH dependent, with reoxidation of Fe(II)-CBS to Fe(III)-CBS being favored at low pH conditions (8). Although the physiological implications of this regulatory behavior in CBS are uncertain,
this protein joins the growing list of heme sensor proteins, in which enzymatic or DNA-binding activity is controlled by the ligation or redox status of the heme.

In addition to the N-terminal heme-binding domain, CBS has a C-terminal inhibitory domain; the inhibition is lifted upon binding of another regulatory molecule, S-adenosylmethionine (AdoMet) (9,10). Truncation of the AdoMet-binding domain leaves a fully active catalytic core that is less prone to aggregation than the full-length enzyme, and is useful for spectroscopic and mechanistic studies (11). Catalysis is inhibited by CO binding, as in the full length protein. The truncated protein in which the last 143 residues of the C-terminal domain have been deleted (CBS-Δ143) is dimeric (whereas the full-length protein forms tetramers and higher oligomers). The crystal structure of the catalytic core has revealed the arrangement of the heme- and PLP-binding domains, which are ∼20Å apart (Figure 1) (12,13).

In the present study, we have investigated CO binding to truncated CBS, with the aim of elucidating the mechanism of regulation by this gas. Our principal findings are that the dimer displays equilibrium anticooperativity in CO binding, that the rate of CO binding is controlled by pre-dissociation of the endogenous Cys52 ligand, and that rebinding of Cys52 is relatively slow and independent of pH, between 7.6 and 10.5, implying a perturbed pK_a for the dissociated Cys52. These results support a model in which interaction between the heme and PLP cofactors is mediated by a hydrogen bond between Cys52 and the Arg266 residue, which is connected via an intervening helix to the PLP binding site.

Materials and Methods

Enzyme purification and CO equilibrium constant measurements

The truncated dimer, CBS-ΔC143, was purified as described previously (11). The binding constants for CO for the truncated enzyme were determined exactly as described for the full-length enzyme (5).

Sample Preparation

For Raman spectroscopy, aliquots of Fe(III)-CBS protein were transferred to NMR tubes and diluted using specified buffers to achieve a typical concentration of 25 μM in heme. The tubes were sealed with rubber septa and parafilm and purged with high purity nitrogen gas for 2 h. Sodium dithionite was added to reduce the iron to the Fe(II) form under anaerobic conditions. To prepare the CO bound form of CBS, following the reduction step above, the samples were immediately purged with CO for 3-5 min. For the rapid-mixing experiments, Fe(II)-CBS for the stopped-flow experiments was prepared with the same procedure as for Raman experiments. The final concentration was 14 μM before mixing. CO saturated buffers for use in rapid-mixing experiments were prepared by first degassing with alternate application of vacuum and N_2 flushing followed by 2 h of CO purging. Solutions saturated with CO are 1mM in dissolved CO. Buffer with lower concentrations of CO was obtained by mixing the CO saturated buffer with an appropriate volume of N_2 saturated buffer. The buffers used were phosphate at pH 7.6, Tris at pH 8.6, and glycine-NaOH at pH 10.6. These latter two pH values were above those at which significant anaerobic reoxidation of Fe(II)-CBS has been observed and the reoxidation of Fe(II)-CBS is slow at pH 7.6 (8) and so did not interfere in the conversion to the CO adduct, which was stable.

Stopped-flow measurements of CO association rates

A solution of CBS (75 μl, 14 μM) in buffer (Tris, pH 8.6) was rapidly mixed with an equal volume of buffer solution (Tris, pH 8.6) containing dissolved CO, using a stopped-flow instrument (HI-TECH Scientific SF-61 DX2 Double Mixing Stopped-Flow System) with a diode array detector. The concentration of CO was varied by dilution of CO-saturated buffer.
A total of 300 scans were acquired over a period of 4500s. The spectral range observed was from 300 nm to 700 nm. All reactions were carried out at 24.5±1 °C.

Global analysis of the time-dependent changes in the spectra was carried out using MATLAB v.7.0.4 on the data from 380 nm to 650 nm.

**Time-resolved resonance Raman measurements of photo-recombination**

The set-up has been described in detail previously (14,15). 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 LBO crystal to obtain pump pulses at 419 nm. Probe laser pulses at 426 nm were generated by a second Ti:S laser with the same characteristics. The optimum pump laser power to achieve maximum photolysis (i.e., no further increase in the intensity of the ν₄ resonance Raman band of the five-coordinate heme at the earliest time) was 140 mW. Photolysis from the probe laser was minimized by keeping its power at ca. 1.0 mW. The time delay between pump and probe pulses (0.20 - 300 μs) was controlled by a DG535 delay generator.

The sample solution in an NMR tube was spun for efficient mixing of the sample. Spinning ensured that the volume sampled was replaced between probe pulses and that there was no permanent product formation.

The pump and probe beams were spatially overlapped and then focused on to the sample with a pair of cylindrical lenses. The scattered light was collected and focused onto a single spectrograph (SPEX 1269, 3600 grooves/mm) equipped with a gated IPDA (intensified photo diode array) detector. Ten scans, each of 30 s acquisition time, were averaged.

Spectra were calibrated with N, N-dimethylformamide, acetonitrile, and methylcyclohexane. Spectral deconvolution was carried out with the GRAMS/AI v.7.00 software. Parameters for curve fitting included use of a mixed Gaussian/Lorentzian (1:9) band shape and the incorporation of fixed bandwidths: 9 cm⁻¹ (1372 cm⁻¹); 12 cm⁻¹ (1354 cm⁻¹) and 11 cm⁻¹ (1361 cm⁻¹). The spectra were divided by control spectra, obtained by reversing the order of the pump and probe pulses, to compensate for any slow changes in sample composition.

**Measurement of relative Raman cross sections**

Relative Raman cross sections were determined in two ways. In one experiment, samples of Fe(II)-CBS and of its CO adduct were made at the same concentration and spectra were recorded under identical conditions. The laser power at the sample was 0.2 mW. Measured intensities of the ν₄ band were used to obtain the relative cross sections. To obtain cross section of CBS-5c relative to CBS-CO, the laser intensity was increased step-wise to cause different amounts of photolysis. The intensities of ν₄ were then used to calculate the relative cross section using a linear least squares procedure, under the constraint that total heme concentration is constant. In another experiment, CBS was reduced under nitrogen atmosphere and the spectrum of Fe(II)-CBS was recorded. Following this, the sample solution was kept under 1 atm CO for 2 h. CO binding was monitored by recording the absorption spectrum periodically. When CO binding was complete, the Raman spectrum of CBS-CO was recorded. In both experiments, intensities were determined by deconvolution of the spectrum in the ν₄ region into three bands at 1354, 1361 and 1372 cm⁻¹ as described above for the TR3 measurements.
Results

Equilibrium constants for CO binding

Equilibrium dissociation constants were measured by titration of Fe(II)-CBS with CO (not shown). Two dissociation constants are obtained: 3.9(2.0) μM and 50(8) μM, which are similar to those reported for wild-type human CBS, 1.5(0.1) μM and 68(14) μM (5). Thus, CO binding to one subunit reduces the CO binding affinity of the second subunit.

CO association rates

Figure 2 shows the spectra at various times after mixing CO with Fe(II)-CBS in the stopped-flow apparatus. The heme Soret band shifts from 449 nm in Fe(II)-CBS to 419 nm in the CO adduct. However, a side-reaction was discovered, which we attribute to a small amount of O\textsubscript{2} in the Fe(III)-CBS solution prior to dithionite addition. When the O\textsubscript{2} was allowed to increase, complete bleaching of the 449 nm Fe(II)-CBS absorption band was observed, and yet subsequent addition of CO led to complete recovery of the 419 nm Fe(II)-CBS-CO absorption band (Supporting Information, Figure S1). Presumably, radical species resulting from the reaction of O\textsubscript{2} with dithionite attack the Fe(II)-CBS heme, but the nature of the product, which reverts back to the normal CO adduct, is currently a puzzle. The extent of bleaching could be reduced, but not eliminated by a freeze-pump-thaw cycle on the Fe(III)-CBS solution.

In the stopped-flow experiments, the extent of bleaching was minor, but somewhat variable, and produced an extraneous rapid initial increase in the 419 nm absorbance due to CO reaction with the bleach product. To eliminate the influence of this artifact on the course of the CO binding to authentic Fe(II)-CBS, we applied global analysis to extract the spectral component that changed over time due to the binding of CO to Fe(II)-CBS (Figure 3a). The kinetics of the change in this spectral component (Figure 3b) could be fit to two successive exponentials with the fast phase having a much larger amplitude, on average four times greater, than the slow phase (Supporting Information, Table S1).

The successive pseudo-first-order rate constants, k\textsubscript{obs} (Table 1) differ by an order of magnitude. The fast phase was [CO] dependent and increased non-linearly with [CO], showing saturation behavior (Figure 4). This is the behavior expected when the rate of CO association is limited by the prior dissociation of an endogenous ligand (Cys52 in the case of CBS) (18). The kinetic scheme, illustrated in Figure 5, leads to the equation

\[ k_{obs} \approx k_{cys}k_{CO}[CO] / (k_{cys} + k'_{cys}[CO]) = k_{cys}[CO] / (k_{cys} + k'_{cys}[CO]) \]

(1)

when CO dissociation is much slower than binding, k\textsubscript{CO} ≪ k'\textsubscript{CO}[CO], and when Cys52 dissociation is much slower than re-binding of either ligand, k\textsubscript{cys} ≪ k\textsubscript{cys} + k'\textsubscript{CO}[CO] (16). The CO saturation limit gives the Cys52 dissociation rate constant, k\textsubscript{cys}, while the curvature is determined by the ligand rebinding rate ratio, k\textsubscript{cys}/k'\textsubscript{CO}. The fitting of equation (1) to the data for k\textsubscript{obs} in Figure 4 gives k\textsubscript{cys} = 0.166(8) s\textsuperscript{-1} (standard error in the last significant figure given in parentheses) and k\textsubscript{cys}/k'\textsubscript{CO} = 109(18) μM at pH 8.6. An independent estimate of the latter is available from flash photolysis (next section). The slow phase of the CO binding was [CO] independent and had a much smaller amplitude than the fast phase. We attribute it to a sub-population of Fe(II)-CBS molecules which are unable to bind CO, perhaps because the Cys52 ligand is not primed to dissociate (see Discussion), and requires a conformation change to do so. This conformation change is slow, occurring in hours (k\textsubscript{obs} ≈ 0.0011 s\textsuperscript{-1}, Table 1).
Ligand rebinding after flash-photolysis

Resonance Raman spectroscopy was used to assess rebinding rates for CO and Cys52 after flash-photolysis of the CO adduct, at equilibrium with 1 mM CO. The ν₄ heme resonance Raman band has distinguishable positions for the native protein (bound Cys52), 1361 cm⁻¹, the CO adduct, 1372 cm⁻¹, and the five-coordinate (5c) heme intermediate, 1354 cm⁻¹. Figure 6 shows the time evolution of the ν₄ band envelope at different delay times between pump (419 nm) and probe (426 nm) lasers. Deconvolution of this envelope into the three components is illustrated for the 200 ns delay spectrum.

The deconvoluted band areas were converted to concentrations after determining the relative cross sections. Comparison of pure solutions of CBS and the CO adduct revealed the ν₄ cross sections to be the same at 426 nm. The cross section of the five-coordinate heme photoproduct was found to be 1.7-fold larger, via a least squares fit of the intensities under the constraint of constant heme concentration.

Figure 7 shows the time evolution of the relative fractions of CBS-CO, CBS(5c) and Fe(II)-CBS. The intensities were corrected for probe laser photolysis by subtraction of the probe-only spectrum, and normalization with respect to the probe-only intensity of the CBS-CO band. Nearly 70% of the photolyzed CO recombines geminately, so that the initial fraction of CBS (5c) is ~30%. This intermediate decays and is replaced by CBS-CO and by Fe(II)-CBS, but the former grows faster than the latter. The data are well described by single exponentials, with time constants of 45(15) and 55(12) μs for CBS(5c) and CBS-CO, for pH 8.6 solutions (solid curves in Figure 7b); similar results are obtained at pH 7.6 and 10.5. The formation rate for the slowly evolving Fe(II)-CBS, is best determined from the rate difference between CBS(5c) decay and CBS-CO formation, since

\[ k_{\text{obs}} = k'_{\text{CO}} [\text{CO}] + k_{\text{cys}} \]  

The derived Fe(II)-CBS rate constants, are the same within experimental error for all these pH values (Table 2).

There is no evidence for two kinetic phases in the photo-induced rebinding data. Since geminate recombination accounts for 70% of the heme, we can not exclude the possibility that the kinetic traces are monitoring only one of the two hemes in the CBS dimer. However, the \( k_{\text{cys}}/k'_{\text{CO}} \) ratio obtained from the rebinding data, 220(170) μM at pH 8.6, is within experimental error of the value determined from the stopped-flow CO binding experiment. Thus the same elementary rates are monitored in the two experiments, one starting with Fe(II)-CBS an the other with Fe(II)-CBS-CO, suggesting equal rates for the two hemes. Table 3 lists the derived values of the rate constants for the CBS-CO system.

Discussion

Ligation Dynamics

CBS belongs to the class of heme proteins in which exogenous ligand binding requires displacement of an endogenous ligand, unlike hemoglobin or myoglobin, in which exogenous ligands bind directly to five-coordinate heme. Recently investigated proteins of this class include rice hemoglobin, rHb1 (20), in which CO replaces an endogenous histidine, and the gene activator protein CooA (17) from the bacterium Rhodospirillum rubrum, in which CO replaces the N-terminal amine group, which belongs to a proline residue.
As might be expected, the exogenous ligand affinity is diminished by the binding affinity of the endogenous ligand. Thus the equilibrium constant for CO association is much lower for these proteins than it is for myoglobin, for which values in the range of 27-41 μM^{-1} are reported (17). In CooA, a dimeric protein showing positive cooperativity, successive equilibrium constants are 0.17 and 1.25 μM^{-1} for the first and second binding steps, with a geometric mean of 0.46 μM^{-1} (18). The affinity is even lower for CBS; the equilibrium constants are 0.25 and 0.02 μM^{-1} for the first and second binding steps, with a geometric mean of 0.07 μM^{-1}. The lower mean value may reflect the extra energy required to displace an anionic ligand, relative to a neutral one. Likewise the rate constant for endogenous ligand dissociation is lower for CBS, 0.0166 s^{-1}, compared with 0.2 s^{-1} and 0.07 s^{-1} for CooA, and 1900 s^{-1} for rHb1.

The recombination rate of the endogenous ligand is faster for CBS than for rHb1, 4000 s^{-1} vs 500 s^{-1}, again as might be expected for an anionic ligand attacking the positively charged Fe (II). For CooA, two rates are deduced from the requirement for cooperative CO binding, 4000 s^{-1} and 84 s^{-1}, which bracket the rHb1 value and the faster rate is the same as the value for CBS.

The equilibrium constant for Cys52 association in CBS is calculated from the forward and reverse rate constants to be 2.4 × 10^5. This is much larger than the Pro association constants derived for CooA, 20,000 and 1200, and nearly a million times larger than the His association constant in rHb1, 0.27.

On the other hand, the rate of CO rebinding to the five-coordinate photoproduct for CBS, 18 μM^{-1}s^{-1} is in between the rates for rHb1 (6 μM^{-1}s^{-1}) and CooA (32 μM^{-1}s^{-1}). Thus the dynamical differences among these three six-coordinate heme proteins lie in the nature of the endogenous ligand being replaced.

In contrast to CooA, the equilibrium binding of CO is anti-cooperative for CBS. However, there is no evidence for kinetic inequivalence between the two binding sites of the dimer, either in CO association to Fe(II)-CBS, or in CO rebinding to photolysed Fe(II)-CBS-CO. The inequivalent equilibrium constants must therefore stem from inequivalent CO dissociation rates, which have not been measured in this study. The CO dissociation rate must diminish for the second heme when the first heme loses CO, perhaps due to an allosteric change that limits egress of CO from the heme pocket.

**Mechanism of Enzyme Regulation**

The heme group in CBS is 20 Å distant from the PLP active site, yet enzyme activity is diminished 1.7-fold when Fe(III) heme is reduced to Fe(II) (7), and is abolished altogether by CO binding to the heme (5). An allosteric mechanism is presumed to be responsible for this regulatory behavior. Helix 8 separates the heme and PLP sites (Figure 8) and is a likely candidate for an allosteric element. It has been noted that this helix supports Thr257 and Thr260 on the PLP side and Arg266 on the heme side (4). The two threonine residues donate hydrogen bonds to the PLP phosphate group, while Arg266 donates a H-bond to the Cys52 ligand. Thus the Arg266-Thr257/260 segment may act as a lever between the heme and the PLP site, modulating the activity of the latter via mechanical tension that is mediated by the hydrogen bonds at either end.

An important finding in this connection is the absence of any significant pH effect on the photodissociation dynamics. Essentially the same rebinding rates are observed when the solution pH ranges from 7.6 to 10.5 (Table 2). It might have been expected that the Cys52 thiolate would be subject to protonation after it is displaced by CO. The thiolate pKₐ is ~8.8 in aqueous solution (21). If the displaced Cys52 were protonated at pH 7.6, its recombination rate should have slowed substantially, relative to pH 10.5, but this did not happen. We conclude
that Cys52 remains unprotonated, and that its $pK_a$ is shifted below 7.6 by interactions in the binding pocket. The obvious interaction is the hydrogen-bond with Arg266, which can convert to a salt-bridge when the Cys52 thiolate anion is displaced from the positive Fe(II). This interaction can explain the otherwise surprising fact that it is the anionic Cys52 ligand which is displaced by CO, rather than the neutral His65 ligand on the other side of the heme. The Cys52 thiolate is primed for displacement by the availability of a salt-bridge interaction. We speculate that the slow conformation change, represented by the low-amplitude [CO]-independent phase of the stopped flow kinetics (above), arises from a subpopulation of Fe(II)-CBS molecules in which Arg266 is misoriented for H-bonding to Cys52, which is therefore resistant to displacement by CO.

The salt-bridge could account for enzyme inhibition if its formation reorients the Arg266 sidechain, and with it, the Arg266-Thr257-Thr260 lever. The resulting movement of Thr257 and Thr260, which are anchored to the PLP phosphate group, may misalign the pyridoxal end of the PLP, interfering with an optimal geometry for catalysis. It is possible that a more subtle shift of the Arg266-Thr257-Thr260 lever could also account for the 1.7-fold reduction in activity between Fe(III) and Fe(II) forms of the heme. The Arg266-Cys52 H-bond is expected to weaken significantly when Fe(II) is oxidized to Fe(III), because of the reduced negative charge. This weakening could shift the Arg266-Thr257-Thr260 lever slightly, inducing a suboptimal alignment of the PLP. Thus the proposed allosteric mechanism could account for heme regulation of PLP activity by both redox and ligand displacement processes.

**Physiological Implications**

Does CO binding regulate CBS activity *in vivo*? This is quite possible since the reported range of physiologically relevant CO concentrations, 3-30 μM (19), is bracketed by the two dissociation constants of human CBS, 1.5 and 68 μM (5) of full-length human CBS, implying that the reduced enzyme is normally half-saturated with CO. It has also been suggested that heme redox state is a regulator of CBS (7) since the activity doubles when the reduced form is oxidised to Fe(III)-CBS. The two effects are not mutually exclusive, since the reduction potential of Fe(III)-CBS would be raised by CO binding to Fe(II)-CBS. The physiologically relevant active and inactive states might be Fe(III)-CBS on the one hand and Fe(II)-CBS-CO on the other.

The relatively slow rate of CO binding complicates the issue. At 10 μM CO, the half-time for CO binding is expected to be 500 s from the present data on the truncated protein, and it may be affected by interactions with other proteins or with effector molecules *in vivo*. However, a slow binding rate might act as a filter to avoid unproductive responses to transient changes in local CO levels, as has been suggested for CooA (18).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Diagram of a dimer of human CBS lacking the C-terminal domain (from crystal structure PDB 1JBQ) showing the relative disposition of the heme and PLP cofactors. The two subunits are shown in green and purple respectively. The helix that connects Arg266 in the heme domain to Thr257/Thr260 in the PLP domain is highlighted.
Figure 2.
Electronic absorption spectra for Fe(II)-CBS (pH 8.6) after stopped-flow mixing with 1000 μM CO, at 8, 23, 53, 113, 218, 308, 413, 518, 608, 1013, 2018, 3023, 4013, 4493 s.
Figure 3.
Stopped-flow kinetics after mixing Fe(II)-CBS (14 μM, pH 8.6) with CO containing buffer. (a) Spectral component from global analysis that changed over time due to the binding of CO to Fe(II)-CBS. (b) Kinetic traces for the spectral component due to CO binding to Fe(II)-CBS at [CO] = 125, 200, 300, 500 μM.
Figure 4.
Observed rate constants of CO association with Fe(II)-CBS from stopped-flow experiments for the fast ($k_{ob1}$) and slow ($k_{ob2}$) phases as a function of the concentration of CO.
Figure 5.
Kinetic scheme for ligand replacement in CBS.
Figure 6.
ν₄ band region of RR spectra at the indicated times following CBS-CO photolysis at pH 8.6. The dotted curves illustrate the band deconvolution.
Figure 7.
Progress curves for CBS-CO, CBS-5c and Fe(II)-CBS populations obtained from the change in the ν₄ RR band intensities following CBS-CO photolysis, at (a) pH 7.6, (b) pH 8.6 and (c) pH 10.5 (see text for details). The solid lines are single exponential fits to the data, for CBS-5c and CBS-CO. The curves for Fe(II)-CBS are calculated from the derived rate constants (see text).
Figure 8.
Model for the proposed mechanism of CO deactivation of CBS via Cys52--Arg266 salt-bridge formation and consequent PLP displacement.
Table 1
Apparent rate constants for CO association with CBS observed in stopped-flow experiments at various concentrations of CO.

| [CO] μM | \( k_{\text{obs1}} \) (s\(^{-1}\)) | \( k_{\text{obs2}} \) (s\(^{-1}\)) |
|---------|-----------------------------------|---------------------------------|
| 500     | 0.0133(3)                         | 0.00107(4)                     |
| 300     | 0.0127(2)                         | 0.00104(4)                     |
| 200     | 0.0106(2)                         | 0.00127(6)                     |
| 125     | 0.0087(2)                         | 0.00121(6)                     |

\(^a\)Standard errors with significant figures of the last decimal place are given in parentheses.
Table 2  
Rate constants (s\(^{-1}\)) from the pump-probe Raman spectrocscopy of CBS-CO photolysis.

| pH  | CBS-5c                | CBS-CO                | CBS-Cys (calc)  |
|-----|-----------------------|-----------------------|-----------------|
| 7.6 | 1.8(0.3)\(^a\) × 10^4 | 1.32(0.16) × 10^4     | 4.6(1.4) × 10^3 |
| 8.6 | 2.2(0.7) × 10^4       | 1.8(0.4) × 10^4       | 4.0(2.2) × 10^3 |
| 10.5| 2.1 (0.3) × 10^4      | 1.49(0.16) × 10^4     | 6.4(1.6) × 10^3 |

\(^a\)Standard errors with significant figures of the last decimal place are given in parentheses.
Table 3
Rate constants for internal ligand (L) dissociation and reassociation for CBS (L = Cys) at pH 8.6 and for two other hexacoordinate heme proteins rHb1 (L = His) and CooA (L = Pro N terminus). Also listed are the rate constants for CO binding to the 5-c heme intermediate.

|                | CBS      | rHb1\textsuperscript{a} | CooA\textsuperscript{b} |
|----------------|----------|--------------------------|--------------------------|
| $k_{-1}$ (s$^{-1}$) | 0.0166(8)$\textsuperscript{c}$ | 1900 | 0.2 | 0.07 |
| $k_{+1}$ (s$^{-1}$) | 4.0(2.2) × 10$^3$ | 500 | 4.0 × 10$^3$ | 84 |
| $K_L$ | 2.4 × 10$^5$ | 0.27 | 2.0 × 10$^4$ | 1.2 × 10$^3$ |
| $k_{CO}$ (μM$^{-1}$s$^{-1}$) | 18(4) | 6.0 | 32 | 32 |

\textsuperscript{a}Reference (16), rHb1 is rice hemoglobin, the sixth ligand in rHb1 is His.

\textsuperscript{b}Reference (18); the two sets of values are identified with successive binding steps in the dimeric protein. CooA is a CO-sensing heme protein from the bacterium \textit{Rhodospirillum rubrum}.

\textsuperscript{c}Standard errors with significant figures of the last decimal place are given in parentheses.

$K_L = k_{+1}/k_{-1}$