Definition of the Redox States of Cobalt-precorrinoids: Investigation of the Substrate and Redox Specificity of CbiL from *Salmonella typhimurium*†

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ABSTRACT: The enzyme CbiL from the facultative anaerobe *Salmonella typhimurium* exhibits a high degree of homology to CobI from the aerobe *Pseudomonas denitrificans* (29% identity; 51% conservation obtained by a Blastp search of the ncbi database). As CobI catalyzes the third methylation in the aerobic pathway to vitamin B_{12} it is proposed that CbiL catalyzes the analogous step in the anaerobic pathway. Potential metallo and metal-free substrates were characterized and their redox states defined by a combination of physicochemical techniques (MALDI-MS, NMR, UV/vis, IR, and EPR) and then used to investigate the function of CbiL. CbiL exhibited an absolute requirement for the presence of a metal ion (Co(II), Ni(II), or Zn(II)) within the tetrapyrrole substrate. CbiL had no preference for the redox state of its cobalt tetrapyrrole substrate, methylating both the reduced form, Co(II) 2,7-dimethyl-dipyrrocorphin (Co(II)-precorrin-2), and the oxidized form, Co(III) 2,7-dimethyl-isobacteriocorin (Co(III)-factor-II). In contrast CbiL had a marked preference for the oxidized Ni(II) and Zn(II)-2,7-dimethyl-isobacteriochlorin (Ni(II) and Zn(II)-factor-II). Removal of the metal ion from a product of CbiL (Zn(II)-factor-III) allowed characterization by ^{13}C NMR, identifying the tetrapyrrole as 2,7,20-trimethyl-isobacteriochlorin (factor-3), indicating that CbiL methylates at C_{20}, the same site as that methylated by CobI. Competition experiments, utilizing isotopic labeling to distinguish otherwise identical mass substrates and products, revealed that oxidized Co(III) or Ni(II)-factor-II were equally good substrates, whereas Co(II)-precorrin-2 was much preferred over Ni(II)-precorrin-2. Excess Ni(II)-precorrin-2 did not decrease CbiL methylation of Co(II)-precorrin-2, implying that CbiL has a low affinity for Ni(II)-precorrin-2. These results are interpreted on the basis of tetrapyrrole ruffling occurring on the optimization of the metallo-N bond distances. The greater flexibility of the reduced precorrin-2 ring system allows greater deformation on accommodating the bound metal ion, the distortions imposed by bound Ni(II) or Zn(II) ions being larger than Co(II). The resulting distortions imposed on the precorrin ring could then decrease catalysis by causing a departure from the optimal substrate conformation required for CbiL. On oxidation of the Ni(II) or Zn(II)-precorrin-2, the increased stiffness of the ring could then constrain the metallo-factor-II conformation toward that of the usual substrate, allowing greater methylation by CbiL. In contrast to its counterpart CobI in the aerobic pathway of B_{12} biosynthesis, which methylates the metal-free precorrin-2, these studies show CbiL to be the first methylase unique to the anaerobic pathway, methylating a metallo-precorrin-2 substrate. Implications of CbiL specificity for the mechanism of the anaerobic B_{12} pathway are discussed.

Since the discovery of B_{12} synthesis in anaerobic bacteria, a new mechanism of ring contraction has been sought as a counterpart to the aerobic pathway without the participation of molecular oxygen in this process. With the knowledge of early cobalt insertion in the pathway of anaerobic bacteria at the stage of precorrin 2 or 3 (*1*) (see Scheme 1 for structure), it has been proposed that the acetate side chain of the precorrin provides the oxygen atom via delta-lactone formation while the central Co ion provides the redox function driving ring contraction (*2*). Therefore a prerequisite for the anaerobic pathway is the early insertion of Co(II) into its tetrapyrrole ligand prior to ring contraction. Previous work on whole cell systems has indicated that both Co(III)[1]-factor-II and Co(III)-factor-III can give rise to cobalamin (*1*). Additional genetic evidence has implicated CysG and/or CbiK in early cobalt insertion (*3*, *4*). Some of the later intermediates have now been identified and characterized, supporting the role of a delta-lactone during ring contraction (*5*, *6*) and subsequent loss of a 2 carbon fragment as acetaldehyde (*7*) rather than acetic acid as in the aerobic pathway (*8*, *9*) (see Scheme 2). The proposal that the central cobalt ion can act as a redox center both accepting and donating electrons is based on the theory that the HOMO[2]

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[1] The cobalt ion in cobalt factor-II is nominally given the redox state (III) for convenience, although it is probably better described as Co(III)-$O_2^{-\bullet}$.

[2] Abbreviations: ALA, aminolevulinic acid; ALAD, aminolevulinic acid dehydratase; EPR, electron paramagnetic resonance; cosynthatase, uroporphyrinogen III synthase; deaminase, porphobilinogen deaminase; HOMO, highest occupied molecular orbital; LUMO, lowest occupied molecular orbital; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; NMR, nuclear magnetic resonance; SAM, *S*-adenosyl methionine; TFA, Trifluoroacetic acid; TOF, time-of-flight; TLC, thin-layer chromatography; TPP, tetraphenylporphyrin; Uro, uroporphyrin; Uro'gen, uroporphyrinogen.

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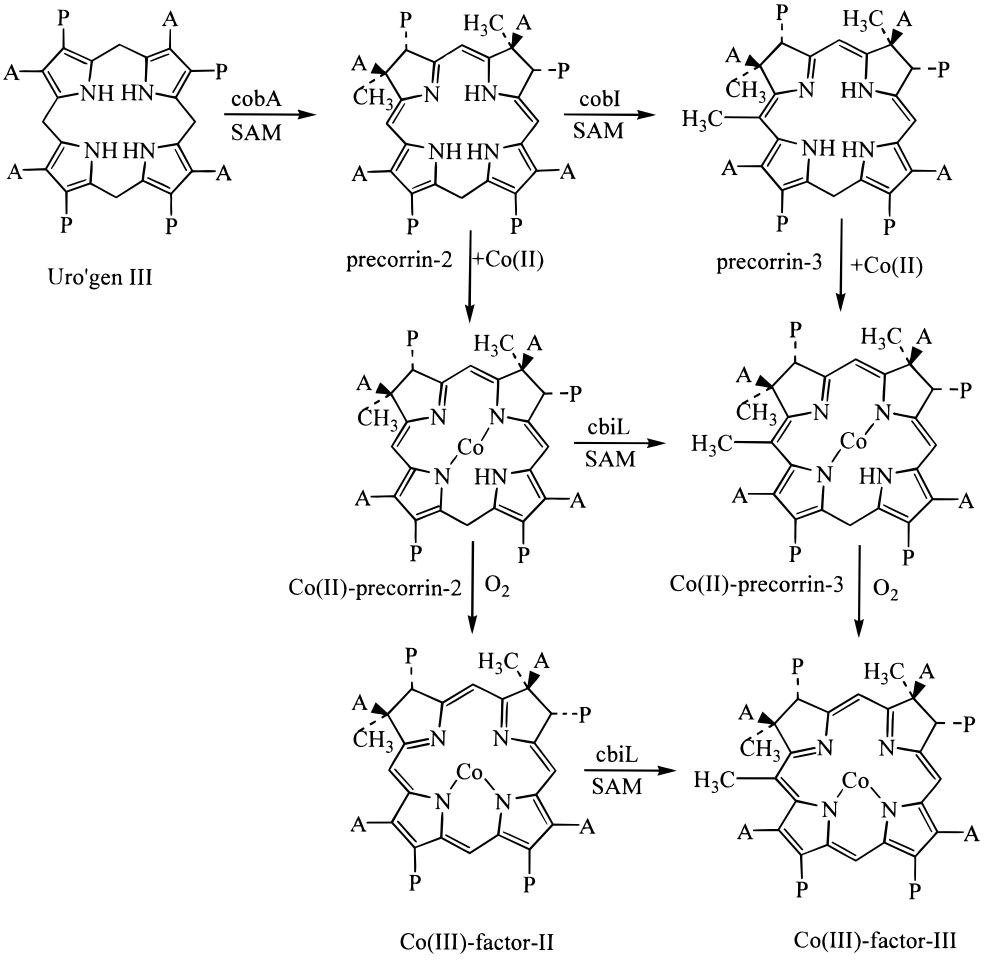
Scheme 1: In Vitro System for the Generation of Cobalt Tetrapyrrole Intermediates[a]



[a] Uro'gen III was synthesized from ALA using ALAD, deaminase, and cosynthase: A = CH_2COOH, P = CH_2CH_2COOH.

and LUMO energy levels of the tetrapyrrole ligand and metal ion can interact, allowing electron shuttling. A theoretical interpretation of orbital mixing of the tetrapyrrole ligand and central metal ion in model systems has been documented (*10*). Electron shuttling within a cobalt−porphyrin cation radical system has recently been demonstrated (*11*). Central to the understanding of the role played by the ligated cobalt ion is the means of determining the redox state of both the tetrapyrrole ligand and its complexed metal ion. In addition to the potential effect of the complexed metal ion on the redox properties of the metallo-tetrapyrrole complex, both its aromaticity and conformation are also altered (*12*, *13*). The alteration in aromaticity controls the site of methylation in model chemical systems while the altered conformation, arising from ruffling of the tetrapyrrole on optimizing the N-metal bonds, would dictate new interactions compared to the free ligand (*12*, *13*). Both of these effects would be either exploited or accommodated by the subsequent enzymes of the anaerobic B_{12} pathway.

To define the redox state of a metallo-tetrapyrrole, a combination of ^{13}C NMR (to determine the redox state of the ligand) and EPR (to determine if the system is paramagnetic and the location of any unpaired electrons, given by the *g* value and any hyperfine coupling (*14*)) is required. Several practical difficulties attend the visualization of a particular redox state of cobalt tetrapyrroles.[3] First, the presence of a paramagnetic metal ion center within the tetrapyrrole may broaden the NMR signals required to determine the redox state of the ligand (*15*). Second, Co(II)-tetrapyrroles can be extremely sensitive to oxygen, readily forming Co(III)-$O_2^{-\bullet}$ species which are also EPR active (*14*). In addition, spontaneous disproportionation between the central metal ion and the tetrapyrrole ligand may also be possible, thereby confounding the assignment of a fixed redox state, as has been observed for Ag(III)-octaethylporphyrin (*15*) and Ni(III)-tetraphenylporphyrin (*16*).

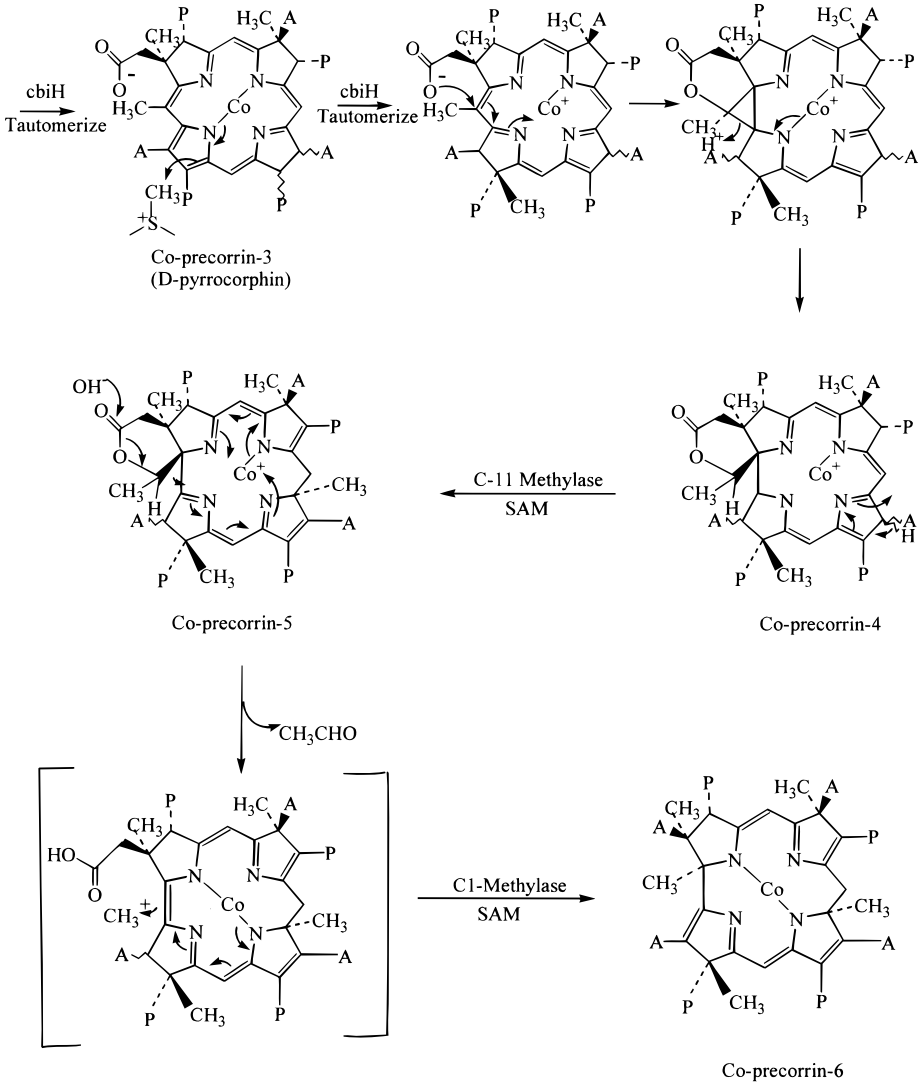
This work utilizes a combination of physical chemical characterizations (MALDI-mass spectrometry, NMR, UV/vis, IR, and EPR) to define the redox state of the early cobalt tetrapyrrole intermediates in vitamin B_{12} biosynthesis. The substrate and redox state preference of the first methyl transferase unique to the anaerobic vitamin B_{12} pathway, CbiL from *S. typhimurium*, was also investigated.

MATERIALS AND METHODS

In Vitro Generation of Metallo-tetrapyrroles. Porphobilinogen was presynthesized from 2 to 10 mg of ALA on incubation with 5 mg of ALAD in 5 mL of 150 mM potassium phosphate buffer pH 8 for 2 h. Purified enzymes, deaminase (0.4 mg) and cosynthase (2 mg), were then added along with 100 mL of 50 mM potassium phosphate buffer

[3] Cobalt tatrapyrroles will be used as a generic term covering all of the possible redox states of the ligand and bound cobalt ion.

Scheme 2: The Proposed Role of the Central Cobalt Ion in the Ring Contraction Step in the Anaerobic Pathway of Vitamin B_{12} Biosynthesis



pH 8 and a 1 L extract of *Escherichia coli* overexpressing CobA. The mixture was then degassed by freeze-thawing under vacuum and left under an argon atmosphere (0.2 ppm O_2) for 10 h generating the yellow intermediate precorrin-2. Similarly precorrin-3 was generated from precorrin-2 by further addition of a degassed 1 L extract of *E. coli* overexpressing CobI. Cobalt derivatives of these precorrins were generated by the addition of $CoCl_2$ (1 mM) followed by a 30 min incubation (Scheme 1). The color of precorrin-2 turned from yellow to brown on the addition of Co(II), while exposure to oxygen resulted in the color change from brown to green. Ni(II) derivatives were generated by the addition of 5 mM $NiCl_2$ followed by an overnight incubation; little color change was observed unless exposed to O_2 when a greenish color appeared. Addition of 1 mM $ZnSO_4$ to precorrin-2 overnight gave a color change from yellow to light green, becoming deep red on exposure to oxygen; these color changes indicated the formation of a Zn(II)-precorrin-2 and Zn(II)-factor-II, respectively. Neither uro III nor uro'gen III was able to incorporate Ni(II) or Co(II) ions during this time, and they were found to be rapidly degraded to a peak of $M_r = 712$ by 4 successive reductions in mass of $M_r = 58$, possibly by a loss of a methylene and a carboxylate group. This degradation was essentially complete after 24 h.

Esterification and Purification of Metallo-tetrapyrroles. Aqueous samples of metallo-precorrins were frozen under an argon atmosphere in a sealed vessel with liquid nitrogen. Upon solidification the vacuum created on freezing was released by flushing in argon. The sample was then quickly attached to the freeze-drier with no further precautions. After freeze-drying the sample, the vacuum was released by flushing with argon and sealed with Parafilm and then imported into an argon glovebox. Esterification of the freeze-dried metallo-precorrins (50 mg) was achieved by adding 20 mL of either 1.6%, 2.5%, or 5% H_2SO_4 to methanol under argon and leaving the mixture for various times to esterify (values given in text). Prolonged esterification at 5% H_2SO_4 resulted in the progressive loss of the metal ion from the metallo-precorrin. Esterification in the presence of even low levels of O_2 resulted in the generation of species of molecular weight higher than that expected for the fully esterified precorrin; the nature of these species was not determined. However, ring opening of γ-lactones (known to form on esterification of precorrin in the presence of O_2) by acidic methanol has been shown to generate hydroxymethyl esters in a related system (*17*) and would be

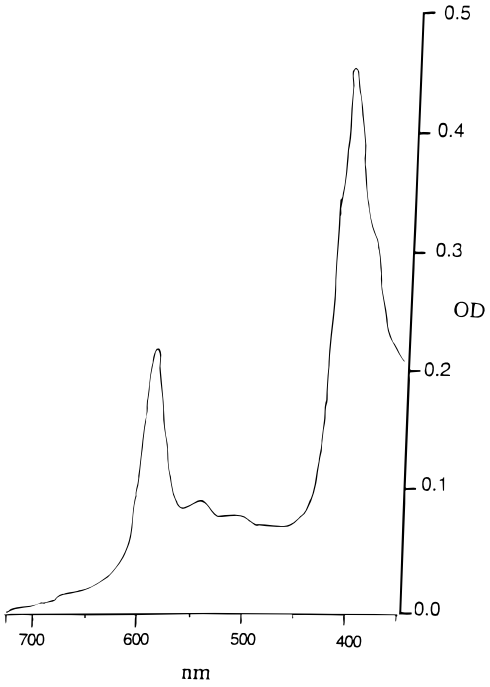


FIGURE 1: UV/Vis of octamethyl Co(II)-precorrin-2. TLC purified octamethyl Co(II)-precorrin-2 was dissolved in dichloromethane and the spectrum taken between 750 and 350 nm. The Soret band at 400 nm has previously been shown to have $\epsilon = 5.7 \times 10^4$ L mol^{-1}.

consistent with the increases of M_r observed. Consequently esterification of precorrin-2 was always included as a control to ensure the methylation step was O_2-free. After esterification dichloromethane was added to the extract sample followed by neutralization with K_2CO_3. Dichloromethane extracted samples were loaded onto TLC plates and developed with 95% dichloromethane and 5% methanol. All bands were excised and extracted with methanol and analyzed for the presence of metallo-precorrin by MALDI-MS. All bands found to contain metallo-precorrin were subsequently analyzed as described.

MALDI-MS Analysis of Tetrapyrroles and Their Metallo Derivatives. Mass analyses were performed on a PerSeptives Biosystems Voyager Elite XL or a Voyager benchtop linear MALDI-TOF-MS utilizing a 337 nm Laser Science, Inc., nitrogen laser. Matrix/analyte solutions were prepared by the addtion of 5 μL of the extracted derivatized metallo or metallo-free tetrapyrrole (40−200 μM) to an equal volume of the MALDI matrix, α-cyano-4-hydroxy-cinnamic acid (30 mg/mL in methanol), and 5 μL of 1% aqueous TFA. The matrix/analyte solution (1.5 μL) was then deposited onto the MALDI plate, vacuum-dried, and submitted for MALDI-MS analysis. Mass axis calibration was achieved externally using substance P ($M_r = 1347$), known matrix ions, and alkali metal ions (Na^+) as references. All samples analyzed by MALDI-MS were prepared in air and are therefore observed at the factor oxidation state, that is, isobacterioclorin.

^{13}C NMR Analysis of Cobalt Tetrapyrroles. During NMR experiments Co(II)-precorrins (80−180 μM in deuterated chloroform) were kept under argon and spun by a nitrogen stream in a 500 MHz NMR spectrometer overnight to ensure a complete anaerobic environment. ^{13}C NMR spectra were recorded on a Bruker ARX-500 spectrometer operating at a frequency of 125.77 MHz for carbon. Samples were run in a 5 mm dual C/H probe operating at 22 °C, employing the standard one-pulse sequence with WALTZ-16 proton decoupling. Typically, 30000−50000 scans were recorded for each experiment, the acquired 32 K FID weighted exponentially with a 10−15 Hz line-broadening function prior to Fourier transformation, and the resulting spectra were referenced to the residual $CDCl_3$ signal at 77.0 ppm.

EPR of Metallo-tetrapyrroles. Metallo-precorrins (80−180 μM in deuterated chloroform) analyzed by EPR (Bruker) were kept under argon until frozen in liquid nitrogen. Once frozen at liquid nitrogen temperature, samples could be stored or exposed to air without subsequent oxidation. Samples were run at 10 K, cooled by liquid He, and temperature-controlled by an Oxford ITC4 unit. Power studies were conducted to ensure that maximum signal intensities were being observed without saturation. Spin concentrations were determined with reference to a 1 mM Cu(II) standard.

RESULTS

Esterification and Purification of Metallo-tetrapyrroles. Co(II)-precorrins were esterified to enable purification by TLC (see Methods). The time course of esterification studied

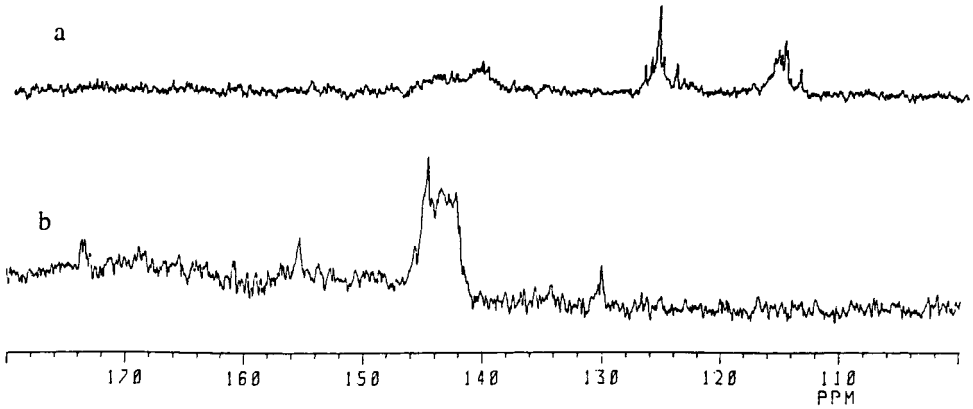


FIGURE 2: (a) ^{13}C NMR spectrum of Co(II)-precorrin-2. The NMR of Co(II)-precorrin-2 (145 μM in $CDCl_3$) derived from [^{13}C-4]-ALA exhibited two sets of peaks around 114−118 ppm and 124−128 ppm similar to precorrin-2. The reduced definition is attributable to the proximity of the paramagnetic Co(II) ion. (b) ^{13}C NMR of Co(III)-factor-II. Oxidation of Co(II)-precorrin-2 (205 μM in $CDCl_3$) derived from [^{13}C-4]-ALA resulted in the loss of two sets of peaks around 114−118 ppm and 124−128 ppm and the appearance of a set of signals at 142−146 ppm similar to factor-II. The reduced definition is attributable to the paramagnetic species likely arising from a Co(III)-$O_2^{-\bullet}$ bond.

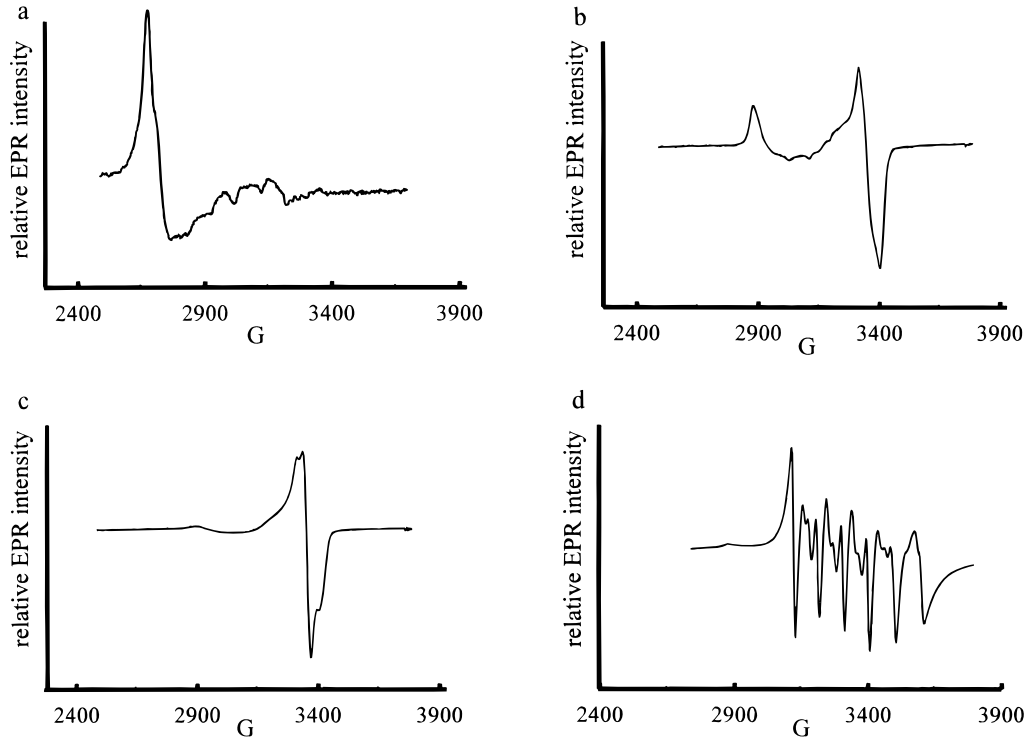


FIGURE 3: (a) EPR spectrum of Co(II)-precorrin-2. The EPR spectrum of Co(II)-precorrin-2 (130 μM in $CDCl_3$) obtained at 10 K by a Bruker EPR machine (center field 3148 G, sweep width 1300 G, 9.428 GHz, modulation frequency 100 kHz, modulation amplitude 11.8 G, receiver gain 1×10^5) shows a low spin Co(II) signal ($G = 2.3$) with hyperfine coupling ($A = 80$ G) and a molar spin ratio of 0.63. (b) EPR spectrum of partially oxidized Co(II)-precorrin-2. The EPR spectrum of Co(II)-precorrin-2 (130 μM in $CDCl_3$ exposed to air for 10 s and refrozen) obtained at 10 K (9.422 GHz, receiver gain 5×10^4, other settings as 3a) exhibited a reduction in the Co(II) signal and the appearance of a radical signal ($G = 2$) with a molar spin ratio of 0.67. (c) EPR spectrum of oxidized Co(III)-factor-II. The EPR spectrum of the oxidized Co(III)-factor-II (230 μM octamethyl Co(II)-precorrin-2 in $CDCl_3$ exposed to air for 15 min and refrozen) obtained at 10 K (9.422 GHz, receiver gain 5×10^4, other settings as 3a) exhibited the loss of the Co(II) signal and the concomitant increase of the radical signal ($G = 2.0$) with a molar spin ratio of 0.67. (d) EPR spectrum of contaminating artifact. Under certain conditions of Co(II)-precorrin generation and derivatization (long incubations with Co(II) and long esterification times) a distinct EPR spectrum could be observed which was little changed on exposure to air. This signal was found not to be uniquely associated with Co(II)-precorrin-2 following subsequent TLC, an equal signal intensity being found throughout the TLC gradient.

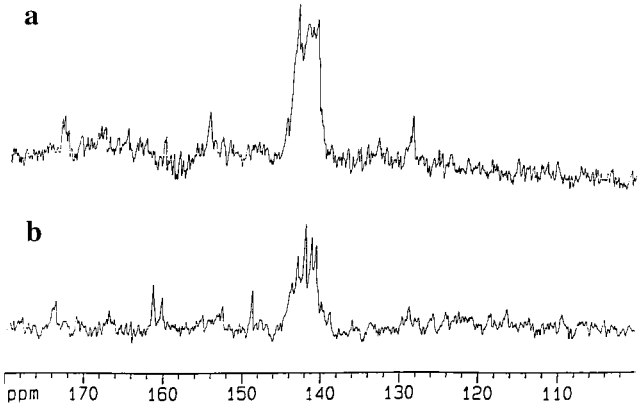


FIGURE 4: (a) ^{13}C NMR spectrum of Co(III)-factor-II. Oxidation of Co(II)-precorrin-2 (205 μM in $CDCl_3$) derived from [^{13}C-4]-ALA gave a set of signals at 142−146 ppm. Poor resolution is attributable to the paramagnetic species which is likely arising from Co(III)-$O_2^{-\bullet}$. (b) ^{13}C NMR spectrum of Co(III)-factor-II on addition of KCN. The addition of KCN to Co(III)-factor-II (205 μM in $CDCl_3$) derived from [^{13}C-4]-ALA resulted in the resolution of the set of signals at 142−146 ppm due to the formation of a diamagnetic species (EPR silent, data not shown).

by MALDI-MS revealed that both precorrins and Co(II)-precorrins took a similar time to become fully esterified giving M_r values of 980 and 1039, respectively (8 units higher due to ^{13}C labeling by ALA). The appearance of an intermediate prior to full esterification having a M_r 14 units lower did not show evidence for lactone formation by IR. This is in contrast to esterifications carried out in the presence of O_2 and, therefore, most likely indicates a heptamethyl derivative with a free carboxyl group. Overnight incubations with sulfuric acid concentrations less than 2% did not result in the generation of the fully esterified derivative whereas this was the case using 2.5% sulfuric acid. Esterification using 5% sulfuric resulted in the generation of the fully esterified product within 4 h. However, prolonged esterification at 5% sulfuric acid concentration resulted in the progressive loss of the metal ion from the metallo-precorrin.

Following TLC of the octamethyl derivatives, two major bands containing Co(II)-tetrapyrroles were identified by MALDI-MS, an upper bluish band and a lower greenish band of lower intensity; both gave rather similar UV/vis absorption with a strong visible absorption at 580 nm ($\epsilon = 2.9 \times 10^4$ L mol^{-1}) (Figure 1). Exposure of the blue band to O_2 resulted in a color change to green. A heptamethyl derivative of Co(II)-precorrin also isolated gave a similar UV/vis spectrum. Interpretation of the system would indicate a Co(II) redox state (Soret 400−410 nm; G. Müller, personal communication) as opposed to Co(III) (Soret 420−425 nm; ref *18*; G. Müller, personal communication). However, UV/vis interpretations can be complicated by the nature of the axial ligand and solvent effects; for example the presence of methanol can cause a 35 nm red shift of the Soret band

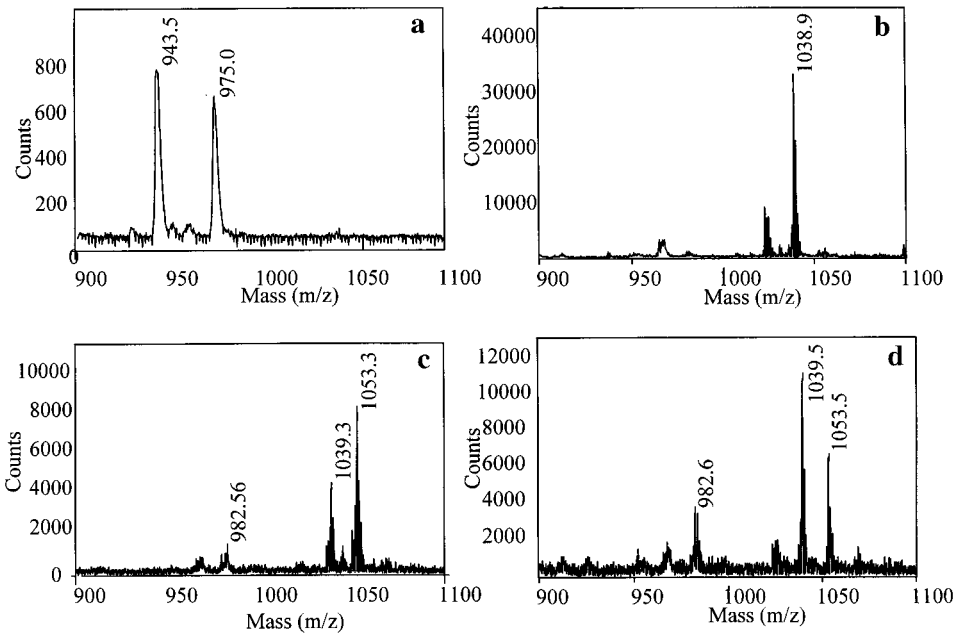


FIGURE 5: (a) MALDI-MS analysis of factor-II following incubation with CbiL. The incubation mixture of factor-II with CbiL and SAM was esterified, extracted, and prepared for MALDI-MS analysis. Only significant peaks at $m/z = 943$ (Uro III) and 976 (factor-II) were observable indicating that CbiL cannot methylate factor-II (similar results were obtained with precorrin-2, data not shown). (b) MALDI-MS analysis of Co(II)-precorrin-2. ^{13}C-[4]-ALA derived precorrin-2 was incubated with Co(II) esterified, extracted, and prepared for MALDI-MS analysis. The major peak observed at $m/z = 1038$ corresponds to Co(II)-precorrin-2 (similar results were obtained with Co(III)-factor-II, data not shown). (c) MALDI-MS analysis of Co(II)-precorrin-2 following incubation with CbiL. The overnight incubation mixture of [^{13}C-4]-ALA derived Co(II)-precorrin-2 with CbiL and SAM was esterified, extracted, and prepared for MALDI-MS analysis. Major peaks observed at $m/z = 1038$ and $m/z = 1053$ correspond to Co(II)-precorrin-2 and Co(II)-precorrin-3, respectively. (d) MALDI-MS analysis of Co(III)-factor-II following incubation with CbiL. The overnight aerated incubation mixture of [^{13}C-4]-ALA derived Co(III)-factor-II with CbiL and SAM was esterified, extracted, and prepared for MALDI-MS analysis. Major peaks observed at $m/z = 1038$ and $m/z = 1053$ correspond to Co(III)-factor-II and Co(III)-factor-III, respectively.

of Co(III) octaethyl porphyrin from 370 to 405 nm (*19*). Consequently UV/vis spectroscopy cannot be used unambiguously to define a previously uncharacterized system, although once the axial ligands have been defined for that system, the associated UV/vis spectra can be used as a means of confirmation. However, the UV/vis spectrum of Co(III)-factor-II can be used to eliminate the possibility that a cation radical species is generated on oxidation by O_2, as the visible bands at 580 nm are still distinct (*20*).

^{13}C NMR Characterization of the Initial Redox State of the Precorrin in ^{13}C-Labeled Co(II)-precorrin-2. Subsequent ^{13}C NMR of TLC purified octamethyl Co(II)-precorrin (derived from [^{13}C-4]-ALA) gave poorly resolved spectral lines although it was evident that the bluish band corresponded to precorrin-2 in the reduced state (Figure 2) whereas the greenish band was in the oxidized factor-II state (Figure 2). Subsequent EPR studies indicated that both species were paramagnetic. Exposure of the blue solution of Co(II)-precorrin-2 to O_2 resulted in the oxidation of the precorrin-2 ligand to the chromophore of factor-II. The poor NMR resolution observed may result from paramagnetic line broadening as similarly observed in other paramagnetic metallo-porphyrins (*15*, *21*, *22*). Both the reduced octamethyl and heptamethyl ester derivatives of Co(II)-precorrin-2 gave two sets of NMR signals expected for a reduced precorrin at around 115 and 127 ppm. However, on oxidation of both of these compounds, the signals at 115 and 127 ppm were replaced by a new set of signals at 140−146 ppm indicative of the oxidized form, namely, the isobacteriochlorin.

Characterization of the Initial Redox State of Metallo-precorrin-2 Intermediates by EPR. The EPR signal of Co-precorrin-2 clearly demonstrated a Co(II) EPR signal with a g value of 2.3 and hyperfine coupling to the cobalt nuclear spin with an A value of 80 G (Figure 3a) which is within the range of values of 80−100 G and 80−150 G observed for other Co(II) porphyrins and Co(II)-corrins, respectively (*23*, *24*). The molar spin equivalent of 0.63 (±0.15) indicates the presence of an unpaired electron within the Co(II)-precorrin-2 system. The unpaired electron of $g = 2.3$ exhibiting hyperfine coupling indicates that the location was on the cobalt ion, hence allowing the assignment of the initial substrate as Co(II)-precorrin-2.

As with other Co(II) and Ni(III) porphyrins (*16*), Co(II)-precorrin-2 did not show any superhyperfine coupling to the pyrrole nitrogens indicating that the free electron associated with the cobalt atom is in the d_{z^2} orbital perpendicular to the nitrogens in the plane of the porphyrin ring and hence unable to couple into their nuclei. Superhyperfine coupling has only been observed with other Co(II)-porphyrins on addition of nitrogen ligands in the axial position, which is in plane with the d_{z^2} orbital, hence permitting nuclear coupling (*25*). In contrast to Co(II) and Ni(III) porphyrins, Ni(I)-porphyrins contain the unpaired electron in the $d_{x^2-y^2}$ orbital where superhyperfine coupling to the in-plane porphyrin nitrogens is possible (*26*). As expected for a d^8 metal ion Ni(II)-precorrin-2 and Ni(II)-factor-II were EPR silent.

Effect of O_2 on the Oxidation State of Co(II)-precorrin-2. Exposure of Co-precorrin-2 to O_2 resulted in the loss of the EPR Co(II) signal and the appearance of a radical signal of

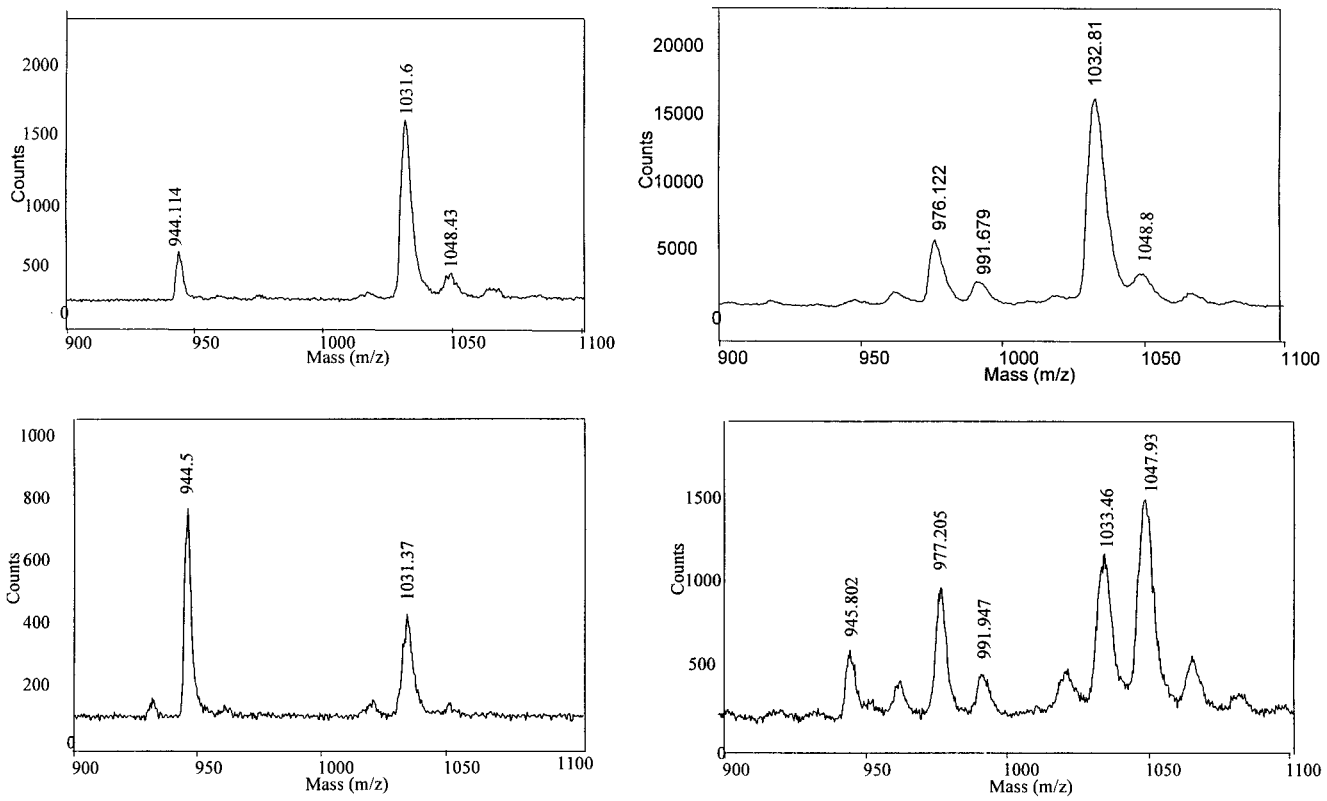


FIGURE 6: (a) MALDI-MS analysis of Ni(II)-precorrin-2. Ni(II)-precorrin-2 was prepared and derivatized as in Methods. MALDI-MS spectrum of the extract gave major peaks at $m/z = 944$ (uroIII) and $m/z = 1032$ (Ni(II)-precorrin-2); the identity of the minor peak at 1048 was not determined. (b) MALDI-MS analysis of Ni(II)-precorrin-2 following incubation with CbiL. Ni(II)-precorrin-2 was prepared as in Methods and incubated with CbiL and SAM overnight. The mixture was then esterified and prepared for MALDI-MS. MALDI-MS spectrum gave major peaks at $m/z = 975$ (precorrin-2) and $m/z = 1032$ (Ni(II)-precorrin-2). The minor peak at $m/z = 1048$ was similar to that found in Ni(II)-precorrin-2 alone. The peak at $m/z = 991$ indicates that some Ni(II)-precorrin-3 had been generated but the Ni(II) ion was lost on esterification. (c) MALDI-MS analysis of Ni(II)-factor-II. Ni(II)-factor-II was prepared from Ni(II)-precorrin-2 by a 1 h exposure to air and derivatized as in Methods. MALDI-MS spectrum of the extract gave major peaks at $m/z = 944$ (uroIII) and $m/z = 1031$ (Ni(II)-factor-II). The identity of a minor peak at $m/z = 1048$ was not determined. (d) MALDI-MS analysis of Ni(II)-factor-II following incubation with CbiL. Ni(II)-factor-II was prepared as in c, degassed, and incubated with CbiL and SAM under argon overnight. The mixture was then esterified and prepared for MALDI-MS. The MALDI-MS spectrum obtained gave major peaks at $m/z = 945$ (uroIII), $m/z = 975$ (factor-II), $m/z = 1033$ (Ni(II)-factor-II), and $m/z = 1048$ (Ni(II)-factor-III). The peak at $m/z = 991$ indicates that some Ni(II)-precorrin-3 had been generated but the Ni(II) ion is lost on esterification.

g value = 2.0, the intensity of which depended on the level of exposure to O_2. The resulting radical species generated by O_2 gave a similar molar spin equivalent 0.67 ($\pm$0.15) as the original reduced form of Co-precorrin-2 (Figure 3b,c). A possible origin of this radical species is the generation of Co(III)-$O_2^{-\bullet}$ which is also EPR active. However, the observed spectrum is different from those of other Co(III)-$O_2^{-\bullet}$ porphyrins documented in the literature (*14*) lacking any fine structure.

Although the possibility of O_2 bridged dimers was not rigorously eliminated the observed EPR spectrum was qualitatively the same over the concentration range 2−300 μM.

Depending on the esterification conditions, a further EPR spectrum could be generated arising from an unknown species that did not remain associated with the Co(II)-precorrin after an additional TLC. This EPR spectrum was therefore attributed to the generation of a radical impurity (Figure 3d).

Effect of KCN on the Redox Characteristics of Co(II)-precorrin-2 and Its Oxidized Equivalent. Addition of KCN to Co(II)-precorrin-2 at 0.2 ppm O_2 did not result in any color change, any increase in resolution of the NMR spectrum, or any alteration of the redox state of the precorrin or metal ion. However, addition of KCN to Co(III)-factor-II did result in the increased resolution of the ^{13}C NMR signals present in the O_2 oxidized Co(III)-factor-II (Figure 4a,b). The associated EPR spectrum of KCN treated Co(III)-factor-II was silent indicating that a redox event had occurred converting the species to a diamagnetic species and consequently increasing the NMR resolution.

Effect of Metal Ligation by Precorrin-2 and Factor-2 on Methylation by CbiL. Incubation of CbiL and SAM with precorrin-2 or factor-II did not result in subsequent methylation of these possible substrates (Figure 5a). However, with the prior insertion of Co(II) into precorrin-2, CbiL was able to methylate this substrate as indicated by the resulting MALDI-MS of the product (Figure 5b,c). The oxidized Co(III)-factor-II was similarly methylated (Figure 5d), and therefore, CbiL appears to have no absolute requirement for the redox state of its cobalt tetrapyrrole substrate; however, the effect of the metal ion on the tetrapyrrole aromaticity and hence its methylation properties, as demonstrated in model systems (*12*), remains to be determined.

Investigation of the Metal Selectivity of CbiL. On incubation of Ni(II)-precorrin-2 with CbiL and SAM only a small amount of metallo-free precorrin-3 ($M_r = 991$) was observed (Figure 6a,b). As CbiL cannot methylate precorrin-2, the

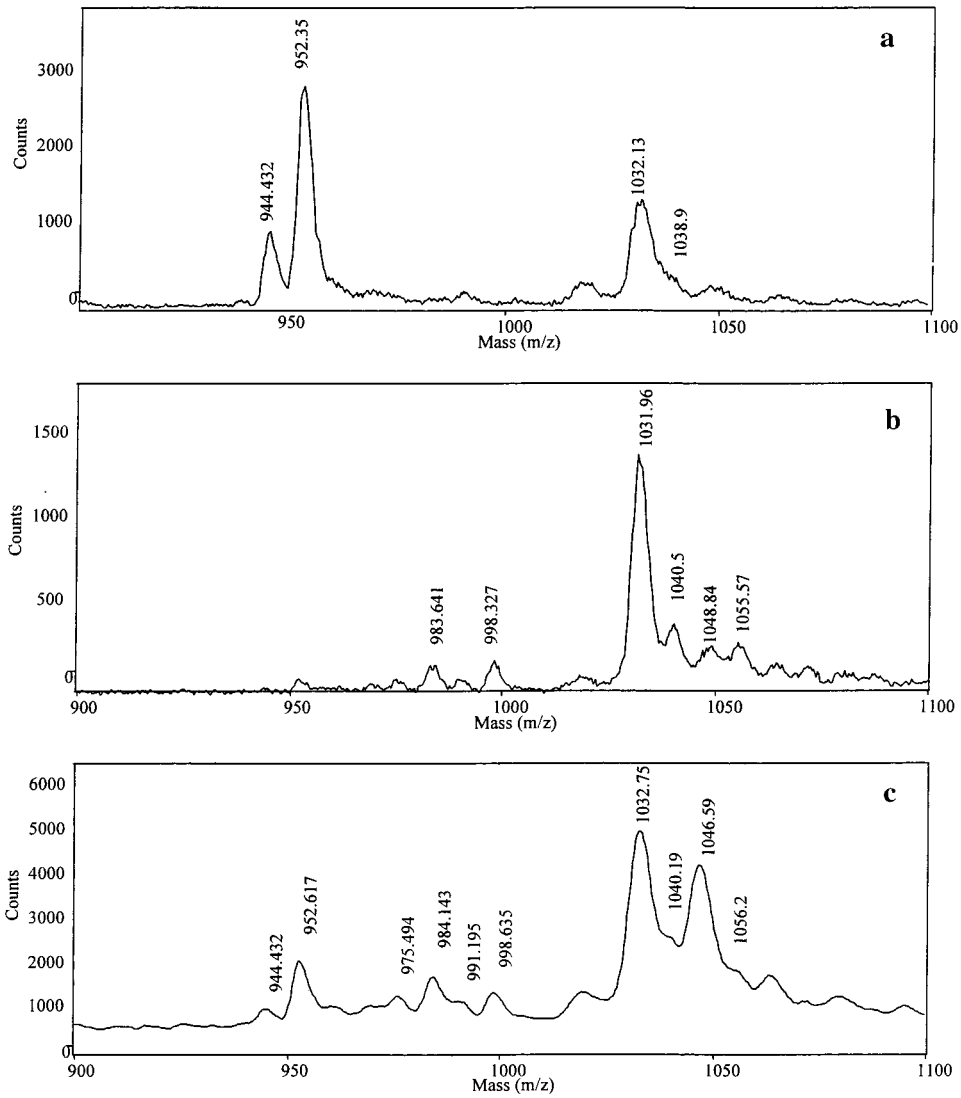


FIGURE 7: (a) MALDI-MS analysis of the initial ratio of ^{12}C-Ni(II)-precorrin-2 and ^{13}C-Co(II)-precorrin-2. Ni(II) and Co(II)-precorrins were generated separately as described in methods from ^{12}C and ^{13}C-ALA, respectively. The two metallo-precorrins were then mixed and the initial ratios determined by MALDI-MS following esterification and extraction. Peaks were observed at m/z = 944 and 952 (^{12}C and ^{13}C uroIII) and m/z = 1032 and 1039 (^{12}C-Ni(II)-precorrin-2 and ^{13}C-Co(II)-precorrin-2). (b) MALDI-MS analysis of ^{12}C-Ni(II)-precorrin-2 and ^{13}C-Co(II)-precorrin-2 mix following incubation with CbiL. The initial mix of ^{12}C-Ni(II)-precorrin-2 and ^{13}C-Co(II)-precorrin-2 shown in part a was incubated with CbiL and SAM overnight. The mixture was then esterified, extracted, and analyzed by MALDI-MS. Peaks were observed at m/z = 945 and 952 (^{12}C and ^{13}C uroIII), m/z = 984 (^{12}C-precorrin-2), m/z = 999 (^{13}C-precorrin-3 generated from ^{13}C-Co(II)-precorrin-3 by loss of Co(II)), m/z = 1032 and 1041 (^{12}C-Ni(II)-precorrin-2 and ^{13}C-Co(II)-precorrin-2), m/z = 1048 (peak similar to that in 7a), and m/z = 1056 (^{13}C-Co(II)-precorrin-3). (c) MALDI-MS analysis of a ^{12}C-Ni(II)-factor-II and ^{13}C-Co-factor-II mixture following incubation with CbiL. The initial mix of ^{12}C-Ni(II)-factor-2 and ^{13}C-Co(III)-factor-2 shown in part c was incubated with CbiL and SAM overnight. The mixture was then esterified, extracted, and analyzed by MALDI-MS. Peaks were observed at m/z = 945 and 952 (^{12}C and ^{13}C uroIII), m/z = 976 and 985 (^{12}C-factor-II and ^{13}C-factor-II), m/z = 992 and 999 (^{12}C-factor-II and ^{13}C-factor-III generated by loss of metal ion from their respective metallo derivatives), m/z = 1033 and 1041 (^{12}C-Ni(II)-factor-II and ^{13}C-Co(III)-factor-II), and m/z = 1047 and 1056 (^{12}C-Ni(II)-factor-III and ^{13}C-Co(III)-factor-III).

precorrin-3 generated must have arisen via Ni(II)-precorrin-3 following loss of the Ni(II) ion during the esterification process. Incubation of Ni(II)-factor-II with CbiL and SAM resulted in a much increased level of methylation indicating that CbiL had a marked preference for the oxidized redox state of this substrate (Figure 6c,d). Competition experiments were performed by co-incubation of CbiL and SAM with Ni(II)-precorrin-2 and Co(II)-precorrin-2 derived from ^{12}C and ^{13}C-ALA respectively to allow molecular weight tagging of these two otherwise identical mass substrates. Comparing the ^{12}C/^{13}C ratio in the products and the remaining substrate to the initial substrate ^{12}C/^{13}C ratio would reveal any substrate preference of CbiL. CbiL was still able to methylate Co(II)-precorrin-2 in the presence of a 5-fold excess of Ni(II)-precorrin-2 to an extent similar to that of Co(II)-precorrin-2 alone, as evidenced by the appearance of Co(II)-precorrin-3 (M_r = 1056) and precorrin-3 derived from ^{13}C-ALA (M_r = 999) which could only have arisen via Co(II)-precorrin-3 (Figure 7a,b). CbiL was found to methylate Ni(II) and Co(III)-factor-II to similar extents indicating no preference for the metal ion in the oxidized substrate (Figure 7c).

Incubation of Zn(II)-precorrin-2 with cbiL also resulted in some methylation of this substrate. As with the Ni(II)-factor-II, the oxidized Zn(II)-factor-II was a much better substrate for CbiL than Zn(II)-precorrin-2 giving 10 times more product Zn(II)-factor-III. However, following esteri-

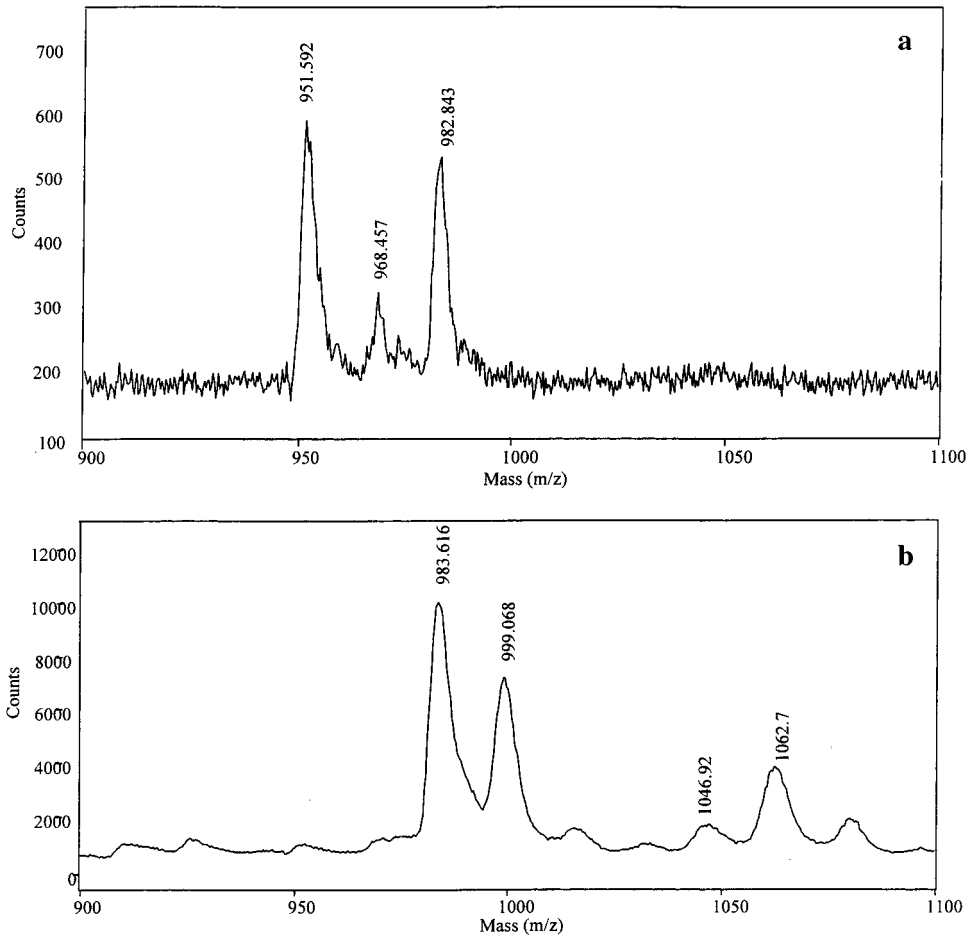


FIGURE 8: (a) MALDI-MS analysis of Zn(II)-precorrin-2. ^{13}C-labeled Precorrin-2 was incubated overnight with 1 mM $ZnSO_4$ under an argon atmosphere then freeze-dried and esterified with 2% H_2SO_4/methanol for 12 h. On analysis by MALDI-MS, major peaks were observed at $m/z = 952$ (UroIII) and 983 (precorrin-2) and a minor peak was observed at $m/z = 969$ (precorrin-1). (b) MALDI-MS analysis of Zn(II)-factor-III. ^{13}C-labeled Zn(II)-precorrin-2 was oxidized to Zn(II)-factor-II and incubated with CbiL and SAM under an argon atmosphere for 14 h. The sample was then freeze-dried and esterified as in part a. On analysis by MALDI-MS, major peaks were observed at $m/z = 984$ (factor-II), 999 (factor-III), and 1063 (Zn(II)-factor-III)

fication with 5% H_2SO_4/methanol only the metal-free substrate and product were observed. Further reduction in the concentration of H_2SO_4 to 2% still only yielded 30% of the product with Zn(II) bound, although Zn(II) was lost from the substrate (Figure 8a,b). Therefore it appears that the Zn-(II)-precorrinoid complexes are significantly more acid sensitive than the equivalent Ni(II) complexes, which required 12 h at 5% H_2SO_4 for significant demetalation. The metal lability of these precorrins is in line with previous studies which found that Zn(II) metallo-porphyrins were much more acid-labile than Co(II) and Ni(II) metallo-porphyrins (*27*). The extreme metal lability of Zn(II)-factor-III, formed by the methylation of Zn(II)-factor-II by CbiL, allowed the preparation of the metal-free product after esterification with 5% H_2SO_4 for 6 h. By using ^{13}C-5-ALA, the site of methylation by CbiL was identified as C_{20} on the alteration of the chemical shift of C_{20} on methylation from 95 ppm to 105 ppm (*28*). On this basis, the site of methylation of nickel and cobalt tetrapyrrole substrates of CbiL was assumed to be the same. The UV/vis spectrum of Co(III)-factor-III prepared via CobI or CbiL was identical again suggesting that the site of methylation is identical.

Investigation of the Redox Preference of CbiL for Its Cobalt Tetrapyrrole Substrate. To determine if CbiL had a preference for a particular redox state of its cobalt tetrapyrrole substrate, both the reduced and oxidized substrates were coincubated with CbiL. The substrate redox state was tagged by synthesizing the reduced substrate from ^{12}C-ALA and the oxidized form from ^{13}C-ALA. The initial substrate ^{12}C/^{13}C ratio was then determined by MALDI-MS (Figure 9a). MALDI-MS of the mixture following incubation with cbiL and SAM revealed similar ^{12}C/^{13}C ratios in both the products and the remaining substrate (Figure 9b), indicating no significant preference of CbiL for the redox state of the cobalt tetrapyrrole, in contrast to that observed for Ni(II) and Zn-(II) tetrapyrroles.

DISCUSSION

The importance of metallo-porphyrins in biochemistry has long been acknowledged. More recently it has been appreciated that, in contrast to the static redox state of Fe(II) in the heme of hemoglobin, many other metallo-porphyrins are required to cycle through several redox states during the course of their biochemical function. This redox cycling is exemplified by the iron ion in the heme of P450 of catalase and peroxidase enzymes (*29*) and the cobalt ion of vitamin B_{12} in methyl transfer reactions. More recently the redox cycling of the nickel ion in the prosthetic group F430 of methyl-coenzyme M reductase was demonstrated during the generation of methane by the methanogenic bacterium

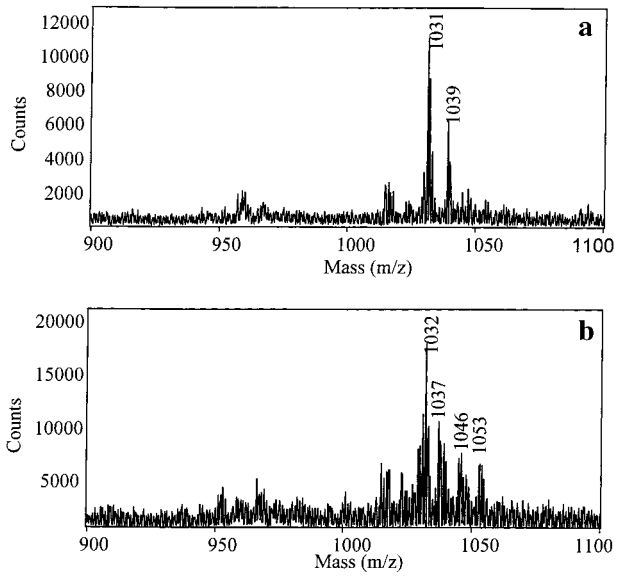


FIGURE 9: (a) MALDI-MS of the initial ratio of ^{12}C-Co(III)-factor-II and ^{13}C-Co(II)-precorrin-2. Co(II)-precorrin-2 was generated separately from ^{12}C-ALA and ^{13}C-ALA as described in Methods. ^{12}C-Co(II)-precorrin-2 was then oxidized to ^{12}C-Co(III)-factor-II by a 1 h exposure to air and then degassed and mixed with ^{13}C-Co(II)-precorrin-2 under an argon atmosphere. Following esterification and extraction, the initial ratio of ^{12}C-Co-factor-2 to ^{13}C-Co(II)-precorrin-2 was determined by MALDI-MS. Peaks were observed at $m/z = 1031$ (^{12}C-Co(III)-factor-2) and 1039 (^{13}C-Co-(II)-precorrin-2). (b) MALDI-MS of a ^{12}C-Co(III)-factor-II and ^{13}C-Co(II)-precorrin-2 mixture following incubation with CbiL. ^{12}C-Co(III)-factor-II and ^{13}C-Co(II)-precorrin-2 mix shown in 8a was incubated overnight with CbiL and SAM under an argon atmosphere. Following esterification and extraction, the mixture was analyzed by MALDI-MS. Peaks were observed at $m/z = 1032$ (^{12}C-Co(III)-factor-II), $m/z = 1037$ (^{13}C-Co(II)-precorrin-2), $m/z = 1046$ (^{12}C-Co(III)-factor-III), and $m/z = 1053$ (^{13}C-Co(II)-precorrin-3).

Methanobacterium thermoautotrophicum (*30*). An additional proposed biochemical role employing metallo-porphyrin redox chemistry is in the biosynthesis of cobalt-corrin from a cobalt-precorrin precursor in the anaerobic vitamin B_{12} biosynthetic pathway (*2*). In this case the cobalt ion is proposed to replace the redox function played by O_2 in the aerobic pathway. To be able to demonstrate a redox role of the cobalt ion the redox states of intermediates along the pathway have to be documented. The first step of this undertaking has now begun with the assignment of the redox state of the first cobalt tetrapyrrole intermediate of the anaerobic pathway with the use of NMR and EPR indicates that it has a reduced dipyrrocorphin ring containing a Co-(II) ion.

A requirement for the redox chemistry of the cobalt ion in ring contraction during corrin synthesis is the ability of electrons to shuttle back and forth from the cobalt ion and tetrapyrrole ligand (Scheme 2). The proposed disproportionation of Co(II)-precorrin-3 may well be analogous to the disproportionation of Ni(III) tetraphenylporphyrin (Ni(III)-TPP$^+$) to the free radical cation Ni(II)-TPP$^{\bullet+}$ (*16*). Both of these Co(II) and Ni(III) tetrapyrroles would exhibit a similar EPR spectrum upon disproportionation converting from a metal signal at $g = 2.3$ to a radical signal at $g = 2.0$ upon an electron transfer between the d_{z^2} and the porphyrin π^* orbitals. Interestingly, electron transfer between Co(II) and its cationic radical macrocycle has recently been demonstrated (*11*). This electron transfer would usually not be observable by EPR due to spin coupling between the Co(II) and cationic radical macrocycle prior to electron transfer and due to spin pairing after electron transfer. However, the use of a coupled Co(II)/Co(III)-porphyrin system allowed the use of EPR to follow electron transfer initiated by the addition of a strong axial ligand to the cobalt ion. It could be envisaged that the enzyme catalyzing the ring contraction step of the anaerobic pathway of B_{12} biosynthesis might well control the axial ligand properties of the Co(II)-precorrin and thereby control the electron shuttling process required during the chemistry.

On investigating substrate requirements for CbiL activity it was found that this enzyme had an absolute requirement for the presence of metal ion (Co(II), Ni(II), or Zn(II)) within the precorrin. It was earlier shown (*31*) that CbiL was able to methylate precorrin-2 at a very low level (<5%). However, it is possible that a small amount of a metallo-precorrin-2 was present in the incubation mixture, probably due to the presence of Zn(II) in the buffer used. This small amount of Zn(II)-precorrin-2 could then be methylated by CbiL to Zn(II)-precorrin-3 which was then esterified. As found in the present study, overnight esterification with 5% sulfuric acid/methanol results in the complete loss of the Zn-(II) ion from the Zn(II)-precorrin. Thus the product, Zn-(II)-precorrin-3 was isolated as metal-free precorrin-3, giving the misleading appearance of direct methylation of precorrin-2 in previous experiments. The requirement of a metallo-substrate for CbiL could be rationalized on the basis of the expected conformational change of the macrocycle ring required to optimize the metallo-N bonds (*12*). On interacting with the substrate binding site the ruffled metallo-macrocycle ring would be easily distinguishable from the more planar metal-free precorrin. The importance of the ease of macrocycle ruffling could explain the low affinity of Ni-(II)-precorrin-2 for CbiL compared to the greater affinity of the stiffer macrocycle ring of the oxidized Ni(II)-factor-2. The stiffer oxidized macrocycle resisted the ruffling by Ni-(II) to a greater extent likely confining the conformation toward that of the usual cobalt tetrapyrrole substrate. With our new ability to assign redox states for potential substrates of CbiL it was found that this enzyme had no absolute requirement or preference for a particular redox state of its cobalt tetrapyrrole substrate indicating that, in contrast to Ni(II)-precorrin-2, the flexibility of the macrocycle ring of Co(II)-precorrin-2 does not greatly impinge upon its binding affinity for CbiL. However, the lack of any requirement for a particular redox state of the cobalt tetrapyrrole substrate of CbiL does not imply that enzymes further down the pathway are similarly indifferent to the redox state of their substrates. Indeed a specific redox requirement for ring closure would surely be required. The lack of discriminatory effect of CbiL upon the redox state of its cobalt tetrapyrrole substrate most probably reflects the fact that it always encounters the reduced form due to the anaerobic nature of the environment required to support anaerobic bacterial growth. However, the possibility that CbiL may exercise a much smaller kinetic discrimination between the redox states of its cobalt tetrapyrrole substrates remains to be investigated. The marked preference for CbiL for the oxidized Ni(II)-factor-II substrate does not necessarily imply a similar preference for the oxidized Co(III)-factor-2 as substrate conformation is determined by both the redox state of the

tetrapyrrole ring and the nature of the bound metal ion. However, as shown here, CbiL would easily be capable of distinguishing between Ni(II) or Zn(II) and Co(II) forms of the reduced substrate. The failure of CbiL to methylate Ni-(II)-precorrin-2, a potential intermediate in $Factor_{430}$ biosynthesis (*32*), would thus allow the biosynthesis of $Factor_{430}$ in methanogens which also biosynthesize cobalt-corrins, by avoiding diversion of the potential Ni(II)-precorrin-2 intermediate into the corrinoid pathway. A Blastp search of the ncbi protein database with the CbiL sequence from *S. typhimurium* found a homologous protein in two obligate anaerobic methanobacteria, *Mb. thermoautotrophicum* and *Mb. jannaschii* (39% identity, 56% conserved; 26% identity, 47% conserved, respectively). However, these values are similar to the match found with the aerobic *P. denitrificans* CobI (29% identity, 51% conserved) and so preclude any conclusions on the substrate specificity of their CbiL homologues based on sequence comparison. The substrate specificity of these CbiL homologues in these methanogens remains to be determined.

The overexpression of the enzymes of the anaerobic B_{12} biosynthetic pathway and the ability to characterize the redox properties of the cobalt tetrapyrrole intermediates as they are transformed during biosynthesis will now provide the means for elucidating the proposed role of the chelated cobalt ion in ring contraction.

ACKNOWLEDGMENT

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