Isotopic Labeling

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Acidity and Hydrogen Exchange Dynamics of Iron(II)-Bound Nitroxyl in Aqueous Solution**

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Abstract: *Nitroxyl-iron(II) (HNO-Fe^{II}) complexes are often unstable in aqueous solution, thus making them very difficult to study. Consequently, many fundamental chemical properties of Fe^{II}-bound HNO have remained unknown. Using a comprehensive multinuclear (^1H, ^{15}N, ^{17}O) NMR approach, the acidity of the Fe^{II}-bound HNO in [Fe(CN)$_5$(HNO)]$^{3-}$ was investigated and its pK$_a$ value was determined to be greater than 11. Additionally, HNO undergoes rapid hydrogen exchange with water in aqueous solution and this exchange process is catalyzed by both acid and base. The hydrogen exchange dynamics for the Fe^{II}-bound HNO have been characterized and the obtained benchmark values, when combined with the literature data on proteins, reveal that the rate of hydrogen exchange for the Fe^{II}-bound HNO in the interior of globin proteins is reduced by a factor of 10^6.*

Nitroxyl (HNO) is among a small group of redox-related nitrogen oxides such as nitric oxide (NO$^{\bullet}$), nitrosonium cation (NO$^+$), nitrite (NO$_2^-$), and nitrate (NO$_3^-$) which have significant biological functions.[1,2] In recent years, HNO has attracted considerable attention because it is not only involved in a variety of enzymatic reactions, but also potentially a pharmaceutical entity in treating various diseases.[3–11] Therefore, it is important to understand the fundamental chemistry of nitroxyl compounds. To this end, investigations of model heme and nonheme nitroxyl–metal complexes will shed light onto nitroxyl reactivity.[12] Surprisingly, while the HNO adducts of globin proteins are known to be remarkably stable,[13] small molecules of the {Fe^{II}-HNO}^{8}-type in aqueous solution are extremely rare.[14] As a result, many fundamental aspects of {Fe^{II}-HNO}^{8} chemistry remain unexplored. In 2009, Olabe and co-workers[15] reported a convenient preparation of [Fe^{II}(CN)$_5$(HNO)]$^{3-}$ in aqueous solution by two-electron reduction of sodium nitroprusside (SNP; Na$_2$[Fe^{II}(CN)$_5$(NO)]·2H$_2$O) with Na$_2$S$_2$O$_4$. They showed that [Fe^{II}(CN)$_5$(HNO)]$^{3-}$ is reasonably stable under slightly acidic conditions. In the same study, the authors also reported the pK$_a$ value for the HNO acid-base ionization equilibrium in this Fe^{II}-HNO complex to be 7.7. This pK$_a$ value is highly unusual considering the fact that the corresponding pK$_a$(^{1}HNO/^{1}NO$^-$) for free HNO is 23.[16] In addition, Farmer and co-workers[12f] observed that the Fe^{II}-bound HNO in Mb-HNO has a pK$_a$ value of greater than 10. Other experimental observations also suggested that low-spin {Fe^{II}-NO$^-$}^{8} complexes are very basic.[17] In this work, we set out to re-examine the pK$_a$ value of HNO in [Fe^{II}(CN)$_5$(HNO)]$^{3-}$ by using a new ^{17}O NMR approach.

Figure 1a shows that, upon addition of 2.8 equivalents of Na$_2$S$_2$O$_4$ to 0.6 mM [Fe(CN)$_5$(NO)]$^{2-}$(aq), the characteristic ^{1}H NMR signal at $\delta = 20.16$ ppm was observed, together with a new absorption band at $\lambda_{max} = 448$ nm and two new IR bands at $\nu_{NO} = 1352$ and $\nu_{CN} = 2044$ cm^{-1} (data shown in Figures S1 and S2 of the Supporting Information), thus confirming the formation of [Fe^{II}(CN)$_5$(HNO)]$^{3-}$.[15] To further establish the identity of the ^{1}H NMR signal, we made an ^{15}N-enriched sample of [Fe^{II}(CN)$_5$(HNO)]$^{3-}$. As seen from Figure 1b, since the ^{15}N enrichment in the sample is only 60%, the ^{1}H NMR signals from both ^{1}H^{15}NO and ^{1}H^{14}NO are observed simultaneously. This data allowed us not only to measure $^1J(^1H,^{15}N) = (71.3 \pm 0.3)$ Hz, but also to determine very accurately the secondary isotope shift, $^1\Delta H(^{14}N,^{15}N) = (5.3 \pm 0.5)$ ppb. Since the [Fe^{II}(CN)$_5$(HNO)]$^{3-}$ complex was reasonably stable in aqueous solution, having a half-life of several hours (see Figure S3), we also obtained a two-dimensional ^{1}H–^{15}N heteronuclear correlation NMR spectrum, from which the ^{15}N chemical shift for HNO in [Fe^{II}(CN)$_5$(HNO)]$^{3-}$ was determined to be $\delta = 642.8$ ppm (relative to liquid NH$_3$). This ^{15}N chemical shift value is comparable to that observed

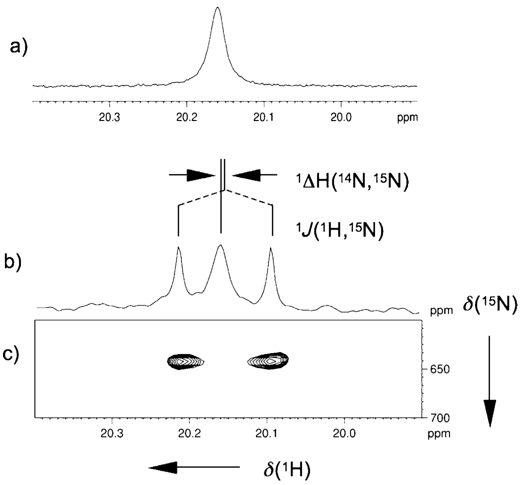

Figure 1. a) ^{1}H NMR spectrum of 0.6 mM [Fe(CN)$_5$(HNO)]$^{3-}$(aq). b) ^{1}H and c) ^{1}H–^{15}N 2D heteronuclear correlation NMR spectra of [Fe(CN)$_5$(H^{15}NO)]$^{3-}$(aq) (60% ^{15}N atom). The pH of the NMR samples was 6.8.

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for the Fe^{II}-bound HNO in the HNO adducts of globin proteins.[13e]

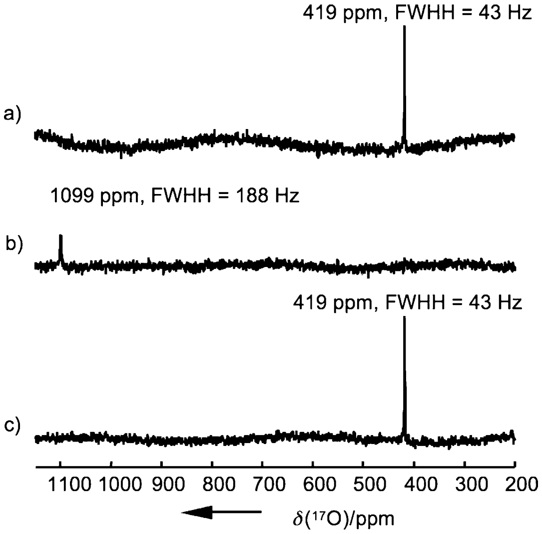

Figure 2. ^{17}O NMR spectra of a) ^{17}O-labeled SNP, b) freshly prepared $[Fe(CN)_5(HN^{17}O)]^{3-}$, and c) fully oxidized $[Fe(CN)_5(HN^{17}O)]^{3-}$ after three days in the NMR tube.

Because direct ^{1}H NMR detection of the proton involved in the acid–base equilibrium is not a reliable method and ^{15}N usually has very long spin-lattice relaxation times, we decided to explore the utility of ^{17}O ($I = 5/2$) NMR spectroscopy. As ^{17}O has a very low natural abundance (0.037 %) we first prepared ^{17}O-labeled SNP and then converted it into $[Fe(CN)_5(HN^{17}O)]^{3-}$. As seen from Figure 2, ^{17}O-labeled SNP exhibits a sharp ^{17}O NMR signal at $\delta = 419$ ppm with full-width at the half height (FWHH) being only 43 Hz. This ^{17}O chemical shift is comparable to that found for the free nitrosonium ion, NO^{+}, at $\delta = 474$ ppm.[18] Upon addition of $Na_2S_2O_4$ to the SNP solution, a much broader ^{17}O NMR signal was detected at $\delta = 1099$ ppm (FWHH = 188 Hz), which can be attributed to $[Fe(CN)_5(HNO)]^{3-}$. This assignment was further confirmed by the UV/Vis and ^{1}H NMR spectra of the same sample (see Figure S4). As also seen from Figure 2, $[Fe(CN)_5(HNO)]^{3-}$ was eventually oxidized back to SNP in a quantitative fashion. The observed and very paramagnetically shifted (deshielded) ^{17}O NMR signal for HNO is in agreement with those reported for the related C-nitrosoarene compounds (Ar-N = O) at $\delta(^{17}O) \approx 1250$–$1550$ ppm.[18,19] The larger ^{17}O NMR line width observed for HNO is also expected on the basis of the fact that C nitrosoarenes have ^{17}O quadrupolar coupling constants, $[C_Q(^{17}O)]$ on the order of 15 MHz,[19] and that aldehydes (H-C=O) also have $C_Q(^{17}O) \approx 11$ MHz.[20] In contrast, the nitrosonium ion $[N{\equiv}O]^{+}$ is isoelectronic to $C \equiv O$ which has $C_Q(^{17}O) = 4.43$ MHz.[21] This data taken together is the first time that NMR parameters for all three magnetic nuclei in HNO are reported for nitroxyl compounds.

Having established the ^{17}O NMR signature for the Fe^{II}-bound HNO, we turned attention to the measurement of its pK_a value. To this end, we recorded the ^{17}O NMR spectra for $[Fe(CN)_5(HNO)]^{3-}$ as a function of pH. Remarkably, the ^{17}O NMR signal does not show any noticeable change over a pH range from 5.46 to 10.51, neither the signal intensity nor its chemical shift (data are shown in Figure S5). Unfortunately, $[Fe(CN)_5(HNO)]^{3-}$ becomes too unstable above pH 11 to be studied by NMR spectroscopy. Nevertheless, the ^{17}O NMR results clearly show that the HNO ligand in $[Fe(CN)_5(HNO)]^{3-}$ has a pK_a value of greater than 11.

To reconcile the discrepancy between our new ^{17}O NMR results and the earlier ^{1}H NMR observation of Olabe and co-workers,[15] we carefully re-examined the ^{1}H NMR properties of $[Fe(CN)_5(HNO)]^{3-}$. We discovered that the hydrogen atom in HNO undergoes a pH-dependent, rapid exchange with water. If the pre-saturation method was used for water suppression, the ^{1}H NMR signal for HNO disappears rapidly from pH 6.70 to 7.96, just as previously reported.[15] However, if the WATERGATE sequence was used, the ^{1}H NMR signal becomes progressively broader as the pH increases above pH 6.70, but it retains its integrated area (see Figure S6). These observations immediately suggest that the apparent titration behavior reported by Olabe and co-workers[15] is an artefact resulting from the hydrogen exchange between HNO and water, and not arising from the acid-base equilibrium of HNO. Indeed, the hydrogen–deuterium (H–D) exchange experiments indicate that the H–D exchange is complete within seconds.

To gain further insights into the hydrogen exchange dynamics in the Fe^{II}-bound HNO, we recorded the ^{1}H NMR spectra of $[Fe^{II}(CN)_5(HNO)]^{3-}$ over the pH range of 1.44–9.58 (see Figure S7). The hydrogen exchange rate, k_{ex}, can be directly determined from the observed NMR line width, $(1/\pi T_2)_{obs}$ [Equation (1)].

$$(1/\pi T_2)_{obs} = (1/\pi T_2)_{non\text{-}ex} + (k_{ex}/\pi) \quad (1)$$

Herein, $(1/\pi T_2)_{non\text{-}ex}$ is the intrinsic line width in the absence of hydrogen exchange. Here we estimated $(1/\pi T_2)_{non\text{-}ex}$ to be 13.7 Hz from the ^{1}H NMR spectrum of $[Fe^{II}(CN)_5(HNO)]^{3-}$ obtained at pH 5.53, at which the hydrogen exchange is negligible. The V-shaped data seen in Figure 3 resemble the well-known acid/base catalyzed hydrogen exchange process in amides, including proteins, and can be fitted to Equation (2).[22]

$$k_{ex} = k_H[H^{+}] + k_{OH}[OH^{-}] + k_0 \quad (2)$$

Herein, k_H and k_{OH} are the rate constants for the acid- and base-catalyzed reactions, respectively, and k_0 is that for direct exchange with water. As seen in Figure 3 a, the data can be fitted quite well with the first-order rate process at both sides of the minimum point. Our analysis yields: $k_0 = 0$, $k_H = 2.5 \times 10^{4}$ and $k_{OH} = 2.0 \times 10^{7}\,M^{-1}s^{-1}$.

We also performed variable-temperature (VT) ^{1}H NMR experiments for $[Fe^{II}(CN)_5(HNO)]^{3-}$ at pH 1.44 and 9.45 (Figure 3 b) and obtained the activation energy for the base- and acid-catalyzed hydrogen exchange to be 12.1 and 7.6 kcal mol^{-1}, respectively. These activation energies are much smaller than those for amides: 23 and 17 kcal mol^{-1} for base- and acid-catalyzed exchange, respectively.[22c] Over-

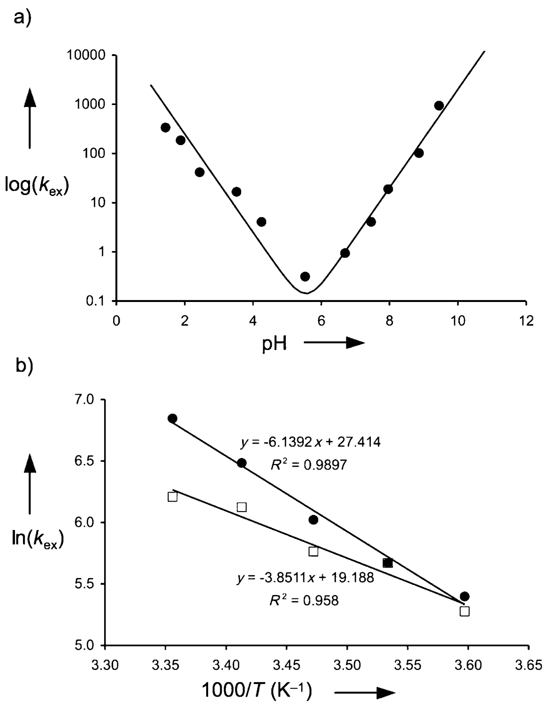

Figure 3. a) Dependence of the exchange rate, k_{ex}, on pH. b) Arrhenius plot of the exchange data for the Fe-bound HNO in $[Fe(CN)_5(HNO)]^{3-}$ obtained at pH 9.45 (filled circles) and 1.44 (open squares). All experiments in (a) were performed at 298 K.

all the hydrogen exchange in HNO is strikingly similar to those in amides and in the hydroxyl groups of protein side chains.[23] It is rather tempting to speculate an intrinsic link between hydrogen exchange dynamics and the acidity of these functional groups (Fe^{II}-HNO: $pK_a > 11$; amides: $pK_a > 15$; serine and threonine: pK_a 13; tyrosine: $pK_a > 10$). For hydrogen exchange between HNO and water, we propose the following mechanisms for base and acid catalysis [Equation (3) and [4], respectively].

$$H{-}N{=}O + OH^- \rightleftharpoons {}^-N{=}O + H{-}O{-}H \tag{3}$$

$$H{-}N{=}O + H^+ \rightleftharpoons H{-}N{=}O^+H \rightleftharpoons H^+ + {}^-N{=}O^+H \tag{4}$$

In the base-catalyzed exchange, hydroxide removes the proton from HNO to produce the singlet NO^- anion, which is then re-protonated by water. In the acid-catalyzed exchange, protonation of the oxygen atom in HNO acidifies the nitrogen to allow water to remove the NH proton, thus producing the singlet NOH tautomer, which returns to HNO by reversing the steps.

More interestingly, Farmer and co-workers[13f] reported that, in the Mb-HNO adduct, the H–D exchange rate is about $3.5 \times 10^{-5}\,s^{-1}$ at pH 8. For $[Fe^{II}(CN)_5(HNO)]^{3-}$ at the same pH, we found that k_{ex} is $20\,s^{-1}$, as seen from Figure 3. This means that, when the Fe^{II}-bound HNO is located in the interior of a protein, the hydrogen exchange rate between HNO and water is reduced by a factor of 10^6! Possibly, the strong hydrogen bonding between HNO and the protein, as proposed by Farmer and co-workers[13f] and by Zhang and co-workers,[24] may also contribute to this large reduction in the hydrogen exchange rate. Regardless, our work on hydrogen exchange dynamics in $[Fe^{II}(CN)_5(HNO)]^{3-}$ provides a benchmark for a naked (thus fully exposed to solvent) Fe^{II}-bound HNO, which will be useful for further interpretation of any protein data.

To better understand the NMR parameters observed for Fe^{II}-HNO complexes, we performed quantum chemical calculations (computational details were given in the Supporting Information). Figure 4 shows the DFT-optimized molecular structures for $[Fe^{II}(CN)_5(HNO)]^{3-}$ and $[Fe^{II}(CN)_5(NO)]^{4-}$, corresponding to the protonated and deprotonated states of the Fe^{II}-HNO complex. The obtained structural features are similar to those computed for the related Fe^{II}-HNO complexes.[25] Table 1 summarizes the computed ^1H, ^{15}N, and ^{17}O chemical shifts for the two nitroxyl complexes and for free HNO. In general, the computed NMR chemical shifts for $[Fe^{II}(CN)_5(HNO)]^{3-}$ are in reasonable agreement with our experimental data. In addition, our computational results are also consistent with those reported by Zhang and co-workers[26] on ^1H and ^{15}N chemical shifts. As mentioned earlier, since $[Fe(CN)_5(HNO)]^{3-}$ is too unstable at pH values greater than 11, we were unable to obtain ^{15}N and ^{17}O chemical shifts for $[Fe^{II}(CN)_5(NO)]^{4-}$. However, two recent reports on analogous Fe^{II}-NO^- complexes suggest that the ^{15}N chemical shift for the deprotonated Fe^{II}-bound nitroxyl is about $\delta = 1100$–1200 ppm (relative to liquid

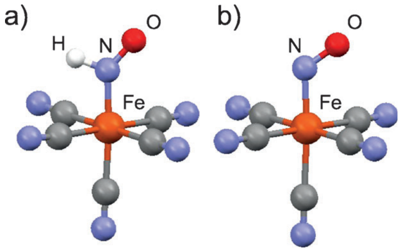

Figure 4. DFT optimized molecular structures of a) $[Fe(CN)_5(HNO)]^{3-}$ and b) $[Fe(CN)_5(NO)]^{4-}$. Selected bond lengths [Å] and angles [°]: a) N-H 1.037, N-O 1.242, Fe-N 1.816; Fe-N-O 137.9; b) N-O 1.239, Fe-N 1.890; Fe-N-O 124.8.

Table 1: Experimental and computed ^1H, ^{15}N, and ^{17}O NMR chemical shifts for nitroxyl compounds.

Compound	Method	^1H δ [ppm]	^{15}N δ [ppm]	^{17}O δ [ppm]
$[Fe(CN)_5(HNO)]^{3-}$	Exptl	20.2	640	1099
	G-09	23.1	776.4	1132.8
	ADF	22.2	610.2	973.2
$[Fe(CN)_5(NO)]^{4-}$	G-09		1769.5	1896.9
	ADF		1672.9	1761.6
HNO	G-09	31.2	1297.7	2376.4
	ADF	36.4	1324.5	2402.5

NH_3).[25d,e] Considering the fact that magnetic shielding calculations are known to overestimate the paramagnetic shielding contribution (thus producing higher chemical shift values) especially for systems exhibiting low-lying excited states,[26] the computed NMR chemical shifts shown in Table 1 are reasonable. The exceedingly large reduction in both ^{15}N and ^{17}O chemical shifts, upon coordination of HNO to Fe^{II}, is entirely consistent with the observation in C-nitroso compounds and can be largely attributed to the paramagnetic shielding contributions resulting from magnetic-field induced mixing between occupied and unoccupied MOs.[19,27] In the present cases, the most relevant ligand-centered MOs are the occupied π^*_h and unoccupied π^*_v. In $\{Fe^{II}\text{-}HNO\}^8$, the strong π-backbonding from Fe^{II} significantly reduces the paramagnetic shielding contributions (thus resulting in smaller chemical shifts) as compared to a free HNO. A lesser degree of π-backbonding is expected in $\{Fe^{II}\text{-}NO^-\}^8$ relative to that in $\{Fe^{II}\text{-}HNO\}^8$. In addition, π^*_h becomes the HOMO and the HOMO–LUMO gap is also smaller in $\{Fe^{II}\text{-}NO^-\}^8$. Both these factors cause an increase in paramagnetic shielding in $\{Fe^{II}\text{-}NO^-\}^8$ as compared to $\{Fe^{II}\text{-}HNO\}^8$.

In summary, we have obtained for the first time NMR data for all three magnetic nuclei (^1H, ^{15}N, ^{17}O) in a Fe^{II}-bound HNO. The ^{17}O NMR results unambiguously show that the pK_a value of the Fe^{II}-bound HNO is greater than 11. We have also discovered that HNO undergoes rapid hydrogen exchange with water in aqueous solution and this process is catalyzed by acid and base in a remarkably similar fashion as those previously known for amides and for hydroxyl groups in protein side chains. We speculate that the striking similarity in the hydrogen exchange dynamics among these functional groups may also reflect their weak acidity. We hope that this report will encourage others to consider ^{17}O NMR spectroscopy as a viable new technique for probing nitroxyl–metal interactions.

Experimental Section

Chemicals were obtained from Sigma–Aldrich unless stated otherwise: sodium nitroprusside (SNP, $Na_2[Fe(CN)_5NO]\cdot 2H_2O$), sodium hydroxide (NaOH), sodium dithionite ($Na_2S_2O_4$), ^{15}N-labeled sodium nitrite ($Na^{15}NO_2$, 98% ^{15}N), ^{17}O-labeled water ($H_2{}^{17}O$, 41.1% ^{17}O, purchased from CortecNet), ethylenediaminetetraacetic acid (EDTA, $C_{10}H_{16}N_2O_8$), sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), Sodium chloride (NaCl), potassium cyanide (KCN), and ion-exchange resin (Amberlite IR-120, strongly acidic form).

$Na_2[Fe(CN)_5(^{15}NO)]\cdot 2H_2O$ was prepared in aqueous solution by mixing commercial SNP (260 mg) with 1.1 molar equivalents of NaOH and 1.9 molar equivalents of $Na^{15}NO_2$. The reaction mixture was kept at room temperature for 10 min, following by addition of 285 μL of 4 M acetic acid. The reaction mixture was concentrated to a paste on rotary evaporator. The residual material was dissolved in 2 mL of 4 M acetic acid and the solvent was evaporated on rotary evaporator. This process was repeated once. The residue was treated with 1,4-dioxane (4 mL); solid material was collected by filtration, washed with ethanol (2 × 3 mL), dried under vacuum, to give the title compound as red powder (153 mg, 59%). The ^{15}N enrichment in the product was 60%. ^{15}N NMR (50.6 MHz, D_2O): $\delta = 371$ ppm (ref. to liquid NH_3); ^{13}C NMR (125.6 MHz, D_2O): $\delta = 134.81$ ppm (eq) and 132.84 ppm (ax).

$Na_2[Fe(CN)_5(N^{17}O)]\cdot 2H_2O$ was prepared by mixing 74.0 mg commercial SNP and 9.9 mg NaOH in 300 μL $H_2{}^{17}O$ (41.1% ^{17}O). The work-up procedures were the same as that used in preparing the ^{15}N-labeled SNP. The ^{17}O enrichment in the product was 20%. ^{17}O NMR (67.7 MHz, D_2O): $\delta = 419$ ppm (ref. to water), ^{13}C NMR (125.6 MHz, D_2O): $\delta = 134.81$ ppm (eq) and 132.84 ppm (ax).

For the synthesis of $Na_3[Fe(CN)_5(HNO)]$, the literature method[15] was used with minor modifications. Typically, to the 0.6 mM SNP solution prepared freshly in deoxygenated buffer (0.05 M sodium carbonate, containing 0.1 M NaCl, 0.5 mM EDTA and 12 mM KCN; pH 10) was added a solution of $Na_2S_2O_4$ (3 molar equivalents) freshly prepared in 0.2 M deoxygenated NaOH. The pH of the solution was adjusted to 6.8 using Amberlite IR-120 (strongly acidic form). In the solution the complex exhibits a characteristic absorption band at $\lambda_{max} = 448$ nm in the UV/Vis spectrum. ^{15}N- and ^{17}O-labeled complexes were prepared in the same fashion, except that ^{15}N- and ^{17}O-labeled SNPs were used, respectively.

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