Carbon monoxide poisoning is prevented by the energy costs of conformational changes in gas-binding haemproteins

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Carbon monoxide (CO) is a product of haem metabolism and organisms must evolve strategies to prevent endogenous CO poisoning of haemoproteins. We show that energy costs associated with conformational changes play a key role in preventing irreversible CO binding. AxCYTcp is a member of a family of haem proteins that form stable 5c-NO and 6c-CO complexes but do not form O₂ complexes. Structure of the AxCYTcp-CO complex at 1.25 Å resolution shows that CO binds in two conformations moderated by the extent of displacement of the distal residue Leu16 toward the haem 7-propionate. The presence of two CO conformations is confirmed by cryogenic resonance Raman data. The preferred linear Fe-C-O arrangement ($170 \pm 8^\circ$) is accompanied by a flip of the propionate from the distal to proximal face of the haem. In the second conformation, the Fe-C-O unit is bent ($158 \pm 8^\circ$) with no flip of propionate. The energetic cost of the CO-induced Leu-propionate movements is reflected in a 600 mV (57.9 kJmol⁻¹) decrease in haem potential, a value in good agreement with density functional theory calculations. Substitution of Leu by Ala or Gly (structures determined at 1.03 and 1.04 Å resolutions) resulted in a haem site that binds CO in the linear mode only and where no significant change in redox potential is observed. Remarkably, these variants were isolated as ferrous 6c-CO complexes, attributable to the observed eight orders of magnitude increase in affinity for CO, including an approximately 10,000-fold decrease in the rate of dissociation. These new findings have wide implications for preventing CO poisoning of gas-binding haem proteins.

sensors | crystallography | kinetics | ligand discrimination | atomic resolution

Haem is one of the most versatile protein cofactors with involvements in gas transport, electron transfer, catalysis, and signaling. Preventing the irreversible binding of CO to haem is a critical aspect of its biological functionality. As is well known, CO binding to hemoglobin can lead to eventual death in environments with excess CO, and more general poisoning of haemproteins by CO would have fatal consequences for cellular metabolism and signaling. However, CO is also a normal metabolic product and is now recognized as having a variety of physiological functions in immune regulation and antioxidant defence mechanisms (1). CO is mainly formed by the activity of haem oxygenase, a superfamily of enzymes with a wide distribution found in all three domains of life. The production of low concentrations of endogenous cellular CO requires that the protein environment of ferrous haem is such that the tight binding of CO is discriminated against.

Competing hypotheses have sought to explain why Mb is able to lower the ratio of CO to O₂ affinity to approximately 25 as compared to approximately 20,000 in bare haem (2, 3). Because of the inherent geometric preferences of Fe-C-O (linear) and Fe-O-O (bent) moieties it was initially proposed that Mb discriminated

against CO due to distal pocket steric hindrance, which created a significant energetic cost for bending the Fe-C-O unit. This hypothesis has since been downplayed for Mb in favor of preferential H-bonding to haem-bound O₂ (3). Studies of H-NOX (haem nitric oxide/oxygen sensor) proteins and the NO carriers nitrophorins have suggested that variations in haem conformation and propionate interactions may modulate haem reactivity with diatomic gases (4, 5). Furthermore, the ability of bacterial cytochrome *c'* to discriminate between CO and NO by utilizing opposite haem faces is believed to be driven by steric effects (5). The present study on AxCYTcp, a bacterial cytochrome *c'*, provides a paradigm shift in our understanding of how CO poisoning is prevented in haem proteins.

Results and Discussion

Crystallographic structures of Leu16Ala and Leu16Gly determined at 1.03 and 1.04 Å resolutions, respectively showed that the substitution of Leu16 by the less bulky residues resulted in a haem site where CO binds only in the linear mode with no shift of the haem 7-propionate. In the WT CO-bound structure determined at 1.25 Å, Fig. 1, two binding modes of CO are found[†]. The linear mode of CO binding is accommodated by the movement of Leu16 that results in the flip of haem 7-propionate from the distal to the proximal face of the haem whereas the bent conformation ($158^\circ \pm 8^\circ$ with approximately 40% occupancy) causes a smaller shift in Leu with a commensurate movement of propionate that remains on the distal face (Fig. S1). The presence of dual conformations of CO is confirmed by the resonance Raman (RR) data on cryogenically cooled solution samples but at room temperature only a single conformation consistent with a linear binding mode is observed in the RR data (Table S1). The energetic cost of this linear binding mode is reflected in a decrease in potential of approximately 600 mV on binding CO in the WT protein at room temperature. In the case of the Leu16 variants no change in redox potential on CO binding is observed demonstrating that this energy cost is directly associated with

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[†]Only a single conformation of CO was modeled without a propionate flip in the 1.96 Å resolution structure of CO-bound AxCYTcp (3).

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Fig. 1. Structural comparison of the haem sites of ferric AxCYTcp, ferrous AxCYTcp, and AxCYTcp-CO. (A) As-isolated ferric AxCYTcp at 0.84 Å resolution, note that Arg124 perpendicular to the haem plane, (B) ferrous AxCYTcp at 1.45 Å resolution (note the change in conformation of Arg124), (C) AxCYTcp-CO at 1.25 Å resolution where CO binds in two conformations resulting in the displacement of Leu16. Note that relative to the ferrous structure the haem 7-propionate has flipped to the proximal haem face in the linear binding mode for CO

conformational changes imposed by the linear binding mode in the WT protein.

Crystallographic, Spectroscopic, and Kinetic Studies of the Wild-Type and Mutant Proteins. Remarkably, both the L16A and L16G variants were isolated with CO bound to haem. Typical RR spectroscopic data for L16A are shown in Fig. 2 and Fig. S2, where RR bands sensitive to $^{12/13}\text{CO}$ substitution are compared with the authentic carbonyl complex. The structures of as isolated L16A and L16G determined to 0.95 and 0.89 Å resolution, respectively, are shown in Fig. S3. Kinetic data revealed these proteins to have an unprecedented haem-CO binding affinity, Figs. 3 and 4. For example, relative to the L16A variant, the presence of Leu16 in WT *AxCYTc*p boosts the CO off-rate constant by a factor of approximately 10,000 while lowering the on-rate constant by the same amount (Table S2), preventing irreversible haem-CO formation (vide infra). The two variants represent the case where this natural capability is removed and essentially “irreversible” CO binding occurs. To our knowledge, these variants of *AxCYTc*p are unique examples of a haem protein that is isolated as CO complexes, indicating that the protein is effectively trapped in the CO-bound state. The CO is presumed to originate from an as yet unidentified route for CO formation or atmospheric CO because the strain of *Escherichia coli* used to overexpress the proteins was derived from strain K12 that lacks heme oxygenase.

These single amino acid substitutions also changed the reactivity toward the physiologically important ligand NO, stabilizing a 6c-NO distal adduct (Fig. 4), that in WT *AxCYTcp* is normally only a transient species in the formation of the proximal 5c-NO species. Thus, as well as a role for Leu16 in preventing irreversible haem-CO formation in *AxCYTcp*, the current data support a role in reversible binding of the physiological ligand, NO. This represents a clear-cut and stunning example of how conformational change in haem-centers and the associated potential energy costs can drive ligand release. This strategy may be employed more broadly in haem sensor proteins, for example to transiently respond to the signaling effectors CO, NO.

To unambiguously assign potential effects of CO binding from those associated with redox state we determined high resolution crystal structures for the ferric, ferrous, and the 6c-CO complex of AxCYTep at 0.84, 1.45[±], and 1.25 Å (Table 1 and Table S3) resolution respectively, (Fig. 1A–C). The overall structures are very similar and only the salient features of these structures related to CO binding are discussed here. The heme environment parameters are shown in Table 1. Comparison of the oxidized and reduced structures (Fig. 1A and B) shows no significant changes

Fig. 2. Identification of CO as the distal haem ligand of “as-isolated” L16A from the equivalence of RR vibrations with preformed L16A–CO. Correspondence of the low-frequency room-temperature RR spectrum of as-isolated L16A AxCYTcp (A) with that of its carbonyl complex prepared with ^{12}CO (B). Substitution of the carbonyl complex with ^{13}CO (C) identifies vibrations of the Fe–CO moiety from the ^{12}CO – ^{13}CO difference spectrum. The match of the as-isolated L16A AxCYTcp spectra to the carbonyl complex, in particular the 487-cm^{-1} RR vibration attributable to $\nu(\text{Fe}=\text{CO})$, confirm that CO is the distal ligand.

in the haem, only the change in conformation of Arg124 noted in the earlier study at lower resolution. Relative to its 5-c ferrous state the structure of the *Ax*CYTcp 6c-CO adduct shows that binding of CO causes conformational changes not only in Leu16, as was shown previously (6), but also in the haem 7-propionate group (Fig. 1 *B* and *C*). The CO molecule binds at full occupancy, but in two conformations, to the distal side of the haem resulting in a change in haem conformation from bent to planar. The major Fe-CO conformation (60%) is linear, and the minor (40%) is bent and tilted. In both binding modes the C to Fe distance is 1.73 ± 0.05 Å, forcing the C_{α} - C_{β} bond of Leu16 to adopt two conformations, with 120° and 34° rotation away from its original position (Fig. S1 *A* and *B*). The 120° rotation of Leu16 is associated with the linear binding mode with a bond angle of $170 \pm 7^\circ$ (Table 1) and a flipping of the 7-propionate side-chain

Fig. 3. Reaction of ferrous L16A with CO. Absorption spectra showing the reaction of ferrous L16A (solid line) with 63 μ M CO at 25 $^{\circ}$ C to give the CO complex (dotted line). The *Inset* shows the time course at 420 nm. Overlaid, and mostly obscured, is a single exponential fit.

^aThe slightly lower resolution of ferrous form is due to data being collected on the home-based FR-E+ laboratory source.

Fig. 4. Kinetics and spectral changes associated with dissociation of CO from L16A AxCYT_{cp} in the presence of NO. The release of CO from L16A AxCYT_{cp}–CO was measured in the presence of 1 mM NO at 25 °C to determine the rate constant. Absorption spectra show the reactant (CO complex: λ_{max} 418, 535, 566 nm) and the product (NO complex: λ_{max} 416, 542, 574 nm). The inset shows the 418-nm time trace, along with a single-exponential fit. The presence of NO causes the conversion of the 6c–CO complex (λ_{max} 418 nm) into a species characteristic of a 6c–NO complex (λ_{max} 416 nm). This is in marked contrast to the behavior of native AxCYT_{cp}, where the 6c–NO complex is a transient species in the formation of a stable 5c–NO product (λ_{max} 395 nm).

shifting to the proximal side of the haem to stack against Arg124 that is perpendicular to the haem plane. The second Leu16 conformation is associated with a smaller rotation of the 7-propionate. Significantly, this conformer exhibits a significantly bent Fe–C–O unit with a bond angle of $158 \pm 8^\circ$ (Table 1) comparable to that found in a variety of haemoproteins at atomic resolution structures (7–12).

The crystal structure of WT *Ax*CYTcp-CO (obtained at 100 K) shows two Fe-C-O conformers, but room-temperature RR and kinetic studies are consistent with a single Fe-C-O conformation. To investigate the effect of temperature on haem speciation, additional RR measurements were recorded on frozen solutions of *Ax*CYTcp-CO and L16A-CO protein at approximately 100 K to match the crystallographic sample temperatures. Cooling *Ax*CYTcp-CO to 100 K produced changes in its RR spectrum of $^{12}/^{13}\text{CO}$ -sensitive bands compared to previously reported room-temperature data, consistent with the appearance of dual Fe-CO conformations (Fig. S4, Fig. S5, and Table S1). Spectral deconvolution reveals two $\nu(\text{Fe-CO})$ modes at approximately 493 and approximately 482 cm^{-1} , and two $\delta(\text{Fe-C-O})$ modes at approximately 580 cm^{-1} and approximately 570 cm^{-1} . We assign the approximately 493- cm^{-1} $\nu(\text{Fe-CO})$ mode to the approximately linear Fe-C-O conformer in the *Ax*CYTcp crystal structure (because its frequency is close to that of L16A-CO; see Fig. S5 C and D), whereas the approximately 482 cm^{-1} component is ascribed to the bent Fe-C-O conformer. Thus, at room

temperature, the single Fe–C–O conformer observed in RR spectra of AxCYTcp–CO is assigned to the linear Fe–C–O species. In contrast, cooling L16A–CO, from room temperature to 100 K produced only minor changes in its RR spectrum suggesting that L16A–CO at room temperature retains the single linear Fe–C–O conformer evident in the 100-K crystal structures of the variants. The fact that only a single haem–CO species is observed at room temperature accounts for the observed monophasic kinetics of CO binding and release (see below), and suggests that it is the structure of the linear Fe–C–O conformer that governs these kinetic rates and is responsible for discrimination against CO at physiological temperatures. For 6c–CO complexes of WT and L16A AxCYTcp, a single predominant $\delta(C_{\beta}C_cC_d)$ RR mode is apparent at approximately 385 cm^{-1} at both room temperature and 100 K (Fig. 2 and Figs. S4 and S5), suggesting that propionate flipping does not significantly affect this vibration. The relatively high frequency of the $\delta(C_{\beta}C_cC_d)$ mode in AxCYTcp is attributed to H-bonding/electrostatic interactions with the propionate groups.

The structures of the “as isolated” L16A and L16G variants (Fig. S3 A and B) revealed that the distal ligands were a mixture of H₂O/CO with 0.5/0.5 (L16A) and 0.3/0.7 (L16G) occupancies, respectively. There is also a noncoordinated water, (W2), in the distal pocket with 0.5 (L16A) and 0.3 (L16G) occupancy. To unequivocally define the CO binding mode and potential changes in protein structure, the structures of the pure CO-bound species were determined. To obtain structures with full occupancy of CO, crystals of the as-isolated variants were either directly saturated with CO or by ascorbate-treatment of ferrocyanide oxidized crystals prior to saturation with CO before data collection. The structures of the L16A–CO (Fig. 5A) and L16G–CO adducts (Fig. 5B) prepared in this way at 1.03 and 1.04 Å resolution respectively showed that CO bound at full occupancy in a single linear mode with no shift of the haem 7-propionate. The removal of conformational restraint associated with Leu16 allows an essentially colinear geometry of Fe–C–O where the angle is $170^\circ \pm 1.8^\circ$ and $177.5^\circ \pm 1.5^\circ$ for L16A and L16G respectively (Table 1). The shape of the 6c haem of these CO-bound species is slightly bent away from the distal side with average Fe–N (pyrrole) distances of 2.01 and 2.04 Å.

To understand how the L16 variants perturbed haem-CO reactivity, rate constants for ligand dissociation (k_{off}) and association (k_{on}) were determined at 25 °C, enabling CO affinities to be quantified from the dissociation constant, $K_D = k_{\text{off}}/k_{\text{on}}$. Stopped-flow measurements of CO binding to L16A and L16G revealed monophasic reactions in which the ferrous haem center (λ_{max} 420, 432_(sh) nm) converts to a 6c-CO product (λ_{max} 418 nm) with no detectable intermediates. Typical data for L16A are shown in (Fig. 3). The pseudo-first-order rate constants, k_{obs} , exhibit a linear dependence on CO concentration within the range measured (63–500 μM), yielding a bimolecular rate constant, $k_{\text{on}} = 1.1(\pm 0.1) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $0.51 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for L16A and L16G respectively (Fig. S6 A and B). The negligible ordinate-intercept indicates that the back reaction (k_{off}) is relatively slow, consistent with direct measurements of k_{off} (vide infra).

Table 1. Heme environment parameters

	Resolution (Å)	Fe–CO	Fe–Ne2 (H120)	Fe–Np	Fe–C–O (°)
WT ferric AxCYTcp	0.84	—	2.09 ± 0.01	2.06 ± 0.01	—
WT ferrous AxCYTcp	1.45*		2.17	2.00–2.07	
6c–CO AxCYTcp	1.25	1.73 ± 0.05	2.02 ± 0.02	2.06 ± 0.02	170 ± 8 & 158 ± 8
L16A–6c–CO	1.03	1.79 ± 0.02	2.02 ± 0.01	2.02 ± 0.01	170.01 ± 1.76
L16A–ASC–6c–CO	1.05*	1.73	2.06	1.99–2.04	175.03
L16G–6c–CO	1.04	1.84 ± 0.03	2.02 ± 0.02	2.05 ± 0.02	177.55 ± 1.51

In all cases other than those indicated by *, SHELX unrestrained refinement was undertaken for ESD calculations.

Fig. 5. Structures of the haem sites of the CO adducts of L16A and L16G variants of AxCYTc. The structures of A) the L16A-CO and B) L16G-CO adducts at 1.03 and 1.04 Å resolution respectively showed that CO binds at full occupancy in a single linear mode with no shift of the haem 7-propionate.

The rate constant (k_{off}) for CO release from L16A and L16G was determined by ligand replacement with NO¹³. The presence of NO causes the conversion of the 6c-CO complex (λ_{max} 418 nm) into a species characteristic of a 6c-NO complex (λ_{max} 416 nm) (13) shown for L16A in Fig. 4. This is in marked contrast to the behavior of native *Ax*CYTep, where the 6c-NO complex is a transient species in the formation of a stable 5c-NO product (λ_{max} 395 nm) (14). The rate of release of CO from the L16A and L16G 6c-CO complexes is monophasic and unusually slow with $k_{\text{off}} = 3.7(\pm 0.6)10^{-6} \text{ s}^{-1}$ and $7.4(\pm 0.1)10^{-6} \text{ s}^{-1}$, respectively). The $k_{\text{off}}/k_{\text{on}}$ ratio for L16A and L16G yields a haem-CO dissociation constants, $K_D = 3.4 \times 10^{-12} \text{ M}$ and $1.5 \times 10^{-11} \text{ M}$, respectively (Table S2), which, to our knowledge, are the highest reported CO affinities of any haem protein or model porphyrin system. The avid nature of CO binding to these variants of *Ax*CYTep explains why the proteins are isolated as the carbonyl complex.

Comparative kinetic measurements of CO binding (Fig. S7A) and release (Fig. S7B) revealed monophasic reactions in AxCYTp. The pseudo-first-order rate constants, k_{obs} , exhibited a linear dependence on CO concentration within the range measured (63–500 μM) (Fig. S6C), with $k_{\text{on}} = 101 \text{ M}^{-1} \text{ s}^{-1}$ and with

a $k_{\text{off}} = 0.028 \text{ s}^{-1}$, yielding $K_D = 2.8 \times 10^{-4} \text{ M}$. Thus the presence of Leu16 in native protein lowers the CO affinity by a remarkable eight orders of magnitude relative to the L16A variant, preventing poisoning by CO, and allowing the reversible binding of its physiologically relevant NO ligand. It is particularly interesting that the L16 mutations cause k_{off} to decrease by four orders of magnitude relative to native *Ax*CYTcp. This is entirely opposite to their effect on the k_{on} value that increases by four orders of magnitude (Table S2).

Energy Potential for CO Binding. To determine whether the electronic (potential) properties of the haem contribute to the much higher CO affinity of L16A and L16G, the oxidative redox behavior of these variants were compared with that of WT *Ax*CYTcp in the presence and absence of CO. For WT *Ax*CYTcp in the absence of CO, haem oxidation, indicated by replacement of the 5c-Fe²⁺ Soret absorption (λ_{max} 426 nm) with that of the 5c-Fe³⁺ state (λ_{max} 404 nm), occurred only above +160 mV (NHE) (Fig. 6). Remarkably different behavior was observed in the presence of CO. As the potential was increased the characteristic 6c-CO Soret absorption (λ_{max} 418 nm) evident at -300 mV (NHE) was progressively replaced by the absorption spectrum of the reduced 5c protein (Fig. 6). These changes continued until +300 mV, whereupon an absorption increase at 404 nm indicated the onset of haem oxidation. This behavior indicates that the potential of the WT 6c-CO adduct is sufficiently low that following oxidative loss of CO the haem is rereduced and the 5c-Fe²⁺ state remains until the potential reaches +300 mV. These data indicate that the binding of CO to *Ax*CYTcp is associated with an energy cost equivalent of approximately 600 mV potential.

In marked contrast to Δx CYTcp, the 418-nm Soret peaks of the L16A-CO and L16G-CO adducts were retained during oxidative titrations until the potential reached +350 and +265 mV respectively. Above this potential the absorption of the ferric L16A and L16G haem (λ_{max} 404 nm) was the dominant feature and no features assignable to ferrous species were observed (representative spectra for L16A are shown in Fig. 6). This behavior indicates that the L16A and L16G substitutions increase

Fig. 6. Spectral changes observed during progressive oxidation of WT AxCYTcP in the presence and absence of CO and for the L16A variant in the presence of CO. Representative spectra during oxidative titration are shown. Arrows indicate the change in peak intensity as the potential is increased by the addition of ferricyanide over the potential range indicated. Note that spectral features characteristic of ferrous haem are revealed (peak b) as CO is oxidatively released from the WT AxCYTcP, indicated by the decrease in the 418 nm peak. As the potential is increased further, oxidized haem (peak a) is observed. This is not apparent in the case of the L16A variant, where as the 418 nm peak decreases, peak b is not seen, and only the oxidized species (peak a) is observed.

Fig. 7. The amplitude dependence of absorption spectra as a function of potential for as-isolated L16A and WT Ax CYTc in the presence and absence of CO. (A) The amplitude of the absorbance maxima of WT (426 nm), WT plus CO (418 nm), L16A (418 and 404 nm) during oxidative titration. These wavelengths correspond to the CO adduct (418 nm), oxidized L16A (404 nm) and reduced WT protein (426 nm). In the case of L16A the open squares correspond to potentials where multiple species contribute to the 418 nm absorbance. The lines are interpolation to the data points. (B) A potential energy profile for the release of CO from WT and the L16A variant and the oxidation of reduced CO-free WT protein. The L16A_{red} species (shown boxed) is not observed in these titrations due to its oxidation potential being lower than that required for CO release. Color-coding for B is the same as for potential curves in A.

the potential of the haem-CO adduct by approximately 600 mV and approximately 515 mV corresponding to 57.9 and 54.5 kJmol⁻¹ respectively, such that when CO is released on oxidation of the haem, the potential is sufficiently positive that rereduction of the haem does not occur. Thus these mutations remove much of the energy cost associated with CO binding when compared to WT AxCYTc, shown in Fig. 7A and schematically in Fig. 7B. The energy cost of linear binding mode in WT clearly results from the movement of Leu and more importantly from the propionate flip into the proximal pocket.

Density functional theory calculations performed using the B3LYP functional and 6-31(d) basis set including a solvation model on WT structures of the linear and bent CO binding modes (Fig. S8) showed the movement of L16 residue in distal pocket and concomitant flipping of the 7-propionate group results in a calculated change in reduction potential of 517 mV.

Concluding Remarks. We show that increasing the “space” in the distal pocket of the haem in AxCYTc by protein engineering of Leu16 increases haem-CO affinity by a remarkable eight orders of magnitude thus resulting in an essentially irreversible binding of CO. We conclude that an increase in k_{off} results from the coupling of conformational change (including propionates) to potential energy costs as demonstrated here for AxCYTc. This identifies a mechanism by which haem-dependent proteins are protected from CO poisoning at low (physiological) concentrations of CO. Similar mechanisms coupling the energetics of structural change with gas release may have broad implications for the functioning of a wide variety of haem systems including haem-based sensors (15, 16).

Materials and Methods

L16A (L16G) Construction. The L16A mutation in AxCYTc was introduced into *E. coli* using the plasmid pTCP-1 as a template carrying the primers L16A_F5'-CAGTCG GCGGCCACGCTGATG-3' and L16A_R5'-CATCAGCGTGGCCGCCGAC-CTG-3'. The L16G_F5'-GTACCGCCAGTCGCGCGGACGCTGATGGCTCGCAC-TTCGGC-3' and L16G_R5'-GCCGAAGTGCAGGCCATCAGCGTGCCGCCGAC-TGGCGGTAC-3', growth of the organism and protein purification used the methods described previously (17). In contrast to the brown color of WT AxCYTc the L16A and L16G variants were cherry-red in color on isolation.

Crystallization and Refinement. Pyramidal shaped crystals were grown from “as-isolated” cherry-red L16A and L16G and brown WT protein. These were obtained at room temperature after 2–7 days by the hanging drop vapor diffusion method, using 2 μ L of 18–24 mg/mL protein in 50 mM Tris-HCl, pH 7.5 with an equivalent volume of reservoir solution containing 50 mM Hepes at pH 7.5 and 2.1 M ammonium sulfate. AxCYTc-CO was produced

by anaerobic soaking crystals in 100 mM ascorbate, 2.4 M ammonium sulfate, and 50 mM Hepes pH 7.5 for 15 min followed by CO saturated solution for a further 30 min. Prior to X-ray data collection all crystals were transferred into 40% sucrose in 2.2 M ammonium sulfate and 50 mM Hepes buffer pH 7.5 for 10 s. In case of ferrous and CO complexes cryoprotectant solutions were deoxygenated. To obtain full occupancy of CO in L16A and L16G crystals of as-isolated or ferrocyanide reoxidized and then ascorbate treated crystals were soaked in CO saturated 2.4 M ammonium sulfate and 50 mM Hepes pH 7.5.

Spectroscopy. RR spectra were recorded at room temperature using 413.1 nm excitation as described previously (18, 19). A laser power of 5 mW (measured at the sample) was used for the preformed 6c-CO complex. The L16A(G) 6c-CO complex (prepared by the reaction of CO with ferrous protein) was prepared by first oxidizing the as-isolated protein to the ferric state with 2,000-fold excess of ferricyanide solution. Excess oxidant was removed from the ferric protein using a P6-DG desalting column. Solutions of ferric L16A(G) were reduced to the ferrous state inside an anaerobic glove box with a five-fold excess of 2-mM sodium dithionite, followed by the removal of reductant using a minispin desalting column (Zeba filter, Pierce). The ferrous protein was then transferred to an anaerobic septum-sealed capillary tube, and the haem-CO complex prepared by introduction of gas (either ¹²CO or ¹³CO) into the headspace using a gas-tight Hamilton syringe. The identity of RR samples was verified by UV-vis spectroscopy before and after exposure to the laser beam using a modified Cary 50 spectrophotometer. A noticeable difference between the WT and L16A(G) samples is that the 6c-CO complex of the L16A(G) variant is much less susceptible to photodissociation in the laser beam. For room-temperature samples (with a 1-atm CO headspace), the 6c-CO RR spectrum of L16A(G) AxCYTc was obtained using 5-mW laser power without any detectable photodissociation, whereas previous RR spectra of the native AxCYTc 6c-CO complex required a lower laser power (<0.5 mW), as well as a reciprocating translation stage, to prevent loss of CO (19). Because the headspace of the as-isolated protein sample did not contain added CO gas, a lower laser power (0.05 mW) and reciprocating translation stage were used to minimize photodissociation. Frequencies were calibrated relative to indene, CCl₄, CD₃CN, NO_(g), CO_(g), and O_{2(g)} as standards, and are accurate to ± 1 cm⁻¹.

Kinetic Studies. Rate constants for CO binding to ferrous L16A and L16G AxCYTc were determined using an Applied Photophysics SX18MV-R stopped-flow spectrophotometer (dead time approximately 1 ms) housed within an anaerobic glove box (Coy Laboratory Products Inc.). A solution of ferrous protein (approximately 4 μ M haem) in buffer [50 mM N-Cyclohexyl-2-aminoethanesulfonic acid (CHES), pH 8.9, containing 0.1 M NaCl] was rapidly mixed with an equal volume of buffer containing dissolved CO, and the reaction monitored with monochromatic light (420 or 435 nm) using a photomultiplier detector. Solutions of CO were prepared by equilibrating CO gas or mixtures of CO and N₂ with buffer at 25.0 °C. The concentration of 1 atm aqueous CO solution was taken to be 1.0 mM (13, 20). Concentrations of dissolved CO (63–500 μ M) were maintained in at least 10-fold excess over

the haem binding sites to ensure pseudo-first-order conditions. Values of pseudo-first-order rate constants were determined from single exponential fits of time courses, and are the average of 3–5 separate kinetic runs. The rate constant for CO release from AxCYTcp was determined by time-resolved optical absorbance measurements using a Cary 50 UV-visible spectrophotometer equipped with a temperature-controlled cuvette holder. Solutions were prepared in 50 mM CHES pH 8.9 containing 0.10 M NaCl, and all kinetic measurements were conducted at 25.0 °C. The CO dissociation rate constant, k_{off} , was determined by ligand replacement with NO, as previously described (13). Typically, a small volume (approximately 5 μL) of concentrated 6c-CO complex (0.5–1.0 mM in haem) was transferred to an anaerobic cuvette containing 700 μL of NO-saturated buffer. Release of CO was monitored by the rate of disappearance of the 418-nm 6c-CO peak or the appearance of the haem-NO absorption. Rate constants were insensitive to variations in the concentration of NO (0.5–2.0 mM).

Electrochemistry. Redox titrations to determine potentials for the heme cofactor were carried out in an anaerobic glovebox (Belle Technology) under a nitrogen atmosphere, with oxygen levels maintained at <5 ppm, as described previously (21). Protein samples (typically 5–10 μM), in the presence of 2 μM phenazine methosulfate, 5 μM 2-hydroxy-1,4-naphthoquinone, 0.5 μM methyl viologen, and 1 μM benzyl viologen as mediators, were titrated with sodium dithionite as a reductant and potassium ferricyanide

as an oxidant. Absorption changes were monitored via a fiber optic absorption probe (Agilent Technologies) immersed in the protein solution and connected to a Cary UV-50 Bio UV-visible spectrophotometer (Agilent Technologies). Potentials were measured using a Hanna pH 211 m coupled to a Pt/calomel electrode (ThermoRussell Ltd.) at $25 \pm 2^\circ\text{C}$. The electrode was calibrated using the $\text{Fe}^{3+}/\text{Fe}^{2+}$ EDTA couple as a standard (+108 mV). A value of +244 mV was used to correct relative to the standard hydrogen electrode. Data were analyzed by plotting the absorbance at the stated wavelength and the midpoint reduction potential for the heme was determined using a single electron Nernst function.

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