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Effect of Ascorbate on the Cyanide-Scavenging Capability of Cobalt(III) *meso*-Tetra(4-*N*-methylpyridyl)porphine Pentaide: Deactivation by Reduction?

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Abstract

The Co(III)-containing water-soluble metalloporphyrin cobalt(III) *meso*-tetra(4-*N*-methylpyridyl)porphine pentaide (Co^{III}TMPyP) is a potential cyanide-scavenging agent. The rate of reduction of Co^{III}TMPyP by ascorbate is facile enough that conversion to the Co(II)-containing Co^{II}TMPyP should occur within minutes at prevailing *in vivo* levels of the reductant. It follows that any cyanide-decorporating capability of the metalloporphyrin should depend more on the cyanide-binding characteristics of Co^{II}TMPyP than those of the administered form, Co^{III}TMPyP. Addition of cyanide to buffered aqueous solutions of Co^{II}TMPyP (pH 7.4, 25–37 °C) results in quite rapid ($k_2 = \sim 10^3 \text{ M}^{-1} \text{ s}^{-1}$) binding/substitution of cyanide anion in the two available axial positions with high affinity ($K'_\beta = 10^{10}$ to 10^{11}). Electron paramagnetic resonance spectroscopic measurements and cyclic voltammetry indicate that cyanide induces oxidation to the Co(III)-containing dicyano species. The constraints that these observations put on plausible mechanisms for the reaction of Co^{II}TMPyP with cyanide are discussed. Experiments in which Co^{III}TMPyP and cyanide were added to freshly drawn mouse blood showed the same sequence of reactions (metalloporphyrin reduction $\rightarrow$ cyanide binding/substitution $\rightarrow$ reoxidation) to occur. Therefore, in cyanide-scavenging applications with this metalloporphyrin, we should be taking advantage of both the improved rate of ligand substitution at Co(II) compared to that at Co(III) and the increased affinity of Co(III) for anionic ligands compared to that of Co(II). Finally, using an established sublethal mouse model for cyanide intoxication, Co^{III}TMPyP, administered either 5 min before (prophylaxis) or 1 min after the toxicant, is shown to have very significant antidotal capability. Possible explanations for the results of a previous contradictory study, which failed to find any prophylactic effect of Co^{III}TMPyP toward cyanide intoxication, are considered.

Graphical Abstract

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Notes

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INTRODUCTION

In a previous study, we demonstrated the efficacy of the metalloporphyrin cobalt(III) *meso*-tetra(4-*N*-methylpyridyl)-porphine penta iodide (Co^{III}TMPyP) as an antidote toward cyanide toxicity in mice.¹ Consequently, the contradictory results of an earlier investigation² suggesting that cobalt(III) porphyrins are ineffective as cyanide antidotes when given to mice prophylactically remain intriguing. While some effort has been expended on Co^{III}TMPyP and other water-soluble cobalt porphyrins in the past,^{2–5} there seems to be no recent work addressing the possible development of these compounds as cyanide scavengers. This is unfortunate given the renewed interest in the availability of cyanide-binding compounds to treat victims of smoke inhalation.^{6–8} We continue with the long-term objective of finding cyanide-binding agents that are cheaper and similarly effective to known antidotal compounds like cobalamin and cobinamide. Herein, hypothesizing that the apparent lack of prophylaxis exhibited by Co^{III}TMPyP in cyanide-intoxicated mice² might be a consequence of the reduction of the complex to the Co(II) form by physiological reductants circulating in the blood, we have (i) undertaken an investigation into the kinetics of reduction of Co^{III}TMPyP by ascorbate, (ii) determined the equilibrium and kinetic cyanide-binding characteristics of the reduced product, Co^{II}TMPyP, (iii) verified that the same reactions, relevant to the antidotal activity of CoTMPyP toward cyanide, occur *in vivo* in the mouse bloodstream, and (iv) reassessed the issue of whether CoTMPyP is antidotal toward cyanide toxicity in mice when given both before (prophylaxis) and after the toxicant.

EXPERIMENTAL SECTION

Materials

All reagents were ACS grade and obtained from Sigma-Aldrich unless otherwise stated. Cobinamide was prepared by acid hydrolysis of hydroxocobalamin and purified essentially by reported chromatographic procedures,^{9,10} and the purity of the final product was assessed by comparison of its electronic absorption characteristics with those of published spectra.^{9,11} The metal-ion-free water-soluble porphyrin 4,4',4'',4'''-porphyrin-5,10,15,20-tetrayltetrakis(1-methylpyridinium) tetrakis(4-methylbenzenesulfonate) (H₂TMPyP) and its cobalt complex [[4,4',4'',4'''-porphyrin-5,10,15,20-tetrayltetrakis(1-methylpyridiniumato)] (2-)] cobalt(III) penta iodide (Co^{III} *meso*-tetra(4-*N*-methylpyridinyl) porphine, Co^{III}TMPyP) were synthesized as previously reported.^{1,4,12} The presence of the target macrocyclic structures was verified by electrospray mass spectrometry: H₂TMPyP, 169.5 calculated for $m/z = C_{44}H_{38}N_8/4^+$; 196.5 found; CoTMPyP, 183.7 calculated for $m/z = CoC_{44}H_{36}N_8/4^+$;

183.6 found.^{1,13} The synthesized complex has previously been determined to be diamagnetic¹² and, therefore, to contain Co(III). As prepared, the product was confirmed to contain Co(III) by electron paramagnetic resonance (EPR). Initially, there were no EPR signals to be observed, but upon reduction of the complex with sodium ascorbate, Co(II) EPR signals became readily detectable. However, the mass spectral data indicates the presence of Co(II) rather than Co(III), so reduction must have occurred on the probe during introduction to the mass spectrometer.^{1,13} In the kinetic and titration procedures, concentrations of Co^{III}TMPyP solutions were determined using the reported extinction coefficient $\epsilon_{437} = 1.7 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Titration

For determinations of cyanide-binding equilibria,¹³ all solutions were maintained in capped (septum sealed, with head volumes minimized) vessels and transfers were made with gastight syringes. Small aliquots of relatively concentrated cyanide solutions (buffered with 5 mM sodium borate, pH 11) were titrated into larger volumes of relatively dilute solutions of Co^{II}TMPyP (buffered with 50 mM sodium phosphate and 1 mM EDTA, pH 7.4) to maintain neutrality at both 25 and 37 °C. As multiple fast and slow cyanide-binding forms are possible, solutions were allowed to equilibrate for ~10 min after the addition of cyanide prior to recording the resultant absorption changes. Binding constants were determined by the construction of Hill plots. The saturation of the Co^{II}TMPyP with cyanide was determined from the absorbance changes, where Y represents the proportion of sites occupied by cyanide (or fractional saturation), which was plotted against the concentration of the free cyanide concentrations. Fits of the data yielded cooperativity values and binding constants for the association of cyanide to Co^{II}TMPyP.

Stopped-Flow Kinetics

An Applied Photophysics laser-flash/stopped-flow spectrometer (LKS.60-SX.18MV-R system) was used to measure rapid reaction kinetics, and the resultant data was fit with PC Pro-K software (!SX.18MV) provided by the manufacturer. All reactions were run under pseudo-first-order conditions and at thermostatically controlled temperatures. The individual rates reported represent the means of at least three runs. The average deviation of these runs was less than 5%. Rate constants were obtained from linear fits to the observed rates versus the ascorbate, or cyanide, concentrations.

Electron Paramagnetic Resonance (EPR)

X-band (9 GHz) EPR spectra were recorded on a Bruker ESP 300 spectrometer equipped with an Oxford ESR 910 cryostat for ultra-low-temperature measurements. The instrument and software (SpinCount) used to analyze the EPR spectra were provided by Professor Michael Hendrich, Carnegie Mellon University. Quantification of EPR signals was performed by simulating the spectra using known (or determined) parameters for each sample in question. Simulations employed a least-squares fitting method to match the line shape and signal intensity of a selected spectrum. Simulated spectra were expressed in terms of an absolute intensity scale, which could then be related to sample concentration through comparison with a Cu^{II}(EDTA) spin standard of known concentration.

Animals, Exposures, and Righting Recovery Determinations

All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (protocol numbers 1008725 and 14094469). Veterinary care was provided by the Division of Laboratory Animal Research of the University of Pittsburgh. Male Swiss-Webster (CFW) mice weighing 35–45 g were purchased from Charles River Laboratories, Wilmington, MA. All animals were 16–20 weeks old and were housed four per cage. The mice were allowed access to food and water *ad libitum*, and experiments commenced after the animals were allowed to adapt to their new environment for 1 week.

Recovery from cyanide intoxication was assessed based upon some of the recommendations of Crankshaw et al.¹⁴ regarding their measurement of the righting reflex but adopting a simpler procedure.¹⁵ Briefly, following intraperitoneal (ip) administration of NaCN (5.0 mg/kg), mice were placed in a dark-colored, but transparent, plastic tube in the supine position. The duration between the cyanide injection (irrespective of whether antidotal CoTMPyP was also given before/after NaCN) and the instance at which the mouse flipped from the supine to the prone position in the plastic tube was taken as the end point.

For collection of blood samples, mice were first euthanized in an atmosphere of carbon dioxide, the thoracic cavities were opened, and blood was drawn by cardiac puncture. The blood was expelled into the bottom of a quartz EPR tube containing 10% (w/v) EDTA through a Teflon “needle” and then frozen by immersion in liquid nitrogen. This entire process could comfortably be completed in 2 min. The cryogenically preserved sample was stored and subsequently transferred to the EPR spectrometer without ever having been thawed.

Other Instrumentation

Shimadzu UV-1650PC and UV-2501PC spectrophotometers were employed for the measurement of electronic absorption spectra and subsequent photometric analysis. Cyclic voltammetry was performed using a Princeton Applied Research Potentiostat/Galvanostat 283. Polished platinum wire was used as both the working and counter electrodes in a three-electrode configuration with a Ag|AgCl|KCl_{sat} reference. All experiments were performed under argon, and potentials are reported with respect to the normal hydrogen electrode (NHE).

Data Analysis

Titration data and animal experiments were analyzed using KaleidaGraph software. A *p*-value < 0.05 was considered to be statistically significant. Data are reported as values ± standard deviation.

RESULTS

Ascorbate Reduction of Co^{III}TMPyP

The reduction of Co^{III}TMPyP to Co^{II}TMPyP has been shown to have a quasi-reversible reduction potential of +0.42 V in 0.1 M H₂SO₄ vs NHE using either cyclic voltammetry (CV) or by spectroelectrochemistry (OTTLE) in the absence of oxygen.^{16,17} Co^{III}TMPyP

was originally shown by Pasternack and Cobb³ and subsequently verified by Chan et al.¹⁶ to exhibit two pK_a values, 6.0 (± 0.1) and 10.0 (± 0.1), for the following acid–base equilibria:

Thus, at neutral pH, the aquo/hydroxo form predominates and the pertinent equation for reduction at pH > 6 as determined by Chan et al.¹⁶ is as follows:

The absence of axial ligands in the product should be taken to mean that the presence of either one, two, or no water molecules is controversial, although Stich et al.¹⁸ have concluded that there are no axial ligands present in reduced corrinoids. Exhaustive electroreduction of $\text{Co}^{\text{III}}\text{TMPyP}$ in an OTTLE cell has confirmed the product to be $\text{Co}^{\text{II}}\text{TMPyP}$, with an associated reduction potential at pH 7.4 of +0.33 V vs NHE.¹⁶ Ascorbate is found in circulating plasma at a concentration of $\sim 60 \mu\text{M}$,¹⁹ and because it has a reduction potential of -0.06 V ,²⁰ it should easily be able to reduce $\text{Co}^{\text{III}}\text{TMPyP}(\text{OH}^-)(\text{H}_2\text{O})$ to $\text{Co}^{\text{II}}\text{TMPyP}$ *in vivo*. We measured the rate of ascorbate reduction of the complex under pseudo-first-order conditions by stopped-flow spectrophotometry, following the disappearance of the 427 nm band of $\text{Co}^{\text{III}}\text{TMPyP}(\text{OH}^-)(\text{H}_2\text{O})$. Linear fits of the observed rates to sodium ascorbate concentrations were used to determine a rate constant of $8.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ at 25 °C and $1.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ at 37 °C (Figure 1). This rate constant for the reduction of $\text{Co}^{\text{III}}\text{TMPyP}(\text{OH}^-)(\text{H}_2\text{O})$ at 37 °C is quite fast so that, even though the circulating level of ascorbate is only $\sim 60 \mu\text{M}$, we would expect essentially full conversion to $\text{Co}^{\text{II}}\text{TMPyP}$ within minutes in the bloodstream.

Cyclic Voltamogram of CoTMPyP in the Presence of Cyanide

CV performed on $\text{Co}^{\text{III}}\text{TMPyP}$ in the presence of excess cyanide (1 mM KCN, 0.1 M NaNO_3 , 50 mM phosphate buffer at pH 7.4 and 25 °C under argon) resulted in the observation of a single i_{pc} at -180 mV that had no corresponding wave (i_{pa}) during the positive potential scan (Figure 2A). The reduction observed during the negative potential scan did not decay on cycling (10–20 cycles), indicating there to be a nonelectrochemical reoxidation of the complex. Previous authors studying the reduction of dicyano $\text{Co}^{\text{III}}\text{TMPyP}$ by γ -radiolysis²¹ and the electrochemical reduction of other metalloTMPyP complexes²² have found the one-electron reduction of these complexes to involve the formation of a π -cation radical, that is, the electron is seemingly centered on the porphyrin ring. Reduction of the coordinated metal ion was reported^{21,22} to require the application of considerably more negative potentials (outside the physiological range) than we have employed here. It is to be noted, however, that these earlier studies were performed in solvent dimethylformamide²² and alkaline aqueous solution (pH > 11),²¹ unlike the more physiologically relevant

conditions employed here. Irrespective of whether the one-electron-reduced complex contains predominantly Co(II) or a porphyrin π -cation radical, nonelectrochemical reoxidation can be explained by reaction schemes such as

where reaction with oxygen can be excluded due to the anaerobic conditions employed. Oxidation mechanisms of Co(II)-containing species involving disproportionation are certainly known.²³ However, Mosseri et al.²¹ have previously suggested that in this particular reaction the products are $\text{Co}^{\text{III}}\text{TMPyP}(\text{CN}^-)_2 + \text{Co}^{\text{III}}\text{TMPyP-H}(\text{CN}^-)_2$, where a hydrogen has been added to the porphyrin to produce a phlorin ring.²⁴

Under similar conditions, cobinamide exhibited a virtually identical CV with a less negative potential, -90 mV, during the negative potential scans and no observable corresponding wave during the positive potential scans (Figure 2B). These results demonstrate that, for the dicyano adducts of both CoTMPyP and cobinamide, there are nonelectrochemical reoxidation pathways for $\text{Co}(\text{II}) \rightarrow \text{Co}(\text{III})$. In both cases, disproportionation mechanisms are plausible, given the known coordination chemistry of Co(II).

EPR Studies

Reduction of $\text{Co}^{\text{III}}\text{TMPyP}$ by ascorbate and subsequent addition of excess cyanide resulted in an X-band EPR spectrum (Figure 3A) with axial symmetry. The observed hyperfine in these signals results from the interaction of the unpaired electron ($\text{Co}(\text{II}) = d^7$) with the ^{59}Co nucleus ($I = 7/2$), having an axis of symmetry with $g_{\parallel} < g_{\perp}$ and hyperfine splitting of $A_{\parallel} > A_{\perp}$ (Table 1). It should be noted that this EPR spectrum is quite different from that previously reported for cyanide-free $\text{Co}^{\text{II}}\text{TMPyP}$ ²⁵ and nothing like the kind of spectrum expected for a π -cation radical as apparently observed in the pulse radiolysis and electrochemical reduction experiments of Mosseri et al.²¹ The spectrum of the $\text{Co}^{\text{II}}\text{TMPyPCN}^-$ adduct was fit by simulation using the program SpinCount (Figure 3B; see Experimental Section for details) in order to obtain g -values and hyperfine splittings (A). This $\text{Co}^{\text{II}}\text{TMPyP-CN}^-$ EPR signal was only transiently observed, with concentrations much less than the starting material, and the signal completely disappeared within a minute or two of the addition of cyanide. We were only able to trap these rapidly disappearing signals by preparing the samples in an inert (argon) atmosphere to exclude oxygen. Rapid disappearance of the Co(II) EPR signal has also been observed in the EPR spectrum of cobinamide after the addition of excess cyanide.^{26,27} This data strongly indicates that cyanide binds to the reduced form of these cobalt complexes and that, subsequently, cyanide then catalyzes the rapid oxidation of Co(II) to the EPR silent Co(III) forms of CoTMPyP and cobinamide. Therefore, insofar as they are comparable, the CV (see above) and EPR findings appear to be fully consistent.

For comparison, we also show the EPR spectrum of Co^{II}TMPyP in the presence of excess (5%, v/v) pyridine prepared in an inert atmosphere (Figure 3C). Unusually, we did not observe any three-line superhyperfine due to the nitrogen in pyridine as previously reported by Evans and Wood,²⁵ but we may have had some additional line broadening obscuring these features as our samples were prepared in 50 mM phosphate buffer rather than water or methanol. A fit to the observed signal by simulation results in the *g* and *A* values tabulated (Table 1). Addition of oxygen to Co^{II}TMPyP and immediate freezing resulted in a substoichiometric EPR signal near *g* ~2 (3400 G) quite similar to that previously observed²⁵ and characteristic of an O₂ adduct, which disappeared within a few minutes of warming the sample to room temperature and then refreezing again for measurement (data not shown).

Mouse Blood EPR

A convenient way of assessing the biological reactions of CoTMPyP following exposure to mouse blood is by EPR spectroscopy, since the majority hemoglobin (Hb) species, deoxyHb and oxyHb, are EPR silent. While the strong absorption characteristics of the hemoglobin derivatives in blood would tend to obscure the electronic absorption spectra of CoTMPyP species, the EPR spectrum of the reduced form should be readily observable. Following the addition of Co^{III}TMPyP to freshly drawn mouse blood, the appearance of an EPR signal at ~2940 G indicated reduction to Co^{II}TMPyP (Figure 3D). In samples frozen within 2 min of preparation, this signal was found by double integration to represent about 25% of the total CoTMPyP added and persisted in samples maintained at ambient temperature for up to 30 min before freezing. No signals that could be associated with any oxygen-bound form of CoTMPyP were observed. The EPR signal obtained from the mouse blood had some features in common with that of the pyridine adduct of Co^{II}TMPyP (Figure 3C), namely, a similar value of *g*_⊥, albeit with little observable hyperfine structure. Addition of an excess of cyanide resulted in the complete disappearance of these signals (Figure 3E), consistent with rapid oxidation to Co^{III}TMPyP(CN)₂ subsequent to cyanide binding. Failure to observe any intermediate Co^{II}TMPyP cyanide complex in the mouse blood is not surprising given the difficulty of trapping such species, as noted above.

Cyanide Binding to Co^{II}TMPyP

In order to better understand the reaction of cyanide with the reduced metalloporphyrin, Co^{II}TMPyP was prepared by ascorbate reduction of the Co(III) form and titrated with cyanide in 50 mM phosphate buffer, 1 mM EDTA, pH 7.4, monitoring the reaction spectrophotometrically. The Co^{II}TMPyP solution was maintained in a septum-sealed cuvette with little-to-no headspace, and known amounts of cyanide solution (weakly buffered in borate at pH ~ 11 to prevent HCN loss) were added using a gastight syringe. Time intervals of 10 min were allowed to pass between cyanide additions for the solution to equilibrate before recording electronic absorption spectra, which were then verified to be stable by repeated scans. The absorption spectra obtained during of the titration of Co^{II}TMPyP with cyanide at 25 °C do not initially display tightly maintained isosbestic points (Figure 4A, dashed and dash-dot traces). However, after the addition of less than ½ an equivalent of cyanide, the observed electronic absorption spectrum is seemingly identical to that of the oxidized Co^{III}TMPyP(OH⁻)(H₂O) species (Figure 4A, long-dash trace) and the subsequent spectra do maintain isosbestic points. The final spectrum is that of the oxidized

$\text{Co}^{\text{III}}\text{TMPyP}(\text{CN}^-)_2$ complex (Figure 4A, dotted trace). Since we wish to report the simplest useful association constant under physiologically relevant conditions, an effective formation constant (K'_β) can be defined as

$$K'_\beta = [\text{Co}^{\text{III}}\text{TMPyP}(\text{CN}^-)_2] / ([\text{Co}^{\text{II}}\text{TMPyP}][\text{HCN}]_2)$$

at pH 7.4, where the hydrogen ion concentration and electron transfers have been ignored. From the spectra (at 454 nm), the fraction of the $\text{Co}^{\text{III}}\text{TMPyP}(\text{CN}^-)_2$ per total metalloporphyrin (fractional saturation, Y) was determined and thus the free cyanide concentration (protonated plus unprotonated) could be calculated. In Figure 4B, the free cyanide is plotted versus the fractional saturation (Y) and the data was fit using a nonlinear regression and the Hill equation:

$$Y = [(\text{CN}^-)^{a_H} / K'_\beta] / [1 + (\text{CN}^-)^{a_H} / K'_\beta]$$

The best fits were obtained with $a_H = 2$, confirming the cooperativity of the cyanide binding, and K'_β was found to be $2.1 (\pm 0.1) \times 10^{10}$ (Figure 4B), very similar to the previously reported¹ association constant for the binding of cyanide to the oxidized species, $\text{Co}^{\text{III}}\text{TMPyP}(\text{OH}^-)(\text{H}_2\text{O})$, $2.1 (\pm 0.2) \times 10^{11}$.

Rate of Reaction of $\text{Co}^{\text{II}}\text{TMPyP}$ with Cyanide To Form $\text{Co}^{\text{III}}\text{TMPyP}(\text{CN}^-)_2$

The reaction of $\text{Co}^{\text{II}}\text{TMPyP}$ with excess cyanide under pseudo first-order conditions was studied at 25 and 37 °C (Figure 5). $\text{Co}^{\text{III}}\text{TMPyP}(\text{OH}^-)(\text{H}_2\text{O})$ was reduced with an excess of ascorbate and then rapidly mixed with excess cyanide; the reaction was followed at 427 nm (absorption maximum of the reduced species) and 454 nm (absorption maximum of the oxidized dicyano species). The reaction was complicated with four phases, all of which were dependent on the cyanide concentration. The last two phases, with rate constants designated k_3 and k_4 , were found to coincide, identically, with the last two phases previously observed for the reaction of cyanide with $\text{Co}^{\text{III}}\text{TMPyP}(\text{OH}^-)(\text{H}_2\text{O})$.^{1,13} These slower phases are not observed in reactions of CoTMPyP with other anionic ligands and appear to be present only when cyanide is in large (>20-fold) excess over the cobalt complex.^{1,13} They could, for instance, involve self-associated cyanide species (e.g., $\text{NC}-\text{H}\cdots\text{NCH}$, $[\text{NC}-\text{H}\cdots\text{NC}]^-$, etc.) forming intermediate adducts with $\text{Co}^{\text{III}}\text{TMPyP}$, which then slowly convert to $\text{Co}^{\text{III}}\text{TMPyP}(\text{CN}^-)_2$. In this plausible scenario, it is the first two phases with rate constants k_1 and k_2 (Table 2) that account for the rate at which free HCN/CN^- is removed from the solution. The present first two rate constants, however, are both significantly faster than those previously observed during the reaction of cyanide with $\text{Co}^{\text{III}}\text{TMPyP}(\text{OH}^-)(\text{H}_2\text{O})$.

When the kinetics of excess cyanide binding to $\text{Co}^{\text{II}}\text{TMPyP}$ were compared in the presence and absence of bovine serum albumin (BSA), a component of plasma, the initial rates were found to be identical for the first 15 min (Figure 6). However, after 20 min, where essentially 80% of the reaction is complete, the cyanide binding in the presence of BSA was somewhat slower. We have previously shown that the last phase of cyanide binding to $\text{Co}^{\text{III}}\text{TMPyP}$ is

dependent upon ionic strength^{1,13} and, therefore, one might expect to observe some changes in cyanide binding with BSA present. However, there is nothing to indicate in any of these kinetic data that the binding of cyanide to CoTMPyP *in vivo* should be anything other than reasonably facile.

Antidotal Activity of CoTMPyP in Mice

Given that the binding constants and their associated rate constants for the reaction of cyanide with both oxidized and reduced forms of CoTMPyP at physiological pH and temperature are favorable, the previously reported² lack of any prophylactic antagonism of this metalloporphyrin toward cyanide intoxication in mice remains paradoxical. Consequently, we reinvestigated the earlier findings (Figure 7). Mice administered NaCN in saline (5.0 mg/kg ip) are typically rendered unconscious within about 2 min. Those animals that subsequently survive (6 of 18 in the present case) remain “knocked down” for around 30 min, during which time they can be placed on their backs, returning themselves to a prone position upon regaining consciousness. If they were given CoTMPyP 1 min after the toxicant, then the metalloporphyrin was antidotal, as the surviving animals (4 out of 6) exhibited a mean knockdown/righting recovery time of only 8 min. If they were given CoTMPyP 5 min before the toxicant, then all of the animals survived (4 out of 4) and only half of them experienced knockdown, with a mean righting recovery time just under 3 min (Figure 7, third column). Clearly, in this experimental mouse model, CoTMPyP exhibits impressive prophylactic antidotal activity toward cyanide intoxication.

DISCUSSION

The reduction of Co^{III}TMPyP(OH⁻)(H₂O) is facile and easily carried out by ascorbate, with a rapid pseudo-first-order rate constant of $1.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ at physiological pH and temperature (Figure 1). The appearance of an EPR signal with characteristics corresponding to the reduced form of Co^{II}TMPyP in mouse blood (Figure 3), where the ascorbate concentration is near 60 μM , lends further support to the argument that the reduced form must be prevalent under physiological circumstances. Therefore, if CoTMPyP is to be used as an antidote to cyanide toxicity, its ability to bind cyanide in the reduced form is of paramount importance. Many of the results presented in this article, however, lead us to conclude that cyanide rapidly catalyzes the conversion of the reduced complex to the oxidized form, Co^{III}TMPyP(CN⁻)₂. The CV results demonstrate that CoTMPyP in the presence of cyanide had a cathodic wave but no corresponding anodic wave (Figure 2), indicating that another, quite rapid, mechanism exists to convert Co^{II}TMPyP(CN⁻)₂ to the Co(III) form. When cyanide was added to Co^{II}TMPyP, only a transient EPR signal corresponding to Co^{II}TMPyP(CN⁻) was observed, indicating oxidation, as Co^{III}TMPyP(CN⁻)₂ is EPR silent (Figure 3).

The mechanism of cyanide-induced oxidation of Co^{II}TMPyP remains unclear, and contrary to earlier studies at pH > 11,²¹ we found no indications of π -anion radical intermediates or end products containing phlorin rings. The EPR spectra (Figure 3) contain only signals attributable to Co(II)-containing products, that is, addition of the additional electron to metal-ion-localized sites. The electronic absorption spectra (Figure 4) look like those of

absolutely normal metalloporphyrins containing fully unsaturated tetrapyrrole rings, whereas the Soret band of the phlorin derivative is approximately one-third of the intensity of the normal porphyrin.²¹ While we cannot exclude the possibility that π -anion radicals and/or phlorins might have been formed as intermediates, or minority stable species, they need not be invoked to explain our data. An attractive notion for oxidation by a disproportionation mechanism is having cyanide act as a bridging ligand in order to facilitate an electron flow, for example

There are many known examples of cyanide forming bridges between metallo-macrocycles^{28,29} and other metal complexes,^{30,31} but if Co(II) disproportionates, it remains to be considered how the Co(I) form is converted to either Co(II) again or on to Co(III). Addition of oxygen to the Co^{II}TMPyP cyanide adduct could result in peroxo-bridged dimers in analogous fashion to the chemistry of [Co(CN)₅]³⁻, which forms Co(III) μ -peroxo dimers³⁰ that may then dissociate in solution. However, when we carried out the reaction of Co^{II}TMPyP with cyanide anaerobically, we found no difference in the rate of reaction, indicating that oxygen is probably not involved in the mechanism. Therefore, oxidation of Co(I) by aqueous protons (reaction 3) remains the most plausible explanation.

Leaving aside the details of the oxidation–reduction components of the net reaction and restricting the discussion primarily to consideration of the ligand exchange processes, it seems that there are two broad types of plausible mechanisms that fit our observations (Schemes 1 and 2). We infer from the observed rate of Co^{III}TMPyP reduction by ascorbate (Figure 1) that oxidation–reduction reactions are faster than the ligand substitutions in these complexes and thus only the latter should be rate-determining. Moreover, we must consider at least two strongly [HCN]-dependent steps (Figure 5) in any reasonable minimal reaction mechanism proposed. Scheme 1 is the simplest, where the parentheses enclosing one axial aquo ligand of the initial Co^{II}TMPyP complex should be taken to indicate uncertainty as to whether this species is five- or six-coordinate. Substitution of the aquo ligand(s) by the first cyanide anion, with the associated rate constant k_1 , leads to the Co^{II}TMPyP(CN⁻) intermediate detected by EPR spectroscopy. Substitution of the second cyanide anion is characterized by the rate constant k_2 , accompanied by rapid oxidation to the final product Co^{III}TMPyP(CN⁻)₂. The alternate possibility, Scheme 2, involves the initial rapid formation of the EPR-detectable Co^{II}TMPyP(CN⁻) intermediate, followed by two discrete rate-determining pathways to final product. One route involves substitution of the second cyanide and accompanying oxidation, as in Scheme 1, but now characterized by k_1 rather than k_2 ; the other route involves first oxidation of Co^{II} to Co^{III} followed by the second cyanide substitution with associated rate constant k_2 . There seem to be two problems with Scheme 2. The electron transfer reaction must be slow enough to be comparable with k_1 ; otherwise, only k_2 would be apparent in the kinetic traces. Also, the present k_2 should be the same rate constant as k_2 in the reaction starting from the oxidized Co^{III}TPMyP(OH⁻)(H₂O),^{1,13} but the numerical results for these two constants differ by an order of magnitude (Table 2). Therefore, Scheme 1 is the favored minimal mechanism.

While most of the present work has focused on the cobalt-porphyrin, CoTMPyP, we have also included a more limited set of comparative observations regarding the cobalt-corrin cobinamide. Hydroxocobinamide is also fairly rapidly reduced to the Co(II) form by ascorbate ($k \sim 1.4 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.4 and 37 °C, data not shown), and the cyclic voltammogram of the dicyano adduct appears to be similar to that of CoTMPyP(CN⁻)₂ (Figure 2). Consequently, it is to be expected that cobinamide and CoTMPyP will both be reduced *in vivo* but that upon reaction with cyanide these compounds will be rapidly converted to their Co(III) forms and bind cyanide with almost the same affinity as that if the starting complexes were the oxidized species. Therefore, the bio-inorganic coordination chemistry takes advantage of both the more facile substitution chemistry of Co(II) compared to that of Co(III) (the latter typically being substitution inert) and the increased affinity of cyanide for Co(III) compared to that for Co(II). It follows that the significance of any trans effect in rendering the axial positions of Co(III)-containing cobinamide substitution labile in therapeutic cyanide-scavenging applications is probably of much less importance than one might previously have thought.

It is fortunate the present results demonstrate unambiguously that CoTMPyP is significantly antidotal toward cyanide toxicity in mice irrespective of whether the decorporating agent (i.e., scavenger) is given either before or after the toxicant (Figure 7). Any other result would question the widely accepted mechanistic ideas regarding the manner in which other putative cyanide scavengers (e.g., cobalamin and cobinamide) ameliorate the toxicity. The failure to detect any prophylaxis by CoTMPyP given 15 min or 1 h before NaCN in an earlier study² remains to be explained. We observed evidence for excretion of the metalloporphyrin as early as 9 min following administration (slightly orange urine) and, therefore, its prophylactic effectiveness can be expected to start declining at about this time, or shortly thereafter, in a limited number of cases. More typically, though, mice urinate when handled for injection purposes, and they may not urinate for the next ~35 min, after which time the urine of animals that had been given CoTMPyP appears distinctly orange. Thus, any potential prophylaxis is likely to be significantly diminished if the CoTMPyP is given 1 h before the toxicant. Our original idea that reduction of Co^{III}TMPyP *in vivo* might lead to deactivation of its cyanide-binding capability is clearly erroneous for the additional reason that we have now shown Co^{II}TMPyP to be a comparable cyanide scavenger (Figures 3–6). In the present animal experiments, we have used a toxicant dose of 0.1 mmol NaCN/kg and an antidote dose 0.05 mmol CoTMPyP/kg (Figure 7). Because the metalloporphyrin binds two cyanide anions,^{1,13} this means we essentially gave enough antidote to decorporate all of the toxicant administered. In the earlier study, with $n = 10$ mice per experimental group, a $2 \times \text{LD}_{50}$ dose of cyanide was employed, but only enough antidote was given to decorporate a $\frac{1}{2} \times \text{LD}_{50}$ dose of the toxicant. In our experience, there is a very small change in percentage (i.e., < 10%) survival of animals to be expected between $2 \times \text{LD}_{50}$ and $(2 - \frac{1}{2}) \times \text{LD}_{50}$ doses of NaCN, which, to determine with acceptable statistical significance, we would anticipate requiring something like an order of magnitude more mice per experimental group than were used in the earlier study.

CONCLUSIONS

We have shown that ascorbate reduces CoTMPyP in a facile manner *in vitro*, with a rate constant of $1.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ at physiological pH and temperature; furthermore, we observe the reduced complex *in vivo*. However, this does *not* preclude cyanide from binding to the reduced form ($K'_\beta = 10^{10}$ to 10^{11}), and we have shown in an animal study that CoTMPyP is clearly prophylactic and therapeutic toward cyanide intoxication. Thus, the earlier suggestion that CoTMPyP has no prophylactic benefit is in error, and our original hypothesis that *in vivo* reduction might limit the cyanide-binding activity of CoTMPyP is incorrect.

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ABBREVIATIONS

H₂-TMPyP 4,4',4'',4'''-porphyrin-5,10,20-tetrayltetrakis(1-methylpyridinium)tetrakis(4-methylbenzenesulfonate)

CoTMPyP [[4,4',4'',4'''-porphyrin-5,10,20-tetrayltetrakis(1-methylpyridiniumato)(2-)]cobalt(III) pentaiodide

EDTA ethylenediaminetetraacetate

BSA bovine serum albumin

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Figure 1.

Kinetics of the reaction of ascorbate with $\text{Co}^{\text{III}}\text{TMPyP}(\text{OH}^-)(\text{H}_2\text{O})$ under pseudo-first-order conditions. The reaction was followed at 427 nm in 50 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, at 25 and 37 °C with excess sodium ascorbate (1–10 mM).

Figure 2.

Cyclic voltammograms of Co^{III}-TMPyP(OH⁻)(H₂O) and cobinamide in the presence of cyanide at pH 7.4 and 25 °C. Samples of (A) Co^{III}-TMPyP and (B) Cbi(OH)⁺ (200 μM) were prepared anaerobically in 0.1 M NaNO₃, 50 mM potassium phosphate buffer, pH 7.4, at room temperature; then, excess cyanide (20 mM) was added. A three-electrode configuration was employed. Polished platinum wire was employed as both the working and counter electrodes, along with a Ag|AgCl|KCl(sat.) electrode used as the reference. Potentials are reported with respect to the normal hydrogen electrode (NHE).

Figure 3.

X-band EPR spectra of ascorbate-reduced $\text{Co}^{\text{II}}\text{TMPyP}$ and mouse blood at 10 K. Samples of $\text{Co}^{\text{III}}\text{TMPyP}$ (1 mM) were prepared anaerobically in 50 mM potassium phosphate buffer, pH 7.4, at room temperature and reduced with excess sodium ascorbate (20 mM), excess cyanide (20 mM) was added, and samples were then rapidly frozen in liquid nitrogen for subsequent recording of spectra. Samples of mouse blood were obtained by heart puncture to which $\text{Co}^{\text{III}}\text{TMPyP}$ (1 mM) and excess cyanide (20 mM) were added, and samples were then rapidly frozen in liquid nitrogen for subsequent recording of spectra. EPR conditions: 9.8 G modulation amplitude, 63.2 μW microwave power, 10 K. (A) $\text{Co}^{\text{II}}\text{TMPyP}\text{-CN}^-$, (B) simulation of A, (C) mouse blood plus $\text{Co}^{\text{III}}\text{TMPyP}$ (1 mM), (D) $\text{Co}^{\text{II}}\text{TMPyP}$ -pyridine, and (E) sample C with excess cyanide (20 mM). Asterisks (*) indicate signals due to cavity contaminants.

Figure 4.

Titration of Co^{II} -TMPyP with cyanide at pH 7.4 and 25 °C. Small aliquots of sodium cyanide solution in 5 mM sodium tetraborate buffer (pH 11) were titrated into a solution of Co^{II} -TMPyP (3.48 μM in 50 mM sodium phosphate buffer, 1 mM EDTA, pH 7.4) using gastight syringes and a 1.00 cm path length septum-sealed cuvette at 25 °C (see Experimental Section for further details). (A) Electronic absorption spectra of Co^{II} -TMPyP species titrated with NaCN. (B) Titration of 3.48 μM Co^{II} -TMPyP with cyanide following the absorbance changes at 454 nm. The solid line represents a nonlinear least-squares fit to the data using the Hill equation.

Figure 5.

Kinetics of the reaction of cyanide with Co^{II}-TMPyP under pseudo-first-order conditions. The reaction was followed at 454 nm in 50 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, at 25 and 37 °C in the presence of excess (>20-fold) potassium cyanide. Cyanide solutions in the drive syringe were in 5 mM sodium tetraborate buffer (pH 11); cyanide concentrations after mixing were 20–2500 μM ; upon completion of reactions, discharged solutions were verified to be pH < 7.45. The observed rates associated with the two fast rate constants (k_1 and k_2) are plotted versus the cyanide concentration at (A) 25 °C and (B) 37 °C. Rate constants (Table 2) were then obtained from the slopes of the plots.

Figure 6.

Comparison of time courses for cyanide binding to $\text{Co}^{\text{II}}\text{TMPyP}$ in the presence (solid circles) and absence (open circles) of BSA. Initial conditions: $3.48 \mu\text{M}$ $\text{Co}^{\text{II}}\text{TMPyP}$ in 50 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA, and 0.1 mM NaCN, 25 °C. The reactions were followed spectrophotometrically at 454 nm.

Figure 7.

Antidotal effect of CoTMPyP on cyanide-intoxicated mice. Righting recovery assessments showing the efficacy of Co^{III} TMPyP (20.8 mg/kg, 20% LD₅₀, ip) in Swiss-Webster mice (males, 16–20 weeks of age) given NaCN in saline (5 mg/kg ip). Control animals (first column; $n = 6$) were given toxicant only. The antidote was given either 1 min after (second column; $n = 4$) or 5 min before (third column; $n = 4$) administration of the toxicant ($t = 0$; all injections were ip). In both cases, a highly significant antidotal capability ($P < 0.01$ compared to control group) was observed.

Scheme 1.

Plausible Scheme for the Binding of Cyanide to and Subsequent Oxidation of Co^{II}TMPyP

Scheme 2. Plausible Scheme for the Binding of Cyanide to and Subsequent Oxidation of Co^{II} TMPyP

Table 1

X-Band EPR g -values and Hyperfine Constants (A , ^{59}Co) of Several Co(II)TMPyP-ligand Species at 10 K, pH 7.4 in 0.1 M Sodium Phosphate Buffer^a

ligand	g_{xy}	A_{xy} (MHz)	g_z	A_z (MHz)
H ₂ O ^b	2.443	162	2.036	270
pyridine	2.242	150.3	2.099	188.2
HCN	2.120	148.0	2.041	215.8
ethanolamine	2.309	67.95	2.132	242.6

^aEPR conditions: 9.8 G modulation amplitude 63.2 mW microwave power. Ligands were in at least 1000-fold excess over Co(II)TMPyP (1 mM).

^bEvans and Wood.²⁵

Table 2

Second-Order Rate Constants and Absorbance Amplitudes for the Formation of $\text{Co}^{\text{III}}\text{TMPyP}(\text{CN})_2$ from $\text{Co}(\text{II})\text{TMPyP}$ at 25–37°C, pH 7.4, in 0.1 M Sodium Phosphate Buffer^a

conditions	phase 1		phase 2		phase 3		phase 4	
	k_1 ($\text{M}^{-1} \text{s}^{-1}$)	A %	k_2 ($\text{M}^{-1} \text{s}^{-1}$)	A %	k_3 ($\text{M}^{-1} \text{s}^{-1}$)	A %	k_4 ($\text{M}^{-1} \text{s}^{-1}$)	A %
pH 7.4, 25 °C	800 (± 90)	22	263 (± 52)	23	9 (± 1)	8	0.35 (± 0.05)	46
pH 7.4, 37 °C	2500 (± 600)	18	800 (± 100)	27				
pH 7.4, 25 °C*	111 (± 7)	6	29 (± 1)	40	9 (± 1)	8	0.35 (± 0.5)	43

^aThe observed kinetics could be resolved into four exponential phases. Rate constants are calculated from linear fits of observed rates to the cyanide concentration as shown in Figure 5 (cyanide was in at least 20-fold excess over the metalloporphyrin). The percent absorbance change (amplitude) for each phase is also given. Uncertainties shown in parentheses are standard deviations. The last row contains the rate constants for the binding of cyanide to the oxidized form of the porphyrin ($\text{Co}^{\text{III}}\text{TMPyP}$) for comparison.^{1, 13}