Altering the Specificity of CooA, the Carbon Monoxide-Sensing Transcriptional Activator: Characterization of CooA Variants That Bind Cyanide in the Fe^{II} Form with High Affinity†

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ABSTRACT: CooA is a carbon monoxide- (CO-) sensing homodimeric heme protein that activates the transcription of genes required for the anaerobic oxidation of CO to CO_2 in the phototrophic bacterium *Rhodospirillum rubrum*. In this study, we demonstrate that mutational alteration of the histidine residue (His^{77}) that serves as a heme ligand in the Fe^{II} form of CooA allows high-affinity binding of cyanide ($K_d \sim 0.4$ mM) to the heme. In contrast, neither these same variants in the Fe^{III} form nor wild-type CooA in either oxidation state was able to bind cyanide even at high concentrations (50 mM). Examination of the pH dependence of spectral changes upon addition of cyanide suggested that the cyanide anion coordinated the heme iron. In addition, the UV−visible absorption spectrum of H77Y Fe^{II} CooA without added effectors is also pH-dependent, suggesting that an ionizable amino acid has become solvent-accessible in the absence of His^{77}. Finally, we demonstrate that the transcriptional activity of H77Y CooA shows a small (1.4-fold) increase in the presence of cyanide, suggesting that the binding of cyanide to this variant promotes the active conformation of H77Y CooA.

The sensing of, and response to, dissolved gaseous molecules by proteins is of considerable biological interest (*1*, *2*). The sensing of nitric oxide (NO)[1] by soluble guanylyl cyclase (sGC) plays an important role in a variety of physiological responses, including smooth muscle vasodilation and neurotransmission (*3*−*5*), while the oxygen (O_2) sensor FixL is involved in the regulation of nitrogen fixation in rhizobia (*6*, *7*). Carbon monoxide (CO) is a metabolic product of the heme degradation pathway (*8*); although CO also binds to sGC, full activation of sGC in the presence of CO alone has not been observed in vitro (*9*). Recent evidence suggests that CO may control the proliferation of hypoxic vascular smooth muscle cells (*10*) and may be involved in intestinal neurotransmission (*11*), suggesting a direct physiological role for this highly toxic gas. It remains unresolved if sGC is the CO sensor that mediates these events in eukaryotic signaling pathways.

Many bacteria have the ability to oxidize CO to CO_2 by an enzyme termed carbon monoxide dehydrogenase (CODH). The purple, non-sulfur, phototrophic bacterium *Rhodospirillum rubrum* can utilize CO as a sole energy source and possesses a CO-inducible CODH that is involved in the dark, anaerobic oxidation of CO to CO_2 (*12*). The gene products required for growth on CO as a sole energy source are encoded by two separately transcribed operons, *cooMKLXUH* and *cooFSCTJ*, and are expressed under the control of a transcriptional activator, CooA. CooA binds CO and, as a result, becomes competent to bind to its cognate promoters in the *coo* operons (*13*, *14*).

CooA belongs to the family of transcriptional regulators that includes the cyclic AMP receptor protein (CRP) and the related fumarate−nitrate reductase activator protein (FNR) (*14*). CooA has been purified from *R. rubrum* (*15*) and from CooA-expressing strains of *Escherichia coli* (*16*, *17*) and contains a *b*-type heme. UV−Visible spectroscopy revealed that CooA is similar to the *b*-type cytochromes in that the ferric (Fe^{III}), ferrous (Fe^{II}), and ferrous−CO (Fe^{II}−CO) forms are low-spin and hexacoordinate (*15*). Because no spin-state transitions occur upon CO binding to CooA [in contrast to the oxygen sensor, FixL (*18*)], an internal protein ligand is likely to be displaced in CooA upon CO binding (*15*). The displacement of this internal protein ligand is believed to trigger a conformational change in CooA that ultimately leads to the capacity to bind DNA in response to CO (*15*), although the molecular mechanism remains unclear.

Electron paramagnetic resonance (EPR) spectroscopy of Fe^{III} CooA revealed that the heme is ligated to the protein through a thiol or thiolate linkage (*17*), presumably by a Cys residue. Recent mutational and spectroscopic studies identified Cys^{75} as one of the axial ligands of Fe^{III} CooA and indicated that a novel redox-mediated ligand switch to His^{77} occurs upon reduction of the heme iron to the Fe^{II} form (*19*−

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[1] Abbreviations: CO, carbon monoxide; NO, nitric oxide; CN, cyanide; CODH, carbon monoxide dehydrogenase; EPR, electron paramagnetic resonance; sGC, soluble guanylyl cyclase; CRP, cyclic adenosine monophosphate receptor protein; FNR, fumarate-nitrate reductase activator protein; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HRP, horseradish peroxidase; MCD, magnetic circular dichroism.

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22). In addition, an interaction between Cys^{75} and His^{77} has been proposed to influence the redox chemistry of the ligand switch (*20*). However, the identity of the ligand trans to both Cys^{75} and His^{77} in the Fe^{III} and Fe^{II} forms, respectively, remains unknown.

Alteration of His^{77} to a variety of amino acids yields CooA variants that bind CO in the Fe^{II} form (*20*, *21*) yet are unable to bind DNA and activate transcription in a CO-dependent manner (*20*). We have recently demonstrated that even though H77Y CooA binds CO, this variant does not undergo the proper conformational change that is presumably necessary to position the DNA binding domain to enable transcriptional activation (*20*). In contrast to wild-type Fe^{III} and Fe^{II} CooA and to H77Y Fe^{III} CooA, H77Y Fe^{II} CooA was found to coordinate imidazole (*20*), suggesting that this variant either has a relatively solvent-accessible Fe^{II} heme iron or possesses an axial ligand that is more easily displaced by exogenous imidazole.

Because H77Y Fe^{II} CooA binds imidazole, we investigated the possibility that CooA variants altered at His^{77} could coordinate other heme-site ligands such as cyanide. Cyanide binds readily to many Fe^{III} heme proteins, although cyanide binding to Fe^{II} heme proteins is less common. For five-coordinate Fe^{II} heme proteins such as sperm whale myoglobin, relatively high concentrations of cyanide under basic conditions are required because of the inherent instability of the cyanide−heme Fe^{II} complex (*23*, *24*). Cyanide coordination to six-coordinate Fe^{II} heme proteins is rare, as there is an additional energetic barrier that involves deligation of an existing metal−axial ligand bond as a prerequisite for cyanide binding. However, Fe^{II} heme proteins that possess relatively stable Fe^{II}−CN adducts have been reported and include horseradish peroxidase (*24*), mixed-valence state cytochrome *c* oxidase (*25*), lactoperoxidase (*26*), microperoxidase (*27*), and the hemoglobin of the mollusc *Scapharca inaequivalvis* (*28*). Due to the lack of detailed structural information of these ferrous cyanide complexes, there does not appear to be a common theme that governs high-affinity cyanide binding to these proteins. However, in *S. inaequivalvis* hemoglobin the lack of polarity in the heme pocket is believed to be a major factor in the stabilization of the bound cyanide (*28*).

In the present study, we demonstrate that a variety of position 77 variants of Fe^{II} CooA coordinate cyanide with high affinity. This behavior is in contrast to that of wild-type CooA, which does not coordinate cyanide in either the Fe^{III} or Fe^{II} form. We have also found that H77Y CooA has altered effector response properties. Although H77Y CooA does not show detectable transcriptional activation in the presence of CO, a small but reproducible response to cyanide is seen. Possible mechanisms of cyanide-specific activation of H77Y CooA are discussed in terms of currently proposed mechanisms of CO-specific activation of wild-type CooA.

MATERIALS AND METHODS

Strains and Plasmids. The construction of strains bearing wild-type CooA (UQ1421) and CooA variants at position 77 in an *E. coli* overexpression system and of strains bearing wild-type CooA and CooA variants with a β-galactosidase reporter system have been described previously (*29*).

Purification of Wild-Type CooA and CooA Variants. The purification of wild-type CooA and the H77Y and H77F variants (to >95% homogeneity) were performed as described previously (*20*). The heme content of CooA preparations was quantified by the reduced pyridine-hemochromogen method (*30*).

UV−Visible Absorption Spectroscopy and Cyanide Binding. Purified H77Y CooA was diluted to ~ 5 μM (in heme) into an appropriate buffer (see below), transferred to a 1-cm path length quartz cuvette fitted with a rubber stopper, and then made anaerobic by repeated degassing and argon flushing by use of a manifold. An anaerobically prepared sodium dithionite (Fluka) solution was then added to a final concentration of 2 mM. Recently purchased potassium cyanide (KCN; Sigma) was prepared fresh before use in anaerobic distilled water.

Anaerobic titrations of KCN to H77Y Fe^{II} CooA were obtained at a variety of pH values in the following buffers (at their respective final concentrations): pH 7.0, 100 mM MOPS; pH 8.0, 100 mM Tris-HCl; pH 9.0, 100 mM Tris-HCl; and pH 10.0, 100 mM glycine/NaOH. Previous studies (*20*) indicated that wild-type CooA and CooA variants altered at position 77 are stable at high pH values (pH 10). Because KCN produces CN^-, a strong conjugate base ($pK_a = 8.6$), upon dissolution, pH fluctuations during the KCN titrations were minimized by use of relatively high buffer concentrations. Moreover, only small concentrations of KCN (as low as 0.1 mM) were required to elicit spectral changes in position 77 Fe^{II} CooA variants (see Results). UV−Visible absorption spectra were obtained at room temperature on a Shimadzu UV-2401PC spectrophotometer (slit width = 0.5 nm). The time of cyanide equilibrium for the lowest concentration of cyanide (0.06 mM) was approximately 10 min (data not shown); therefore samples were incubated for 10 min at room temperature after each cyanide addition before the spectrum was acquired. For the binding curve analyses, absorbance data were reported as normalized absorbance, where the Soret absorbance (e.g., 423 nm at pH 10; see Results) of H77Y Fe^{II} CooA with no cyanide additions was set to $\alpha = 0$ and the maximum absorbance change was set to $\alpha = 1.0$. Binding constants were calculated by Hill analysis, thereby assuming either 100% unliganded ($\alpha = 0$) Fe^{II} CooA or 100% cyanide-liganded ($\alpha = 1.0$) Fe^{II} CooA from the generated saturation curve (see Results).

In Vivo Activation of CooA Variants with Cyanide. In vivo β-galactosidase activity in an *E. coli* reporter system was monitored as a function of added KCN by a modification of the procedure described previously (*20*). Briefly, 200 μL of a saturated culture of strains containing plasmids of wild-type *cooA* or its variants in the reporter background was inoculated into 2 mL of an induction medium, modified from that described (*31*), that contained the following (final concentrations): 80 mM MOPS, pH 7.7, 4 mM Tricine, 0.05% tryptone, 0.1 mM $FeSO_4$, 14 mM NH_4Cl, 5.5 mM $MgSO_4$, 0.5 μM $CaCl_2$, 50 mM NaCl, 10 mM $NaHCO_3$, 80 mM glucose, 0.88 KH_2PO_4, and 10 μM thiamine. The medium was supplemented with the following trace metals at their respective final concentrations: 10 μM $CuSO_4$, 3 μM $CoCl_2$, 80 μM $MnCl_2$, 10 μM $ZnSO_4$, 0.1 μM Na_2MoO_4, 1 μM Na_2SeO_4, and 1 μM $NiCl_2$. Cultures were made anaerobic and then an anaerobic stock solution of KCN was added to final concentrations varying from 0 to 200 μM. Cultures were then incubated with shaking at 30 °C for approximately 6−8 h until they reached an $OD_{600\ nm}$ of ~1.0.

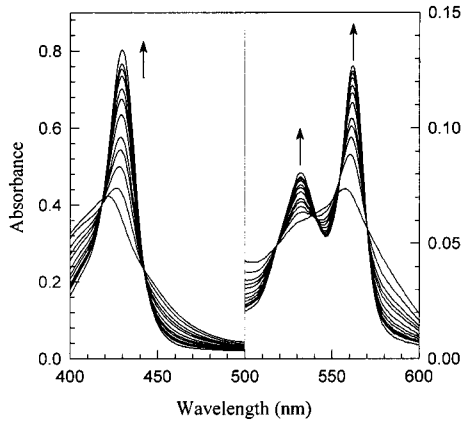

FIGURE 1: Large spectral changes are observed for H77Y Fe^{II} CooA in the presence of cyanide. UV−Visible absorption spectra of ~5 μM H77Y Fe^{II} CooA in 100 mM glycine/NaOH, pH 10.0, upon titration of increasing amounts of exogenous KCN are shown. Arrows depict spectral changes upon KCN additions from 0.06 to 5.0 mM.

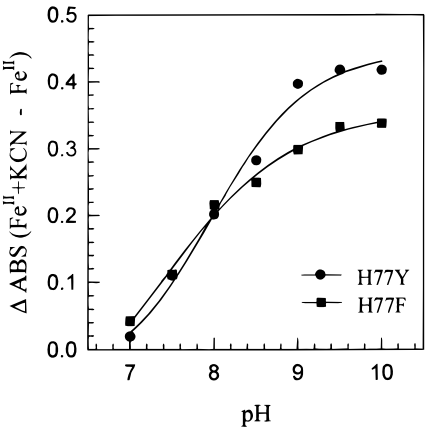

FIGURE 2: Coordination of cyanide to H77Y Fe^{II} CooA is pH-dependent. Difference Soret maxima of H77F and H77Y Fe^{II} CooA−CN adducts are depicted as a function of pH. Protein concentration was ~5 μM and KCN was 2 mM under anaerobic buffering conditions that are described in the Materials and Methods section.

During the induction, there was a marginal effect of added cyanide on *E. coli* viability as judged by similar final $OD_{600\ nm}$ measurements (data not shown). β-Galactosidase activity of induced cells was quantitated by the protocol described by Miller (*32*).

RESULTS

His^{77} Variants Fe^{II} CooA Coordinate Cyanide. In this study, we demonstrate that H77Y Fe^{II} CooA, in contrast to H77Y Fe^{III} CooA and wild-type Fe^{II} and Fe^{III} CooA, coordinates cyanide. UV−Visible absorption spectra of purified H77Y Fe^{II} CooA at pH 10.0 revealed marked spectral changes with increasing additions of relatively small concentrations of exogenous cyanide (Figure 1). There is a general increase and sharpening of the Soret band from 423 to 429 nm with sharp isosbestic points observed at 418 and 442 nm. The latter result suggests the presence of only two major species, the H77Y Fe^{II} CooA−CN adduct and H77Y Fe^{II} CooA. There is a general increase in the intensity of the α (560 nm) and β (532 nm) bands in the visible region of the spectrum, which suggests a six-coordinate low-spin H77Y Fe^{II} CooA−CN adduct.

To ascertain whether cyanide binding to H77Y Fe^{II} CooA was due to the lack of a normal His^{77} residue or imparted specifically by a Tyr residue at position 77, we produced cell-free lysates from CooA-producing *E. coli* strains that contained substitutions at position 77 and screened them for UV−visible absorption spectral changes upon addition of cyanide at pH 7.4. Upon addition of cyanide, cell-free lysates of all substitutions tested (H77E, H77Q, H77C, H77K, H77M, H77A, H77F, H77G, and H77R) showed spectral changes that were indicative of cyanide coordination to Fe^{II} CooA (data not shown). These results suggest that the lack of the normal histidine ligand at position 77 results in the Fe^{II}−CN-binding phenotype of these CooA variants. To further verify that the effects seen were not specific to H77Y, we purified H77F CooA for further analysis as described below. This substitution was chosen because it was similar in size to tyrosine but cannot serve as a ligand.

Upon addition of exogenous cyanide, similar spectral changes have been observed for other Fe^{II} heme proteins such as sperm whale myoglobin (*23*), horseradish peroxidase (*24*), lactoperoxidase (*26*), microperoxidase (*27*), and the hemoglobin of *S. inaequivalvis* (*28*), suggesting that H77Y Fe^{II} CooA is coordinating cyanide. When cyanide was present in concentrations of 2.5 mM and the headspace of the cuvette was flushed and then pressurized with CO, the Fe^{II}−CO spectrum was produced (data not shown), indicating that CO can displace bound cyanide. No changes in the UV−visible absorption spectrum were noted in the presence of high concentrations (up to 50 mM) of cyanide in wild-type Fe^{II} and Fe^{III} CooA, C75S Fe^{II} and Fe^{III} CooA, and H77Y Fe^{III} CooA (data not shown).

pH Studies of His^{77} Fe^{II} CooA and Fe^{II} CooA−CN Variants. Horseradish peroxidase in the Fe^{II} form coordinates the protonated form of cyanide (HCN) (*33*), whereas most other Fe^{II} heme proteins that coordinate cyanide are specific for the anionic (CN^-) form. To distinguish between these possibilities, we examined the ability of H77F and H77Y Fe^{II} CooA to coordinate cyanide (2 mM) as a function of pH (Figure 2). Relatively small but significant spectral changes were observed upon cyanide addition at pH 7.0 and 7.5. However, large spectral changes were observed upon addition of cyanide to H77Y and H77F Fe^{II} CooA at pH values above 8.0. Since the pK_a of HCN dissociation to CN^- and H^+ is 8.6, this result is consistent with H77Y and H77F Fe^{II} CooA coordinating the anionic form (CN^-) of cyanide.

During the course of the pH-dependence experiments of the H77Y and H77F Fe^{II} CooA−CN adducts, we observed that the spectra of these variants also changed with pH in the absence of cyanide. With increasing pH, there was a sharpening and increase of the Soret band of H77Y CooA, with absorbance maxima red-shifting from 422 to 424 nm and isosbestic points at 417 and 453 nm (Figure 3). The pH effects with H77F CooA were generally similar (data not shown). No changes in the UV−visible absorption spectrum were observed as a function of pH in wild-type Fe^{II} and Fe^{III} CooA or H77Y Fe^{III} CooA or H77F Fe^{III} CooA (data not shown). These results suggest that there is an ionizable group within the heme environment of these variants that is either not spectroscopically evident or is solvent-inaccessible in wild-type CooA and Fe^{III} CooA. Interestingly, the Fe^{II} form of horseradish peroxidase also shows pH-dependent UV−

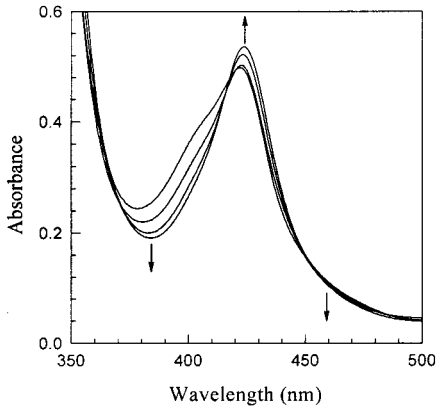

FIGURE 3: UV−Visible absorption spectra of H77Y Fe^{II} CooA are pH-dependent. UV−Visible absorption spectra of H77Y Fe^{II} CooA under conditions of increasing pH (pH 7.0, 8.0, 9.0 and 10.0) are shown, with arrows depicting a general sharpening and slight red shift of the Soret band. Protein concentrations were ~5 μM under buffering conditions that are described in the Materials and Methods section.

visible absorption spectral changes that are centered at ~pH 7.4, which has been attributed to a proton-accepting site in the heme pocket (*34*). The Soret spectral changes due to pH variation observed in H77Y Fe^{II} CooA were relatively minor (<0.1 ΔAbs) compared to the effects of cyanide addition (Figure 2); therefore, the spectral changes upon addition of cyanide are not the result of pH alteration in the buffered CooA samples used in this study.

H77Y Fe^{II} CooA Coordinates Cyanide with High Affinity and Positive Cooperativity. To determine the binding constants of cyanide to H77Y Fe^{II} CooA, we chose pH 10.0 because the observed spectral changes were the largest and the cyanide anion is predominant at this pH. The plot of percent saturation (α) of the H77Y Fe^{II} CooA−CN adduct vs cyanide concentration was not hyperbolic but apparently sigmoidal in shape, suggesting that the binding of cyanide to H77Y Fe^{II} CooA is cooperative (Figure 4A). To determine the extent and nature of cooperativity, the normalized absorbance data were analyzed in a Hill plot (Figure 4B). The slope of the linear portion of the graph was ~1.5, which indicates that the cooperativity of cyanide binding to H77Y Fe^{II} CooA is strongly positive. The point where the linear portion of the Hill plot corresponds to $\log [\alpha/(1 - \alpha)] = 0$ (i.e., when the protein is half-saturated with cyanide; $K_{0.5}$) was equal to 0.37 mM (apparent affinity constant ~ 2700 M^{-1} at pH 10). This result indicates that H77Y Fe^{II} CooA binds cyanide with high affinity when compared to other heme protein Fe^{II}−CN adducts such as those from horseradish peroxidase (770 M^{-1}; *24*), sperm whale myoglobin (2.5 M^{-1}; *24*), and the hemoglobin of *S. inaequivalvis* (17 M^{-1}; *35*). The cooperativity of cyanide binding to H77Y Fe^{II} CooA did not vary with pH (data not shown). However, the affinity of H77Y Fe^{II} CooA for cyanide was pH-dependent with $K_{0.5}$ increasing with increasing pH (data not shown), which, along with the pH dependence of the UV−visible absorption spectra of the H77Y Fe^{II}−CN adduct, strongly suggests that the anionic form of cyanide is preferentially bound.

Human hemoglobin in the Fe^{II} form has also been shown to be cooperative for cyanide dissociation (*36*), although the dimeric hemoglobin of *S. inaequivalvis* binds cyanide noncooperatively in the Fe^{II} form (*35*). The fact that H77Y Fe^{II} CooA binds cyanide with positive cooperativity is somewhat surprising in light of the fact that CRP, a CooA homologue, is negatively cooperative for its effector molecule, cAMP (*37*).

H77Y CooA Is Weakly Responsive to Cyanide In Vivo. We have shown previously that CooA activity (i.e, the ability of CooA to bind DNA and promote transcription) in response to CO can be monitored in vivo by a β-galactosidase reporter assay in *E. coli* (*20*). In this study, we adapted this assay to test if H77Y and H77F CooA become active in the presence of cyanide in vivo. CO-independent β-galactosidase activity was noted in H77Y CooA (Figure 5), which has been observed previously (*21*), although we failed to detect this activity under different growth conditions (*20*). Over this "no effector" background, the strain with H77Y CooA showed a small but highly reproducible increase in activity in response to modest levels of cyanide. The *x*-fold induction

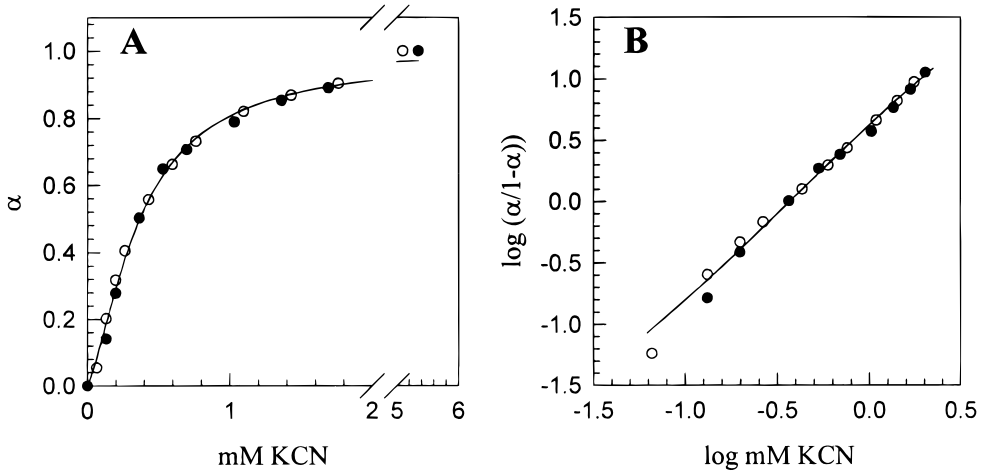

FIGURE 4: (A) Binding of cyanide to H77Y Fe^{II} CooA is cooperative. Upon coordination of cyanide, the normalized absorbance of the Soret maxima of H77Y Fe^{II}−CN CooA increases sigmoidally. Data were obtained with ~5 μM H77Y Fe^{II} CooA in 100 mM glycine/NaOH, pH 10.0, with increasing concentrations (0.06−5.0 mM) of KCN. The solid line represents the best fit line by nonlinear curve-fitting analysis. (B) Cyanide binding to H77Y Fe^{II} CooA is strongly positively cooperative. Hill analysis of cyanide binding to H77Y Fe^{II} CooA data in 100 mM glycine/NaOH, pH 10.0, is shown. Line represents linear regression of the best fit line for the data. The point at which $\log[\alpha/(1 - \alpha)] = 0$, which is equal to the concentration of cyanide at half-saturation, $K_{0.5}$, is ~0.4 mM. The slope, *n* (Hill coefficient), of the fitted line was ~1.5. Filled and open circles represent data from two separate experiments.

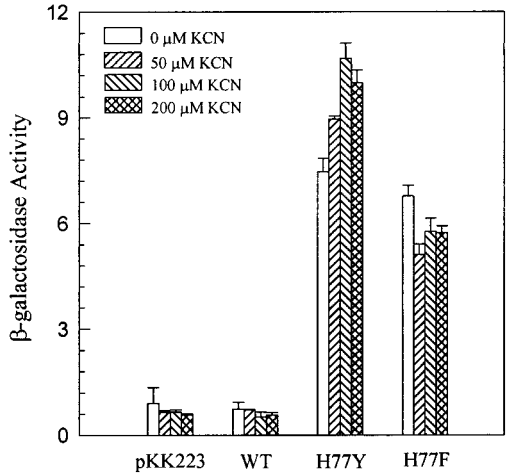

FIGURE 5: H77Y CooA is weakly responsive to cyanide in vivo. In vivo β-galactosidase activity as a function of increasing concentrations of cyanide of selected CooA variants and controls is shown. Error bars represent the standard deviation of data from eight replicate samples.

of β-galactosidase activity for wild-type CooA in response to CO in our system is ~15−20 (*20*), whereas the *x*-fold induction observed for H77Y CooA in the presence of cyanide (typically ~1.4) was much smaller in comparison (Figure 5). However, the response to cyanide was always concentration-dependent to 100 mM and repeatable, with β-galactosidase activity values varying less than 5% in replicate trials. A decrease in β-galactosidase activity was observed for cells incubated in 200 μM cyanide, presumably because of effects of cyanide on cell physiology. Wild-type CooA and H77F CooA in the reporter strain showed no observable changes in β-galactosidase activity in response to cyanide under identical conditions. However, H77F CooA showed similar CO-independent activity (Figure 5), suggesting that relatively bulky amino acid residues at position 77 are the origin of this behavior. Other CooA variants altered at position 77 that were tested in this study (H77C, H77F, H77A, H77K, and H77M) also showed no increase in β-galactosidase activity in the presence of cyanide nor any CO-independent activity (data not shown). These results indicate that the cyanide response is due to the presence of Tyr at position 77 of CooA rather than the lack of the normal His.

DISCUSSION

H77Y and H77F Fe^{II} CooA−CN Adducts. Analysis of heme-containing proteins routinely involves probing the heme iron with exogenous ligands to determine spin-state characteristics and ligand-binding properties. In this study, we demonstrate that H77Y and H77F Fe^{II} CooA coordinate cyanide, which is in contrast to H77Y and H77F Fe^{III} CooA and wild-type Fe^{II} and Fe^{III} CooA. Recent evidence from resonance Raman (*19*) and magnetic circular dichroism (MCD) (*22*) spectroscopy experiments suggests that CO displaces His^{77}. Based on the fact that all position 77 variants appear to bind CO normally (*20*) and CO displaces bound cyanide, a plausible hypothesis is that cyanide also binds to the same side of the heme as does CO. We have also attempted to confirm and extend the results of CN binding to H77Y CooA through resonance Raman spectroscopy but have been unable to definitively identify an Fe−CN stretching band because of weak resonance enhancement and interference from overlapping porphyrin bands (Williams and Spiro, unpublished data).

Although cyanide coordination to heme proteins is more common in Fe^{III} hemes, it is apparent that the axial thiolate bond in the Fe^{III} forms of H77Y, H77F, and wild-type CooA (*17*, *22*) is strong enough to preclude cyanide binding. In wild-type Fe^{II} CooA, which possesses a His as the axial ligand (*20*, *21*), cyanide apparently cannot displace the bound imidazolate (*19*) to form the Fe^{II}−CN adduct. In H77Y and H77F Fe^{II} CooA, the absence of this strong ligand would facilitate cyanide binding. Alternatively, the axial imidazolate might represent an additional barrier to cyanide binding insofar as it increases negative polarity in the heme pocket, thus precluding the binding of the anion. For example, the general lack of polarity in the heme pocket of *S. inaequivalvis* Fe^{II} hemoglobin is thought to facilitate the binding of cyanide (*28*). However, the fact that H77E CooA, presumably with a glutamate anion in the vicinity of the heme pocket, readily binds cyanide (data not shown) implies that polarity is not a major factor in the failure of wild-type CooA to coordinate cyanide.

The presence of an open coordinate position in H77Y Fe^{II} CooA is supported by resonance Raman (*19*) and MCD (*22*) spectroscopies that show that H77Y Fe^{II} CooA exists primarily in the five-coordinate form. While it is possible that Tyr is serving as a weak ligand in H77Y Fe^{II} CooA, we note that all other substitutions at position 77, including those that cannot possibly serve as a ligand (e.g., H77A and H77F), also produce a mixture of five- and six-coordinate species in varying proportions (data not shown). It therefore seems reasonable to suppose that a different residue in CooA is serving as an adventitious ligand in Fe^{II} CooA variants when His^{77} is unavailable. This adventitious ligand allows the presence of a low level of five-coordinate species that presumably can bind cyanide, and cyanide binding shifts the equilibrium to the six-coordinate Fe^{II}−CN adduct.

For heme proteins such as sperm whale myoglobin, the mechanistic steps of cyanide coordination to the Fe^{III} form include (1) the facilitation of uncharged HCN through the protein matrix and (2) deprotonation of HCN by a heme site residue (in this case the distal His^{64}) with subsequent coordination of the cyanide anion to the heme iron (*38*). Thus, an alternative explanation for the pH dependence of unligated H77Y and H77F Fe^{II} CooA may involve the deprotonation of a residue, which causes the observed spectral changes and serves to deprotonate HCN to the cyanide anion. The identity of this residue, however, remains unknown. The high-affinity cyanide binding observed with H77Y Fe^{II} CooA may thus represent a combination of factors that include an increase in the fraction of five-coordinate Fe^{II} heme population, the facilitated deprotonation of incoming HCN to form the cyanide anion, and a decrease in heme-pocket polarity. Further studies to elucidate the nature and identity of the amino acid residues that line the heme pocket that may or may not be contributing to these phenomena is clearly warranted.

Cooperative Cyanide Binding to H77Y Fe^{II} CooA. We have observed that all examined position 77 variants coordinate cyanide, although it remains unclear whether the cooperative nature of cyanide binding to H77Y Fe^{II} CooA

is the result of the lack of the normal His at position 77 or the presence of Tyr at this position. Because of the lack of structural data concerning CooA, the mechanism by which cyanide binding at one subunit influences the affinity for cyanide in the other remains speculative. However, CRP, a CooA homologue, binds cAMP with negative cooperativity (*37*). The negative cooperativity of cAMP binding to CRP can be rationalized physiologically in that only one cAMP molecule bound is sufficient to drive the conformational changes in the protein to create the active state.

The cooperative nature of cyanide binding to H77Y Fe^{II} CooA is interesting in light of the fact that *S. inaequivalvis* hemoglobin binds cyanide noncooperatively yet binds oxygen cooperatively (*35*), while human hemoglobin, in a fashion reminiscent of oxygen, binds cyanide cooperatively (*36*). These differences have been attributed to fundamentally different mechanisms of intersubunit communication between these two proteins. The nature of binding of CO and other small molecules to other His^{77} variants and to wild type has not yet been examined and therefore remains of interest to provide a clearer picture of ligand binding to CooA. We are therefore beginning a more comprehensive analysis of the cooperativity of binding of various ligands to H77Y variants and of CO binding to wild-type CooA.

Cyanide Activation of H77Y CooA. While H77Y CooA shows a reproducible and statistically valid response to cyanide, the *x*-fold activation (∼1.4-fold) is much lower than that of wild-type CooA in response to CO (>15-fold; *20*). The origins of these large differences in the *x*-fold activations in these two systems are at present unclear. However, we hypothesize that the combination of the probable low access of cyanide across the cell membrane (where CO is apolar and readily crosses the membrane) and relatively low level of cyanide (0.1 mM cyanide) tolerated by the cells in the in vivo activation experiment, keeps the cyanide concentrations well below the apparent $K_{0.5}$ of H77Y Fe^{II} CooA (∼0.4 mM). A higher concentration of cyanide in vivo, while avoiding cyanide toxicity to the reporter system, would have to be achieved in order to determine if the *x*-fold activation of cyanide response could be increased in this system.

This report has shown that while cyanide binding to Fe^{II} CooA requires the elimination of the normal His^{77} heme ligand in this state, the partial activation of CooA in response to cyanide binding appears to be specific for H77Y CooA. Because H77Y CooA binds CO but fails to activate in response to CO, there is something about the cyanide/H77Y interaction that mimics the critical features of the CO/wild-type CooA interaction and leads to activation. It is premature to speculate what those features might be until the heme binding region of CooA is better understood in a structural sense. This report also shows that H77Y CooA binds CN with strong positive cooperativity opening the possibility that cooperativity plays an important biological role with this protein.

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