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CO and NO bind to Fe(II) DiGeorge critical region 8 heme but do not restore primary microRNA processing activity

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Abstract The RNA-binding heme protein DiGeorge critical region 8 (DGCR8) and its ribonuclease partner Drosha cleave primary transcripts of microRNA (pri-miRNA) as part of the canonical microRNA (miRNA) processing pathway. Previous studies show that bis-cysteine thiolate-coordinated Fe(III) DGCR8 supports pri-miRNA processing activity, while Fe(II) DGCR8 does not. In this study, we further characterized Fe(II) DGCR8 and tested whether CO or NO might bind and restore pri-miRNA processing activity to the reduced protein. Fe(II) DGCR8 RNA-binding heme domain (Rhcd) undergoes a pH-dependent transition from 6-coordinate to 5-coordinate, due to protonation and loss of a lysine ligand; the ligand bound throughout the pH change is a histidine. Fe(II) Rhcd binds CO and NO from 6- and 5-coordinate states, forming common CO and NO adducts at all pHs. Fe(II)–CO Rhcd is 6-coordinate, low-spin, and pH insensitive with the histidine ligand retained,

suggesting that the protonatable lysine ligand has been replaced by CO. Fe(II)–NO Rhcd is 5-coordinate and pH insensitive. Fe(II)–NO also forms slowly upon reaction of Fe(III) Rhcd with excess NO via a stepwise process. Heme reduction by NO is rate-limiting, and the rate would be negligible at physiological NO concentrations. Importantly, in vitro pri-miRNA processing assays show that both CO- and NO-bound DGCR8 species are inactive. Fe(II), Fe(II)–CO, and Fe(II)–NO Rhcd do not bear either of the cysteine ligands found in the Fe(III) state. These data support a model in which the bis-cysteine thiolate ligand environment of Fe(III) DGCR8 is necessary for establishing proper pri-miRNA binding and enabling processing activity.

Keywords Heme · microRNA · RNA processing · Carbon monoxide · Nitric oxide

Abbreviations

5cHS	5-Coordinate high spin
6cLS	6-Coordinate low spin
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
CHES	N-cyclohexyl-2-aminoethanesulfonic acid
CTT	C-terminal tail
DaCld	<i>Dechloromonas aromatica</i> chlorite dismutase
DEANO	2-(N,N-diethylamino)-diazene-2-oxide
DGCR8	DiGeorge critical region 8
DSD	Dimerization subdomains
dsRBD1	Double-stranded RNA-binding domain 1
dsRBD2	Double-stranded RNA-binding domain 2
DTT	Dithiothreitol
EPES	4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid

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HBD	Heme-binding domain
MCD	Magnetic circular dichroism
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
miRNA	microRNA
MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
NLS	Nuclear localization signal
<i>Ns</i>	<i>Nostdoc</i> sp.
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PPIX	Protoporphyrin IX
Rhed	RNA-binding heme domain
RMSD	Root-mean-square deviation
rR	Resonance Raman
SEC	Size exclusion chromatography
WW	WW-motif

Introduction

MicroRNAs (miRNAs) are non-protein-coding RNAs that play an essential role in post-transcriptional regulation and whose malfunctions contribute to human disease [1, 2]. Although mature miRNAs are about 21 nucleotides in length [1], they are initially generated as longer primary miRNAs (pri-miRNAs) that must be processed through sequential cleavages [3]. In the nucleus, pri-miRNAs are cleaved to produce the intermediate precursor miRNAs (pre-miRNAs) by the Microprocessor complex comprised of the RNase III enzyme Drosha and its RNA-binding partner DiGeorge critical region 8 protein (DGCR8) (Fig. 1a) [3–6]. Drosha alone has no specific pri-miRNA cleavage activity, because it requires its partner DGCR8 for substrate recognition [6, 7]. DGCR8 binds to key structural elements in pri-miRNAs; higher order structures of DGCR8 and Drosha appear to be required for Drosha-mediated cleavage [7–14].

DGCR8 contains a heme cofactor that is essential for DGCR8 function [15]. The recombinant human DGCR8 construct NC1, which lacks the nuclear localization signal (NLS), binds one heme per NC1 dimer (Fig. 1b) [7]. The Drosha/NC1 complex is more active in the Fe(III) heme-bound state than the heme-free state [7, 16–18]. A second recombinant DGCR8 construct that only contains the RNA-binding heme domain of *Xenopus laevis* DGCR8 (Rhed, previously called heme-binding domain, DGCR8 HBD, Fig. 1b) has proved valuable for elucidating functional and spectroscopic characteristics of the DGCR8 heme. Rhed anchors the DGCR8–Drosha complex to the ends of the pri-miRNA hairpin; Fe(III) heme is required for proper association [11]. Dimeric, heme-containing Fe(III) NC1 and Fe(III) Rhed are low spin and 6-coordinate with an unusual bis-cysteine thiolate ligation motif, where one cysteinate ligand (Cys-352, human; Cys-354, frog) is

Fig. 1 a Scheme depicting conversion of pri-miRNA to pre-miRNA. b Recombinant DGCR8 proteins used in this study. *Top* the sequence map of full-length human DGCR8 (amino acids 1–778). *NLS* nuclear localization signal, *DSD* dimerization subdomain, *WW* WW-motif, *Rhed* RNA-binding heme domain, *dsRBD1* double-stranded RNA-binding domain 1, *dsRBD2* double-stranded RNA-binding domain 2, *CTT* C-terminal tail. *Middle* the sequence map of human NC1 expressed protein fragment (amino acids 276–751). *Bottom* the sequence map of His-tagged frog Rhed expressed protein fragment (amino acids 278–498)

derived from each DGCR8 subunit [7, 16]. When the heme is reduced, the Drosha/NC1 complex loses pri-miRNA processing activity [18]. Spectroscopic characterization revealed that dimeric Fe(II) Rhed loses the cysteine ligands upon reduction, replacing them with one or two neutral donors. Reduction and ligand exchange greatly lower the affinities of DGCR8 NC1 and Rhed for Fe(II) heme [18]. Apo NC1 may be prepared from Fe(II) NC1 by size exclusion chromatography, and the apo protein remains a dimer. Together, these data suggest that the unique bis-thiolate-coordinated Fe(III)-heme may play a role in establishing a functional conformation for DGCR8, and that this conformation is disrupted when the heme is reduced.

A precise biological function for heme in DGCR8 remains to be elucidated, and one possibility is that heme-containing DGCR8 may serve as a sensor for signaling gas molecules. For example, it is possible that Fe(II) DGCR8 might be in an inhibited state, and that binding of CO or NO via ligand replacement and downstream conformational change might release this inhibition. Examples of heme-dependent sensors are: mammalian NPAS2, bacterial CooA and RcoM, which sense CO, and mammalian sGC, bacterial H-NOX and DNR, which sense NO [19–26]. Typically, CO or NO binding to heme causes local rearrangement of the heme coordination environment and subsequent global

changes in protein conformation, driving a switch that modulates function. For example, CooA is a CO-sensor that only binds to its DNA promoter in the Fe(II), CO-bound state. Pro/Cys ligated Fe(III) CooA switches its cysteine ligand for a histidine when reduced to Fe(II). CO replaces the more weakly bound Pro ligand when CooA is exposed to CO [20, 21]. Similarly, the mammalian CO-sensing transcription factor NPAS2 undergoes a ligand switch upon reduction, priming the protein for CO activation: Cys-/His-ligated Fe(III) NPAS2 switches its cysteine ligand for histidine when reduced to Fe(II), and CO replaces one histidine [19]. Other CO sensors follow a similar pattern: ligand switching upon reduction, which primes the heme to bind CO by replacing the more weakly bound ligand. At each step in this process, the heme is 6-coordinate. A common theme, possibly relevant to DGCR8, is loss of a heme-bound Cys thiolate upon reduction.

NO sensors function differently. There is a wider variety of ligation motifs that lead to protein activity modulation: NO sensors may have an Fe(II) or an Fe(III) resting state, and may form 5-coordinate Fe(II), 6-coordinate Fe(II), or 6-coordinate Fe(III) NO-bound states. For example, mammalian sGC and some bacterial H-NOX proteins, like that of *Shewanella oneidensis*, have a resting state of a 5-coordinate, His-ligated Fe(II) heme which binds NO at the open coordination site. Upon NO binding, a transient 6-coordinate NO-bound state is formed; subsequently, the strong trans effect of NO causes the sixth ligand to be lost, forming a 5-coordinate Fe(II)–NO adduct [24, 25, 27]. Other H-NOX proteins, like that of *Nostdoc* sp. (*Ns*), have an Fe(III) resting state and must be reduced before binding NO. *Ns* H-NOX forms two stable 6-coordinate NO adducts: one with His trans to the NO, and another with two, trans-oriented NO molecules bound to heme [28]. H-NOX proteins from *Legionella pneumophila* and *Thermoanaerobacter tengcongensis* also form 6-coordinate Fe(II) NO adducts [24, 29, 30]. Other heme proteins form functionally important 6-coordinate Fe(III) NO adducts, including the nitrophorin NO donor proteins, cytochrome *cd*₁ nitrite reductase and fungal nitric oxide reductase [31–38]. These 6-coordinate Fe(III) NO adducts bear either His or Cys trans to NO. Plausibly, DGCR8 might react with NO to form a functionally relevant 5- or 6-coordinate adduct in the Fe(II) or Fe(III) state.

To further probe the DGCR8 heme environment and to test the hypothesis that CO or NO might regulate DGCR8 function, we studied the reactivity of Fe(II) Rhed with CO and NO. For the NO adduct, we tested whether NO could react directly with Fe(III) DGCR8 and retain activity, since NO-mediated reduction coupled with NO binding might occur without driving the protein into an inactive Fe(II) conformation. Herein, we report pH-dependent behavior of Fe(II) Rhed, which alters the proportion of 5- and

6-coordinate heme and implicates a protonatable lysine as a ligand. The 5- and 6-coordinate Fe(II) forms react with CO and NO to form common adducts. Resonance Raman data implicate His as a ligand to Fe(II) Rhed and Fe(II)–CO Rhed. Fe(II)–NO Rhed is 5-coordinate; Fe(III) Rhed reacts directly with NO to form the Fe(II) NO-bound species, but this reaction is slow and reduction by NO is rate-limiting. Neither CO nor NO restore pri-miRNA processing activity to the Fe(II) NC1/Drosha complex. Re-oxidation of Fe(II) Rhed results in partial restoration of the bis-cysteinate Fe(III) species. None of these species [Fe(II), Fe(II)–CO, Fe(II)–NO] are capable of constituting a reversible switch between inactive and active [Fe(III)] states. Our observations do not support a gas sensing function for DGCR8; nevertheless, CO and NO serve as valuable structural probes of the heme environment. Spectroscopic studies of the Fe(II)–CO Rhed and Fe(II)–NO Rhed further illuminate the nature of the unusual heme of DGCR8, and emphasize the necessity for maintaining native Fe(III) bis-Cys coordination for Microprocessor activity.

Materials and methods

Materials

Materials used in buffer preparation and glycerol were purchased from Sigma-Aldrich and used as received. Sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) was purchased from Fluka and stored under Ar(g) at -20°C until used. CO(g) ($\geq 99.0\%$ purity) was obtained from Linde Gas LLC and used as received. The isotopic composition of the ^{13}C -enriched CO used to make FeCO mode assignments was 99 atom % ^{13}C and <2 atom % ^{18}O . Samples for examination by resonance Raman spectroscopy were prepared with ultrapure buffers and distilled–deionized water. NO(g) was generated in situ by mixing NaNO_2 (Sigma-Aldrich), CuCl_2 (MCB Chemicals), and L-ascorbic acid ($\geq 99.0\%$ purity, Sigma-Aldrich) with a previously prepared buffer solution. CO(g) used in the pri-miRNA processing assays was purchased from Airgas. DEANO [2-(*N,N*-diethylamino)-diazonolate-2-oxide diethylammonium salt] was purchased from Enzo Life Sciences (Farmingdale, New York).

Protein isolation

Frog (*Xenopus laevis*) DGCR8 Rhed-His₆ (amino acids 278–498, referred to here as Rhed), globally selenomethionine-labeled frog DGCR8 Rhed-His₆ (referred to here as SeMet Rhed), and human NC1 DGCR8 (amino acids 276–751, referred to here as NC1) (Fig. 1b) were expressed and purified as previously described [16, 18, 39]. Briefly, both Rhed and SeMet Rhed were purified via Ni-affinity

chromatography followed by size exclusion chromatography (SEC) using a HiPrep 26/60 Sephacryl S-200 High-Resolution column. NC1 was purified via SP Sepharose High-Performance cation exchange chromatography followed by SEC. The SEC running buffer contained 20 mM Tris pH 8, 400 mM NaCl, and 1 mM DTT. Purified NC1 was buffer exchanged into 50 mM MES pH 6, 400 mM NaCl, and 1 mM DTT and concentrated using Amicon Ultra centrifugal filters (Millipore).

Spectroscopic sample preparation

Fe(II), Fe(II)–CO, and Fe(II)–NO Rhed were characterized spectroscopically. All Rhed samples (3–136 μ M heme) were prepared in 50–100 mM buffer, 400 mM NaCl, pH as indicated. Because of protein instability, Rhed could not be studied below pH 7.2. Fe(II) Rhed was prepared from the as-isolated Fe(III) Rhed as follows. Fe(III) protein samples were purged of oxygen by flowing Ar(g) through the headspace of a gas-tight sample vessel for 10 min. Anaerobic sodium dithionite and Fe(III) protein solutions were allowed to equilibrate separately at the indicated temperature for 10 min, after which dithionite (1–10 mM final concentration) was added to the Fe(III) protein. Reduction of Fe(III) Rhed is very slow, requiring 1–2 h at an elevated temperature (37–44 °C). Conversion of Fe(III) to Fe(II) Rhed was monitored by visible spectroscopy, and samples were incubated at the indicated temperature until no further spectral change was observed. To produce Fe(II)–CO and Fe(II)–NO Rhed, Fe(II) protein samples were mixed with CO(g) or NO(g) under anaerobic conditions via gas-tight syringe, and reaction progress was monitored by visible spectroscopy. Fe(II)–CO products were prepared by injecting aliquots of CO(g) (300 μ L) into Fe(II) Rhed. Fe(II)–NO products were prepared by injecting smaller aliquots of NO(g) (100 μ L) into Fe(II) Rhed, with more NO added as needed to achieve complete reaction. Fe(II)–NO Rhed was also formed by adding 100 μ L NO(g) to Ar(g)-purged Fe(III) protein at 37 °C, and allowing the NO(g) to reduce and bind to the protein over the course of 2 h. All gases were added under STP conditions.

Electronic absorption spectroscopy

Electronic absorption spectra were recorded on a double-beam Varian Cary 4 Bio spectrophotometer set to a spectral bandwidth of 0.5 nm and equipped with a temperature controller. Spectra for Fe(II), Fe(II)–CO, and Fe(II)–NO Rhed were obtained at varying temperatures (20–40 °C) and varying pHs (7.4–11.0), as indicated. Reduction of Fe(III) to Fe(II) and the formation of CO- and NO-bound species were monitored until no further spectral changes were observed. Rates for reduction of Fe(III) Rhed to Fe(II)

Rhed and for reaction of Fe(III) Rhed with NO to produce Fe(II)–NO Rhed were determined by recording spectra (250–800 nm) every 2 min for 2 h. Rates were determined by plotting the loss of absorbance at 450 nm with time; both reactions could be fit to a first-order rate law to derive the first-order rate constants using IGOR Pro software (Wavemetrics, Inc.; Lake Oswego, OR).

Fe(II) Rhed exhibited pH-dependent changes in spectral intensity. The 424-nm molar absorptivity (ϵ_{424}) was plotted against pH to generate a titration curve. The data were fit to a modified version of the Henderson–Hasselbalch Eq. (1), where $\epsilon_{7.4}$ is the molar absorptivity at pH 7.4 and $\epsilon_{11.0}$ is the molar absorptivity at pH 11.0. The pK_a value was varied to optimize the fit of the data to Eq. (1) using a non-linear least-squares analysis, with an RMSD of 3.5.

$$\epsilon_{424} = \frac{\epsilon_{7.4} + \epsilon_{11.0} \times 10^{pH-pK_a}}{1 + 10^{pH-pK_a}} \quad (1)$$

MCD spectroscopy

Magnetic circular dichroism (MCD) spectra of Fe(II)–CO Rhed were recorded on a Jasco J-715 CD spectropolarimeter with the sample compartment modified to accommodate an SM-4000-8T magnetocryostat (Oxford Instruments). The sample was transferred via gastight syringe into an MCD cell purged with Ar(g), flash-frozen and stored in N₂(l). MCD spectra were recorded at +7 and –7 Tesla over a temperature range from 2.5 to 100 K. Negative polarity data were subtracted from positive polarity data to remove CD contributions, and the resulting spectrum was divided by 2. Electronic absorption spectra showed that glycerol had no effect on the peak maxima at room or liquid-helium temperatures.

EPR spectroscopy

X-band electron paramagnetic resonance (EPR) spectra of Fe(II)–NO Rhed were recorded on a Bruker ELEXSYS E500 equipped with an Oxford ESR 900 continuous flow liquid helium cryostat and an Oxford ITC4 temperature controller. The microwave frequency was monitored with an EIP model 625A CW microwave frequency counter. Scans of 0–10,000 gauss revealed no signals other than those reported.

Resonance Raman spectroscopy

Resonance Raman (rR) spectroscopy was used to characterize Fe(II) and Fe(II)–CO Rhed. Two different spectrometers were used: Fe(II) SeMet Rhed spectra were recorded from stationary samples at 4 °C, and Fe(II) and Fe(II)–CO Rhed spectra were recorded at ambient temperature from continuously spinning samples to minimize photolysis of CO.

Spectra of wild-type Rhed in its Fe(II) and Fe(II)–CO forms were recorded from samples contained in 5-mm NMR tubes spinning at ~15 Hz. Each sample tube was sealed with a septum and the headspace purged with $N_2(g)$, in the case of Fe(II)– ^{13}CO Rhed samples, or $CO(g)$ in the case of natural abundance Fe(II)–CO Rhed samples. Fe(II)– ^{13}CO Rhed samples were prepared by anaerobic transfer of 4 mL of ^{13}CO to the protein sample tube via gas-tight syringe. The samples were heated to 44 °C and sodium dithionite solution was added to a final concentration of 8 mM. The samples were incubated at 44 °C for 1 h, after which reduction and CO binding was complete. Resonance Raman scattering was excited with either 413.1- or 441.6-nm emission from Kr^+ and HeCd lasers, respectively. The laser beam was focused to a line parallel to the spectrograph entrance slit. Scattered light was collected ($f/1$) in the 135° backscattering geometry, passed through a holographic notch filter and polarization scrambler. The laser image was then focused by an f matching lens on the entrance slit of a 0.67-m, $f/4.7$ Czerny–Turner spectrograph fitted with 1200 and 2400 grooves/mm gratings and a liquid N_2 -cooled CCD detector (1340 × 400 array of 20 × 20 μm pixels, 26.8 × 8.0 mm² image area). The spectrometer was calibrated against Raman shifts of toluene, dimethylformamide, acetone, and methylene bromide. Electronic absorption spectra were recorded from the rR samples before and after Raman acquisition to assess whether sample integrity had been compromised by exposure to the laser beam. Laser power at the samples ranged from 1 to 7 mW; no spectral artifacts attributable to photoinduced chemistry were observed at these powers.

Resonance Raman scattering by the Fe(II) SeMet protein was excited by 413.1-nm emission from a Coherent I-302C Kr^+ laser in a 135° backscattering geometry. Low incident laser powers of ≤ 20 mW were focused with a cylindrical lens onto the stationary sample. Laser light was dispersed by an Acton Research triple monochromator equipped with 1200 and 2400 grooves/mm gratings and analyzed with a Princeton Instruments Spec X:100BR CCD camera. The spectrometer was controlled via computer with Spectra-sense software. Samples were prepared in a quartz cuvette, monitored spectrophotometrically and transferred anaerobically in an $Ar(g)$ -purged chamber to a quartz EPR tube via small-bore tubing (connected to a gas-tight syringe), capped and stored on ice. Samples held in a quartz finger Dewar filled with ice water to reduce local heating during Raman data collection. Peak positions were calibrated relative to a Na_2SO_4 peak at 981 cm^{-1} . IGOR Pro software was used to import and process all spectral data. Major vibrational modes are assigned based on comparison with those of other heme proteins and the work of Spiro and Kitagawa [40–42].

Reconstituted pri-miRNA processing assay

Activity assays were performed as previously described [16, 18], with the following modifications to incorporate addition of NO and CO. Fe(III) NC1 was reduced in an anaerobic chamber by incubation with 2 mM sodium dithionite at room temperature for 45 min. An aliquot of the reduced protein was transferred to a cuvette, and the electronic absorption spectrum was recorded to ensure that reduction was complete. To form the CO-bound product, a bolus of CO gas was added to an aliquot of Fe(II) NC1. To form the NO-bound product, an aliquot of Fe(II) NC1 was incubated with freshly prepared 0.2 mM DEANO, an NO-releasing compound. Freshly prepared DEANO solution was compared to a control “dead DEANO” solution; “dead DEANO” was 0.2 mM DEANO allowed to sit at room temperature in a fume hood for 2 h. This “dead DEANO” solution was assumed to contain minimal residual NO. Complete formation of Fe(II)–CO NC1 and Fe(II)–NO NC1 was confirmed by electronic absorption spectroscopy (Fig. S1). Six NC1 samples were assayed for pri-miRNA processing activity with uniformly ^{32}P -labeled pri-miR-380 and pri-miR-9-1 substrates: Fe(II), Fe(II) with “dead DEANO”, Fe(II)–NO, Fe(II)–CO, Fe(III) with “dead DEANO”, and Fe(III). Briefly, each reaction contained: NC1 (25 nM dimer), ^{32}P -labeled pri-miRNA substrate (10,000 cpm), His₆-Drosha^{390–1374} (4 nM), $MgCl_2$ (6.4 mM) and RNaseOUT (2 U/ μl) in 500 mM Tris pH 8.0, 0.2 mM EDTA, 100 mM NaCl, and 10 % glycerol (v/v). The absolute concentrations of pri-miRNAs were much lower than those of the Drosha and DGCR8 proteins; therefore, these reactions were under single turnover conditions. Reactions were incubated for 45 min at 37 °C, stopped with addition of 2× RNA loading dye and analyzed using denaturing gel electrophoresis and autoradiography.

Results

The heme environment of Fe(II) Rhed is pH sensitive

Fe(II) Rhed exhibits pH-dependent changes in the heme coordination environment, as evidenced in the electronic absorption and Soret-excited rR spectra. In the electronic absorption spectra (Fig. 2), the molar absorptivities of the Soret, α , and β bands increase with increasing pH. The rR spectrum (Fig. 3) is also affected by pH, showing a pH-dependent mixture of 5-coordinate high-spin (5cHS) and 6-coordinate low-spin (6cLS) hemes. The electronic absorption and rR spectra reveal that increasing pH favors the 6cLS population. The changes in spectral shape and the increase in molar absorptivities observed in the visible spectra clearly indicate a transition from HS to LS heme

Fig. 2 Electronic absorption spectra of Fe(III) and Fe(II) Rhed at different pHs. *Top panel* electronic absorption spectra of Fe(III) Rhed at pH 8.0 (dashed line) and Fe(II) Rhed at different pHs (solid line at pH 10.0 and dotted line at all other pHs). Representative Fe(II) spectra are shown at pHs 8.0 (EPPS), 8.7 (CHES), 9.2 (CHES) and 10.0 (CAPS). The region from 500 to 700 nm has been multiplied by a factor of 3. Arrows on the α , β , and Soret peaks indicate the change in ϵ with increasing pH. *Bottom panel* the molar extinction coefficient at 424 nm ($\epsilon_{424\text{ nm}}$, closed triangle) as a function of pH and its best-fit curve (dotted line) to Eq. (1); the pHs assessed were 7.4 (MOPS), 8.0 (EPPS), 8.2, 8.4, 8.7, 8.9, 9.2 and 9.4 (CHES), 10.0, 10.8 and 11.0 (CAPS). Protein concentration ranged from 3 to 12 μM heme in 50 mM buffer, 400 mM NaCl, for both panels

(Fig. 2). At low pH, the α/β band region (500–650 nm) shows an overlapping and poorly resolved set of features, consistent with a mixture of species having largely HS character. As the pH is raised, a sharp, intense α band grows in at 558 nm along with the corresponding β band at 527 nm. At pH 10.0, the spectrum in the α/β region shows a well-defined pair of bands at 558 and 527 nm, whose relative intensities are consistent with LS Fe(II) heme. When the Soret band extinction coefficient as a function of pH is fit to Eq. (1), an apparent pK_a of 9.1 ± 0.1 (Fig. 2) is extracted

from the data. Since HS-to LS-transitions in Fe(II) heme proteins are commonly associated with changes in coordination number from 5 to 6, these data implicate the presence of a ligand that is half protonated and half coordinated to the Fe(II) center in its conjugate base form at pH 9.1. This spin- and coordination-state change is confirmed by rR spectroscopy (Fig. 3). At low pH, the HS marker band, ν_3 , occurs at 1469 cm^{-1} ; this feature diminishes and disappears as the pH is increased. Concomitant with this decrease, the LS ν_3 band at 1491 cm^{-1} grows to dominate this region of the spectrum [42–44].

The spectral characteristics and pH-dependent coordination changes provide insight as to the identity of the protonatable ligand in Fe(II) Rhed, eliminating certain amino acids from consideration. The Soret maximum is not near 450 nm; therefore, neither of the two cysteine thiolates bound to Fe(III) Rhed are retained in the Fe(II) state [45]. Furthermore, the fact that there is no pH-dependent shift of the Soret maximum to 450 nm with increasing pH rules out a cysteine thiolate, with an expected $pK_a \leq 8$ [46], as the protonatable sixth ligand. Therefore, cysteine can be ruled out as a candidate ligand to the Fe(II) Rhed heme. The electronic absorption and rR spectral features of Fe(II) Rhed are consistent with neutral amino acid donors, and pK_a of 9.1 for the 5- to 6-coordinate transition implicates arginine or lysine as the ligand [46]. Although arginine, with a free side chain pK_a of 12.48, is a plausible candidate [46], arginine coordination to transition metal centers in proteins is rare. Because of its high pK_a and poor Lewis basicity, arginine only coordinates in highly hydrophobic environments [47, 48].

Lysine is the more likely axial ligand candidate. The observed pK_a is consistent with competition between iron coordination and protonation of a lysine γ -amine group (pK_a of 10.54) [46]. Heme coordination is expected to lower the pK_a relative to that of the free amino acid. Similar pH-dependent behavior, with a measured pK_a of 9, is observed in the electronic absorption and rR spectra of a lysine-coordinated Fe(II) mutant of *Dechloromonas aromatica* chlorite dismutase, R183K DaCld [49, 50]. Although there are no structures of full-length DGCR8 or Rhed, additional circumstantial evidence supporting lysine as the protonatable heme ligand derives from the protein context. Exchange of heme ligands often occurs between nearby amino acids [20, 22, 51, 52]; lysines 342/4, 356/8, 357/9, and 359/61 are adjacent to Cys-352/4 in the linear sequence of human NC1 and frog Rhed proteins, and may plausibly replace Cys-352/4 upon reduction. In contrast, there are no arginines within ten amino acids. Crystal structures of the dimerization subdomains (DSD) of human and frog DGCR8 contain Cys-352/4 at the C-terminal end of the chain. In these structures, the four arginines and the single lysine are not near Cys-352/4 nor are they oriented

Fig. 3 Resonance Raman spectra of Fe(II) Rhed as a function of pH. **a** The high-frequency region of the Fe(II) Rhed 413.1-nm-excited resonance Raman spectrum are shown at pHs 7.8 (HEPES), 8.6 (CHES), 9.0 (CHES) and 9.5 (CHES). Samples containing 30–60 μ M heme were prepared in 50 mM buffers as indicated, 400 mM NaCl, and 3 mM sodium dithionite. Laser power was 8–10 mW at the sample.

b The low-frequency window of the Fe(II) Rhed resonance Raman spectrum under the same conditions described in **a**. **c** The low-frequency window of the Fe(II) Rhed 441.6-nm-excited resonance Raman spectrum under the same sample conditions described in **a** and 1.2-mW laser power at the sample

appropriately relative to the heme iron to replace Cys-352/4 upon reduction [17, 53]. The multiple lysine residues that reside within ten amino acids of Cys-352/4 in the continuing sequence lie beyond the C-terminal end of these structurally characterized fragments, so their positioning within the three-dimensional structure is not known and they may or may not be appropriately oriented. Furthermore, in the three-dimensionally folded protein, any one of the 18 lysines in Rhed may replace Cys-352/4 upon heme reduction.

The identity of the fifth ligand, which is present in both the 5- and 6-coordinate states, is most likely histidine. Current evidence favors a neutral donor, with histidine or methionine as plausible candidates. Electronic absorption and rR spectra of SeMet Fe(II) Rhed are identical to those of the wild-type protein (Table S1; Fig. S2), ruling out the possibility that this fifth ligand is methionine. Two pieces of evidence support the suggestion that the ligand is histidine. First, the low-frequency spectra in Fig. 3b, c reveal that, in addition to the pH-dependent spin-state interconversion evident in Fig. 3a, there is also a pH-dependent change in axial ligation. At low pH where the $1469\text{ cm}^{-1}\nu_3$ band

reports the presence of some 5cHS heme, the 441.6-nm excited low-frequency spectrum in Fig. 3c reveals a band at 229 cm^{-1} , consistent with a $\nu_{\text{Fe-His}}$ mode of a 5cHS Fe(II) heme [42, 54]. This band gains resonance enhancement through B-excitation of such hemes, which typically maximizes near 435 nm [55, 56]. Consistent with this assignment, the 229 cm^{-1} band is absent from the 413.1-nm spectra in Fig. 3b. As this band is not present in 6cLS hemes, its diminution with rising pH is consistent with the simultaneous loss of intensity in the $1469\text{ cm}^{-1}\nu_3$ band. Thus, the rR spectra reveal distribution of the heme in Fe(II) Rhed between 5cHS and 6cLS species, with a single His coordinated at low pH and His and Lys coordinated at high pH. Second, the spectroscopic characteristics of Fe(II)–CO Rhed (vide infra) are also consistent with a proximal His ligand, further supporting this fifth ligand assignment.

Only Fe(II) Rhed exhibits pH-dependent changes in coordination; furthermore, the reduction mediated loss of the Cys thiolate ligands is only partially reversible with re-oxidation. Unlike Fe(II) Rhed, Fe(III) Rhed shows no changes in spectral features in the pH range 7.4–10.8 (Fig. S3). In addition, Fe(III) Rhed does not bind exogenous

ligands at physiologically relevant concentrations; the electronic absorption spectrum remains unchanged to remarkably high concentrations (10–300 mM) of imidazole, indicating that the two thiolate ligands are retained. At 1 M imidazole, an additional Soret-region peak appears at 412 nm, consistent with partial conversion to an imidazole-bound, high-spin Fe(III) species (Fig. S4) [57]. The only conditions under which the thiolate ligands are completely removed from the Fe(III) heme are upon addition of mercury compounds [16]. These observations suggest that the two Cys thiolate ligands in Fe(III) Rhed are tightly bound. However, both thiolate ligands are lost upon formation of Fe(II) Rhed and are exchanged for neutral Lys and His. This reduction mediated ligand exchange process is reversible to a limited extent. When Fe(II) Rhed is re-oxidized, some of the protein visibly precipitates. The remaining soluble portion consists of a clear mixture of species, one of which appears to be the original bis-Cys thiolate-ligated Fe(III) species (Fig. S6).

Loss of the thiolate ligands is accompanied by significant structural rearrangement. Evidence for this conclusion may be derived from thermal denaturation profiles of Fe(III) and Fe(II) Rhed (Table S2; Fig. S6), which reveal that the redox-mediated ligand switch significantly restructures both the heme pocket and the global protein fold. For Fe(III) Rhed (Fig. S6A), absorbance at 451 nm (the Soret maximum) decreases significantly above 41 °C ($T_{\text{onset,heme}}$), a change which we associate with heme pocket thermal denaturation. Above 57 °C ($T_{\text{onset,agg}}$), absorbance at 750 nm increases as the protein aggregates and light scattering causes a general increase in the baseline, which we associate with protein denaturation and aggregation. Fe(II) Rhed (Fig. S6B) is less thermally stable than Fe(III) Rhed. The heme Soret denaturation curve has a more complex shape, consistent with multiple unfolding transitions of the heme pocket. The global protein fold of Fe(II) Rhed is significantly less stable than Fe(III) Rhed, as evidenced by a lower temperature for aggregation onset (48 °C) and a greater change in absorbance at 750-nm indicative of a larger amount of aggregated protein. Taken together, these observations suggest that the native bis-thiolate coordination stabilizes Rhed.

Fe(II) Rhed binds CO

Multiple lines of evidence reveal that 5cHS and 6cLS Fe(II) Rhed react with CO to form a common product in which CO is bound trans to a neutral, N-donor ligand. The electronic absorption spectrum of the product Fe(II)–CO Rhed is the same, whether the reaction is carried out at pH 7.4, where the Fe(II) Rhed heme is largely 5cHS, or at pH 10.0, where the Fe(II) Rhed heme is 6cLS. Addition of CO to Fe(II) Rhed throughout this pH range results in sharpening

Fig. 4 The electronic absorption and MCD spectra of Fe(II)–CO Rhed. *Top* electronic absorption spectrum of Fe(II)–CO Rhed (7.4 μM heme) in 50 mM EPPS, 400 mM NaCl, pH 8.0 at 37 °C. The spectrum from 500 to 800 nm is multiplied by 5. *Bottom* MCD spectrum of Fe(II)–CO Rhed recorded at 50 K. The sample contained 30 μM heme, 7 mM dithionite, 20 mM EPPS, 160 mM NaCl, pH 8.0, and 60 % (v/v) glycerol

of the Soret band and shifting of its maximum to 420 nm. In addition, a pair of well-defined α and β bands appears at 567 and 539 nm, respectively (Fig. 4). The spectral positions and intensities are consistent with a 6cLS Fe(II)–CO species with a neutral ligand trans to the CO. The 420 nm Soret band rules out the presence of a cysteine thiolate trans to CO; a thiolate-bound Fe(II)–CO species would exhibit a Soret band near 450 nm [22, 45, 58]. Furthermore, the spectral similarity between Fe(II)–CO SeMet Rhed and the wild-type protein eliminates methionine as a possible ligand (Table S1). Further evidence for a 6c Fe(II)–CO species with a neutral donor trans to CO is provided by MCD spectroscopy of Fe(II)–CO Rhed at pH 8.0. The MCD spectrum is temperature-independent, as expected for the $S = 0$ ground state of an LS Fe(II) heme, and displays two dominant A -terms with peak crossovers at the Soret and α bands at positions similar to those of other neutral-ligand-bearing 6c Fe(II)–CO hemoproteins (Fig. 4) [22, 51, 52].

Resonance Raman spectra of Fe(II)–CO Rhed at pH 7.4 and 10.0 provide further evidence that CO is trans to a neutral, N-donor ligand and that the coordination environment of the CO-bound product is pH insensitive. The data

Fig. 5 Resonance Raman spectra of the isotopologs of the Fe(II)–CO Rhed complex allows assignments of the Fe–C and C–O stretching vibrations. **a** Low-frequency and **b** high-frequency rR data are shown for Fe(II)– ^{12}CO Rhed, Fe(II)– ^{13}CO Rhed, and their difference spectrum [Fe(II)– ^{12}CO Rhed–Fe(II)– ^{13}CO Rhed]. Samples of 50.2 μM heme concentration were in 50 mM CHES, 400 mM NaCl, pH 10.0. All spectra were recorded using 413.1-nm excitation

confirm that the same Fe(II)–CO Rhed product is formed at pH 7.4 and pH 10.0. The marker bands, ν_2 , ν_3 , and ν_4 occur at 1581, 1496, and 1371 cm^{-1} , consistent with complete formation of the Fe(II)–CO species (Fig. S7). That there are no differences in band positions or intensities in the rR spectra of Fe(II)–CO Rhed formed at pH 7.4 and at pH 10.0 implies that the same CO-bound product is formed regardless of whether Fe(II) Rhed is 5cHS or 6cLS (Fig. S7). Comparison of the resonance Raman spectra of natural abundance Fe(II)–CO Rhed and Fe(II)– ^{13}CO Rhed allowed for the definitive assignment of the $\nu_{\text{Fe-C}}$, δ_{FeCO} , and $\nu_{\text{C-O}}$ bands, which occur at 497, 574 and 1961 cm^{-1} , respectively, for natural abundance Fe(II)–CO Rhed. The same modes give rise to bands at 494, 561 and 1917 cm^{-1} , respectively, for Fe(II)– ^{13}CO Rhed (Figs. 5a, b, S8). The ratios of $\nu_{\text{Fe-C}}$ and $\nu_{\text{C-O}}$ frequencies for Fe(II)–CO Rhed at pH 7.4 and 10.0 place these species on the neutral N-donor proximal ligand line of the $\nu_{\text{Fe-C}}$ versus $\nu_{\text{C-O}}$ correlation plot (Figs. 6, S8). Most examples of proteins in this category bear an imidazole in the axial position trans to CO, implying that Lys is replaced by CO. A typical Lys pK_a of 10.6 would leave that residue protonated at neutral pH and, therefore, cationic. The plotted positions of Fe(II)–CO Rhed at pH 7.4 and pH 10.0 at the right lower end of the correlation line indicate that the CO ligand is in a similar weakly H-bonding distal environment at these two pHs [59]. If the γ -amine ligand of Lys remained in the vicinity of the bound CO, the expected effect of a cationic H-bond donor in the vicinity of the CO ligand would be to place the plotted ratio toward the upper left end of the correlation line. Furthermore, the spectra are predicted to

be pH-dependent, with a larger fraction of the CO-adduct feeling a cationic environment at lower pH. That the plotted frequency ratios for Fe(II)–CO Rhed at pH 7.4 and 10.0 are located at the same lower right position on the correlation line suggests that the protonated γ -amine ion is remote from the bound CO ligand. This observation implicates a conformational reorganization that places the protonated γ -amine so that it forms a charge-neutralizing salt bridge or H-bond remote from the bound CO ligand. Considering all these data together, we infer that at low pH, CO binds at the open coordination site left by the absence of the pH-sensitive Lys ligand. At high pH, CO replaces this Lys, which is more weakly bound (Fig. 7).

Fe(II) Rhed binds NO(g)

5cHS and 6cLS Fe(II) Rhed react with NO(g) to form a common 5c Fe(II)–NO product ($\{\text{FeNO}\}^7$ in the Enemark–Feltham notation [60, 61], data not shown). In the electronic absorption spectrum, Fe(II)–NO Rhed exhibits a broadened Soret band at 390 nm and a broad α/β absorption with a maximum at 539 nm (Fig. S9), consistent with the formation of 5c NO-bound Fe(II) heme [62–64]. EPR confirms that Fe(II)–NO Rhed is 5c. The EPR spectrum displays a characteristic three-lined signal centered at $g = 2.05$ (Fig. 8) due to hyperfine coupling of the unpaired electron with the $I = 1$ ^{14}N nucleus of the NO molecule [65]. Spectral simulation reveals three g values, $g_1 = 2.010$, $g_2 = 2.053$ and $g_3 = 2.087$ and a hyperfine coupling constant of 16.2 G (Fig. 8) [66]. These simulated values are in good agreement with those of other 5c $\{\text{FeNO}\}^7$

Fig. 6 The $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ backbonding correlation plot illustrates the position of adduct at pH 10.0 and 7.4 relative to other protein heme-CO complexes. Rheds, *star*; globins, *open triangle*; cytochrome P450 s, *open inverted triangle*; horseradish peroxidases, *open circle*; cytochrome c peroxidases, *open square*; [50, 59] and references therein. The *bottom line* correlates $\nu_{\text{Fe-CO}}$ with $\nu_{\text{C-O}}$ for 6c Fe-CO complexes in which the sixth ligand is a thiolate or an imidazolate; the *upper line* represents Fe-CO complexes with histidine (neutral imidazole) as the sixth ligand

hemoproteins [63, 64, 67–70]. No spectral changes were observed with changes in pH (data not shown). The simplest NO-binding model is analogous to that proposed for CO binding (Fig. 7): NO binds to the position that is occupied by the weakly bound protonatable ligand in the high pH 6-coordinate Fe(II) state or is vacant in the low pH 5-coordinate Fe(II) state. The strong trans effect of the NO ligand then causes subsequent loss of the pH-insensitive ligand, thus forming the 5c {FeNO}⁷ species. We cannot rule out the possibility that NO may replace the pH-insensitive ligand first, followed by loss of the protonatable ligand.

An identical 5c {FeNO}⁷ product is formed by direct reaction of Fe(III) Rhed with excess NO. The final product has the same electronic absorption spectrum as that formed from the Fe(II) protein (Fig. 9). Direct reaction of Fe(III) Rhed and NO to form Fe(II)-NO Rhed necessarily involves a multi-step process presumed to be: (1) reduction of Fe(III) to Fe(II) by NO, (2) loss of the two

Fig. 7 The CO- and NO-binding models of Fe(II) Rhed at different pHs

Fig. 8 X-band EPR spectrum of the Fe(II)-NO Rhed protein. *Solid line* is the spectrum. The spectrum is an average of 10 scans taken at 25 K, with 9.385-GHz microwave frequency, 4.000-G modulation amplitude, 100-kHz modulation frequency, 60-dB receiver gain, 81.92-ms time constant, and a power of 10.02 mW. *Dotted line* is the best-fit simulation. The sample contained 136 μM heme, 50 mM EPPS, 400 mM NaCl, pH 8.0, 4.4 mM sodium dithionite

Fig. 9 Electronic absorption spectra that follow the reaction of Fe(III) Rhed and NO(g). **a** The electronic absorption spectra taken every 2 min as Fe(III) Rhed is converted to Fe(II)–NO Rhed. Protein (3.7 μ M heme) was in 50 mM EPPS, 400 mM NaCl, pH 8.0. **b** Absorbance at 450 nm versus time, fit to a single exponential decay. Data were fit in IGOR Pro software

thiolate ligands, and (3) NO binding to Fe(II) heme. We note that the observed rate of formation of Fe(II)–NO Rhed from Fe(III) Rhed is slower than the rate of reduction by dithionite; no visible spectral intermediates are observed and the data are fit well by a single exponential. The apparent first-order rate constant for reaction of Fe(III) Rhed with NO is $9.9 \pm 0.2 \times 10^{-3} \text{ min}^{-1}$ (Fig. 9), while that for reduction of Fe(III) Rhed by dithionite is $7.3 \pm 0.3 \times 10^{-2} \text{ min}^{-1}$ (data not shown), under conditions where NO and dithionite are in large excess but their concentrations are not identical. Presumably, NO must reduce and bind the heme in sequential steps, and reduction and/or thiolate ligand loss is rate determining. Reduction must be first order in heme; if we assume that that reduction is also first order in NO or dithionite, the second-order rate constants are $40 \pm 4 \text{ M}^{-1} \text{ min}^{-1}$ for NO and $73 \pm 3 \text{ M}^{-1} \text{ min}^{-1}$ for dithionite. These values may implicate a distinctive mechanism for reductive

nitrosylation in Fe(III) Rhed [61, 71], which may warrant further study.

Neither CO nor NO alter the pri-miRNA processing activity of Fe(II) NC1

We tested the activity of the gas-bound Fe(II) DGCR8 using reconstituted pri-miRNA processing assays and found that neither Fe(II)–CO NC1 nor Fe(II)–NO NC1 is more active than Fe(II) NC1 (Fig. 10). In these assays, ^{32}P -labeled pri-miR-380 and pri-miR-9-1 fragments are incubated with recombinant Droscha and different forms of NC1, and the reactions are analyzed using denaturing gels and autoradiography. As previously reported, Fe(II) NC1 displays low levels of pri-miRNA processing activity (Fig. 10, lanes 2 in both panels) [18]. (Residual activity is often observed in in vitro assays for DGCR8 proteins that are inactive in human cells.) Importantly, no increased formation of pre-miRNA products is observed for either substrate with Fe(II)–CO NC1 or Fe(II)–NO NC1 relative to Fe(II) NC1 (Fig. 10, lanes 4 and 5). In contrast, simultaneously assayed Fe(III) NC1 incubated with “dead DEANO” is as active as Fe(III) NC1 (Fig. 10, lanes 6 and 7). These latter results indicate that the lack of pri-miRNA processing activity of Fe(II)–NO NC1 is not caused by diethylamine, a product of the NO-releasing compound DEANO.

Discussion

The first objective of this study was to identify the Fe(II) Rhed ligands: we conclude that one ligand is an Lys and the other is His. No crystal structure of Rhed exists; however, within ten residues to either side of Cys-352 in the linear sequence, there are 4 Lys and 2 His that are plausible iron-binding amino acids consistent with the observed spectroscopic characteristics of the Fe(II) Rhed. Electronic absorption and rR spectra of Fe(II) Rhed, as well as the previously published MCD spectra [18], are consistent with Lys as the protonatable ligand. The ferrous bis(methylamine) adduct of H93G myoglobin (Mb) has similar Soret, α and β peak positions to that of Fe(II) Rhed (424, 527, and 559 nm for H93G Mb versus 424, 527, and 558 nm for Rhed) [72]. While these peak positions are also similar to ferrous bis(His)-ligated cytochrome b_5 , the position of the Fe(II) Rhed MCD C-term is more similar to the ferrous bis(methylamine) adduct of H93G Mb than that of bis(His) cytochrome b_5 [18, 72]. The type of pH-dependent behavior exhibited by Fe(II) Rhed is observed in three other primary amine-coordinated proteins: R183K DaCld (Lys/His ligation), ferrocycytochrome f (N-terminal amino/His ligation), and the M100K variant of ferrocycytochrome c -550 (Lys/His ligation) [49, 50, 73, 74]. In addition, Fe(II)

Fig. 10 In vitro pri-miRNA processing assays of Fe(II), Fe(II)–CO and Fe(II)–NO NC1. **a** Pri-miR-380 and **b** pri-miR-9-1 were uniformly labeled with ^{32}P . Pri-miRNAs were incubated at 37 °C for 45 min with purified recombinant His₆-Drosha^{390–1374} and 25 nM

various forms of NC1 as indicated in the figure. The reactions were analyzed using 15 % denaturing polyacrylamide gels. Lanes 3 and 6 (label includes “dead DEANO”) are controls to check for any non-specific effects of inactivated DEANO, the NO-releasing compound

Rhed has similar electronic absorption spectral features as Lys-ligated L116K CoxA [75]. The pH dependence of the Fe(II) Rhed rR spectrum provides insight into the identity of the pH-insensitive ligand: studies on model complexes demonstrate that Fe–N stretching bands are better resonantly enhanced by the B band in 5-coordinate species than in 6-coordinate species, where the nitrogen-containing species is imidazole-like. One may infer from the pH-dependent behavior of Fe(II) Rhed, that the Lys ligand is less tightly bound to heme than the His ligand.

We then sought to explore the hypothesis that signaling gases CO or NO might bind to DGCR8 and modulate pri-miRNA processing activity, thus connecting the activity of the Microprocessor complex to the CO or NO signaling pathways. Plausible scenarios for such modulation include: (1) CO or NO binding to Fe(II) DGCR8 restores Microprocessor activity, or (2) direct NO binding to Fe(III) DGCR8 bypasses the inactive Fe(II) state and changes Microprocessor activity. Our previous studies showed that Fe(III) NC1 DGCR8 supports pri-miRNA processing, while the Fe(II) protein does not [18]. Furthermore, the two cysteine ligands that bind to the heme in Fe(III) DGCR8 are lost upon reduction to the Fe(II) state and these cysteine ligands are not fully regained upon re-oxidation. Formation of a CO-bound product via ligand replacement from 6-coordinate Fe(II) heme, as observed herein for Fe(II) Rhed at high pH, is common among CO-sensing proteins; formation of a 5-coordinate nitrosyl adduct, as observed herein for Fe(II) Rhed at low and high pH, is also observed for

some NO sensors, like sGC. Therefore, it appeared plausible that pri-miRNA processing activity might be CO or NO regulated, as the binding of these ligands inevitably lead to heme pocket rearrangement. Our observation that Fe(II), Fe(II)–CO, and Fe(II)–NO NC1 are all nearly inactive in pri-miRNA processing refutes this hypothesis.

Even though these gases do not appear to modulate Microprocessor activity, CO and NO can serve as structural probes of the heme environment and provide further information as to how heme coordination influences DGCR8 function. Earlier studies of Fe(II) NC1 and Fe(II) Rhed showed that these proteins had different electronic absorption spectra at high and low pH, but the origin of these differences was unknown [18]. The pH-dependent ligation of Fe(II) Rhed described herein explains this observation. The differences in electronic absorption spectra are readily explained by the fact that at low pH, the heme was mixture of 5- and 6-coordinate as the Lys ligand became partially protonated, while at high pH, the heme was largely 6-coordinate with the deprotonated Lys ligand bound to heme. The studies herein further reveal that major alterations in the heme environment are necessary for reduction and gas binding. Reduction of the heme proceeds at a slow rate: 1–2 h at elevated temperatures is required to completely reduce heme in Rhed. The result of this reduction process is replacement of the native bis-Cys thiolate ligands characteristic of Fe(III) Rhed with Lys and His ligands in Fe(II) Rhed. Long reduction time at elevated temperature implies that this ligand replacement requires a major reorganization

of the structure within the heme pocket. Furthermore, the binding of CO to Fe(II) Rhed requires a further rearrangement of the heme environment: the spectral features of Fe(II) Rhed are pH-dependent because of the Lys ligand, but those of Fe(II)–CO Rhed are not pH-dependent. The absence of pH dependence in the Fe(II)–CO Rhed spectra suggests that CO replaces Lys and that Lys does not remain in the distal heme pocket. These structural rearrangements of the local heme pocket likely coincide with global rearrangements of protein structure, which in turn impair Microprocessor activity.

Inactivation of pri-miRNA processing activity may occur at any step in the processing pathway: the active complex must include the pri-miRNA substrate, DGCR8 and Drosha, in a catalytically competent assembly. DGCR8 binds to the pri-miRNA via its unique RNA-binding heme domain (Rhed) and its double-stranded RNA-binding domains (Fig. 1b), and triggers pri-miRNA cleavage by Drosha. Importantly, the Rhed makes critical contributions to the RNA binding specificity by contacting the stem–single-strand junctions [11, 14], which are known to be important for recognition of a pri-miRNA hairpin [76–78]. Removal of heme from NC1 does not appear to alter the affinity for pri-miRNA, but the complexes formed are different from those with Fe(III) heme bound [11]. Loss of processing activity upon reduction of the heme iron may be due to aberrant pri-miRNA binding, loss of or aberrant Drosha binding, or inactivation of the Drosha/DGCR8/pri-miRNA complex. Of the characterized DGCR8 heme states, only Fe(III) DGCR8 is capable of fully supporting pri-miRNA processing. The unique bis-Cys coordination environment of the Fe(III) DGCR8 heme plays a role in conferring the correct active conformation upon the DGCR8 protein.

Recent studies on DGCR8 and Drosha provide insight into plausible connections between DGCR8 heme and pri-miRNA binding by the Microprocessor complex. A crystal structure of human Drosha bound to the C-terminal helix of DGCR8 was recently solved; this structure was used to build a model for how Drosha might interact with the pri-miRNA substrate and a pair of DGCR8 dimers. This hypothetical model of the Microprocessor complex implies that the Rhed region of DGCR8 dimers binds the apical stem–loop junction of the pri-miRNA, with the DGCR8 dimers oriented in an asymmetrical fashion [13]. This model is reinforced by biochemical studies, which clearly implicate selective DGCR8 binding to the apical stem–loop junction in a ratio of 2:1:1 (DGCR8 dimer:Drosha:pri-miRNA substrate) [12]. Heme influences the binding of DGCR8 to its pri-miRNA substrate. SEC studies showed that pri-miRNA-bound apo NC1 eluted at a different volume and with a different substrate ratio than Fe(III)-heme-loaded NC1, suggesting that the presence of heme causes a substantial

structural rearrangement of the NC1–pri-miRNA complex [11]. Importantly, both heme-bound and apo NC1 bind to pri-miRNA, but the nature of these binding events must be fundamentally different, implying that Fe(III) heme is essential for the appropriate formation of the RNA-bound Microprocessor complex. The functional importance of heme in a native coordination environment was probed by replacement of heme with Co(III) protoporphyrin IX (PPIX) in cellulo in heme deficient HeLa cells and in vitro with Co(III)PPIX-loaded NC1. Microprocessor activity is nearly the same in Co(III)-loaded protein as in the natively heme-loaded protein [79]. Co(III)PPIX-reconstituted NC1 exhibits a hyperporphyrin spectrum, suggesting that there are two bound Cys(thiolate) ligands in the Co(III) NC1 protein. The suggested presence of the native bis-Cys ligation motif in Co(III) DGCR8 supports the hypothesis that Microprocessor activity is dependent on maintaining these metal-bound ligands. Similar behavior is observed in the heme-containing enzyme cystathionine β -synthase (CBS), which displays normal activity when heme is replaced by Co(III)PPIX [80]; like NC1, CBS activity is dependent on maintaining its Fe(III) thiolate ligand, which is irreversibly disrupted by reducing the heme and/or binding small gases. In both these heme proteins, the specific role of the heme is still unknown but the integrity of the Fe(III) ligation environment is essential to activity.

Conclusion

We postulate that loss of the native bis-Cys ligation motif of Fe(III) DGCR8 is responsible for the loss of Microprocessor activity in the Fe(II), Fe(II)–CO and Fe(II)–NO states. There are two caveats to these conclusions: (1) since the studies reported are on DGCR8 truncates, it is possible that the full-length protein may behave differently, and (2) RNA or Drosha binding may alter coordination of the DGCR8 heme. In the Fe(III) state, the Rhed and NC1 hemes display a hyperporphyrin spectrum consistent with unique bis-Cys ligation [16, 81]. Cys is not bound to the Rhed heme in the Fe(II) and Fe(II)–CO states; these species have different ligation environments from the Fe(III) protein, in which one or two neutral donors are coordinated. In Fe(II)–NO Rhed, neither the two Cys ligands of the Fe(III) state nor the neutral donor(s) of the Fe(II) state remain bound to the heme iron. The heme in Fe(II) Rhed is bound to at least one Lys ligand, since the pK_a of the protonatable sixth ligand is 9.1. The more tightly bound fifth ligand present in Fe(II) Rhed, a histidine, is retained in the 6cLS CO-bound product. Since neither Fe(II), Fe(II)–CO nor Fe(II)–NO NC1 is active, preservation of bis-thiolate ligation appears to be required for pri-miRNA processing activity. Two other observations point to the importance of the native bis-Cys

heme coordination. Reduction of Fe(III) Rhed is slow even at elevated temperatures, presumably due to slow replacement of the two thiolate ligands. Re-oxidation of Fe(II) Rhed does not completely restore the bis-Cys Fe(III) species. Taken together with the fact that none of the Fe(II) forms of NC1 are functionally active, it appears unlikely that either heme reduction or small gas molecule binding are regulatory mechanisms for DGCR8-dependent pri-miRNA processing. The data support a model where maintaining the native thiolate ligands to the DGCR8 heme is essential to Microprocessor function.

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