How corrinoids are synthesized without oxygen: nature's first pathway to vitamin B_{12}

Patricio J Santander, Charles A Roessner, Neal J Stolowich, Marc T Holderman and A Ian Scott

Background: During the biosynthesis of vitamin B_{12}, the aerobic bacterium *Pseudomonas denitrificans* uses two enzymes, CobG and CobJ, to convert precorrin-3 to the ring-contracted intermediate, precorrin-4. CobG is a monooxygenase that adds a hydroxyl group, derived from molecular oxygen, to C-20, whereas CobJ is bifunctional, inserting a methyl group at C-17 of the macrocycle and catalyzing ring contraction. Molecular oxygen is not available to vitamin B_{12}-producing anaerobic bacteria and members of the ancient Archaea, so the question arises of how these microbes accomplish the key ring-contraction process.

Results: Cloning and overexpression of *Salmonella typhimurium* genes has led to the discovery that a single enzyme, CbiH, is responsible for ring contraction during anaerobic biosynthesis of vitamin B_{12}. The process occurs when CbiH is incubated with precorrin-3, but only in the presence of cobalt. CbiH functions as a C-17 methyltransferase and mediates ring contraction and lactonization to yield the intermediate, cobalt–precorrin-4, isolated as cobalt–factor IV. ^{13}C labeling studies have proved that cobalt–precorrin-4 is incorporated into cobyrinic acid, thereby confirming that cobalt–precorrin-4 is an intermediate in vitamin B_{12} biosynthesis.

Conclusions: Two distinct mechanisms exist in nature for the ring contraction of porphyrinoids to corrinoids – an ancient anaerobic pathway that requires cobalt complexation prior to nonoxidative rearrangement, and a more recent aerobic route in which molecular oxygen serves as the cofactor. The present results offer a rationale for the main differences between aerobic and anaerobic biosynthesis of vitamin B_{12}. Thus, in anaerobes there is exchange of oxygen at the C-27 acetate site, extrusion of acetaldehyde and early insertion of cobalt, whereas the aerobes show no exchange of oxygen at C-27, extrude acetic acid and insert cobalt very late in the biosynthetic pathway, after ring contraction has occurred. These parallel routes to vitamin B_{12} have now been clearly distinguished by their differing mechanisms for ring contraction.

Address: Center for Biological NMR, Chemistry Department, Texas A&M University, College Station, TX 77843-3255, USA.

Correspondence: A Ian Scott and Charles A Roessner
E-mail: scott@chemvx.tamu.edu and roessner@chemvx.tamu.edu

Key words: anaerobic vitamin B_{12} biosynthesis, CbiH methyltransferase, cobalt–precorrin-4, ring contraction

Received: **23 June 1997**
Revisions requested: **16 July 1997**
Revisions received: **21 July 1997**
Accepted: **24 July 1997**

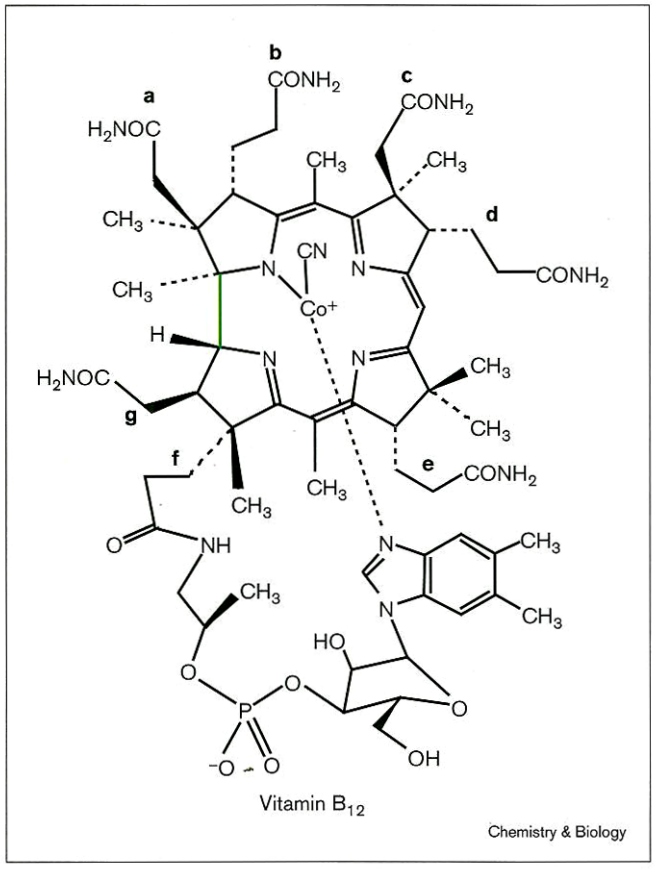
Chemistry & Biology September 1997, **4**:659–666
http://biomednet.com/elecref/1074552100400659

Introduction

One of the most prominent features of the structure of vitamin B_{12} (Figure 1) is that the porphyrinoid macrocycle has undergone extrusion of one of the ring carbons (C-20) via a process termed ring contraction, an event that is unique among the 'pigments of life' derived from uroporphyrinogen III; such compounds also include heme, siroheme, chlorophyl and the methanogenic factor, coenzyme F_{430}. During vitamin B_{12} biosynthesis in the aerobic bacterium *Pseudomonas denitrificans* (for a recent review, see [1]), ring contraction is achieved in two steps (Figure 2, upper scheme) from the metal-free intermediate, precorrin-3. Using a genetically engineered system, consisting of *P. denitrificans* vitamin B_{12} biosynthetic genes overexpressed in *Escherichia coli* [2], it was demonstrated that this process requires two enzymes, the monooxygenase, CobG, which is totally dependent on molecular oxygen [3,4] and the methyltransferase/ring contractase, CobJ. This mechanism probably evolved after the establishment of an oxygen-containing atmosphere ~1.7×10^9 years ago [5].

Vitamin B_{12} is, however, also synthesized under strictly anaerobic conditions in certain other species of bacteria and archaea, such as *Salmonella typhimurium* [6] and *Methanococcus jannaschii* [7], via a more ancient pathway (~3.8×10^9 years old [5]), which presents a paradox of how the mechanism of ring contraction can operate in these organisms under anaerobic conditions. No homolog to the oxygen-dependent CobG enzyme has been discovered in any of the organisms that synthesize vitamin B_{12} anaerobically, including *Propionibacterium shermanii* ([8] and our unpublished observations), *S. typhimurium* [9] and *M. jannaschii*

Figure 1



The structure of vitamin B_{12}. The region that has undergone ring contraction is highlighted in green.

[7], all of which have conserved two other indispensable [10] genes, *cbiD* and *cbiG*, which are not found in *P. denitrificans*. These findings suggest that the microorganisms which synthesize vitamin B_{12} anaerobically share a common pathway that differs in certain aspects from the pathway found in *P. denitrificans*. The pathway that does not require oxygen may also function in some aerobes, however, because analysis of the genes found in the obligate aerobe *Bacillus megaterium* [11] indicates that it uses the same pathway as the three organisms (mentioned above) that synthesize vitamin B_{12} anaerobically.

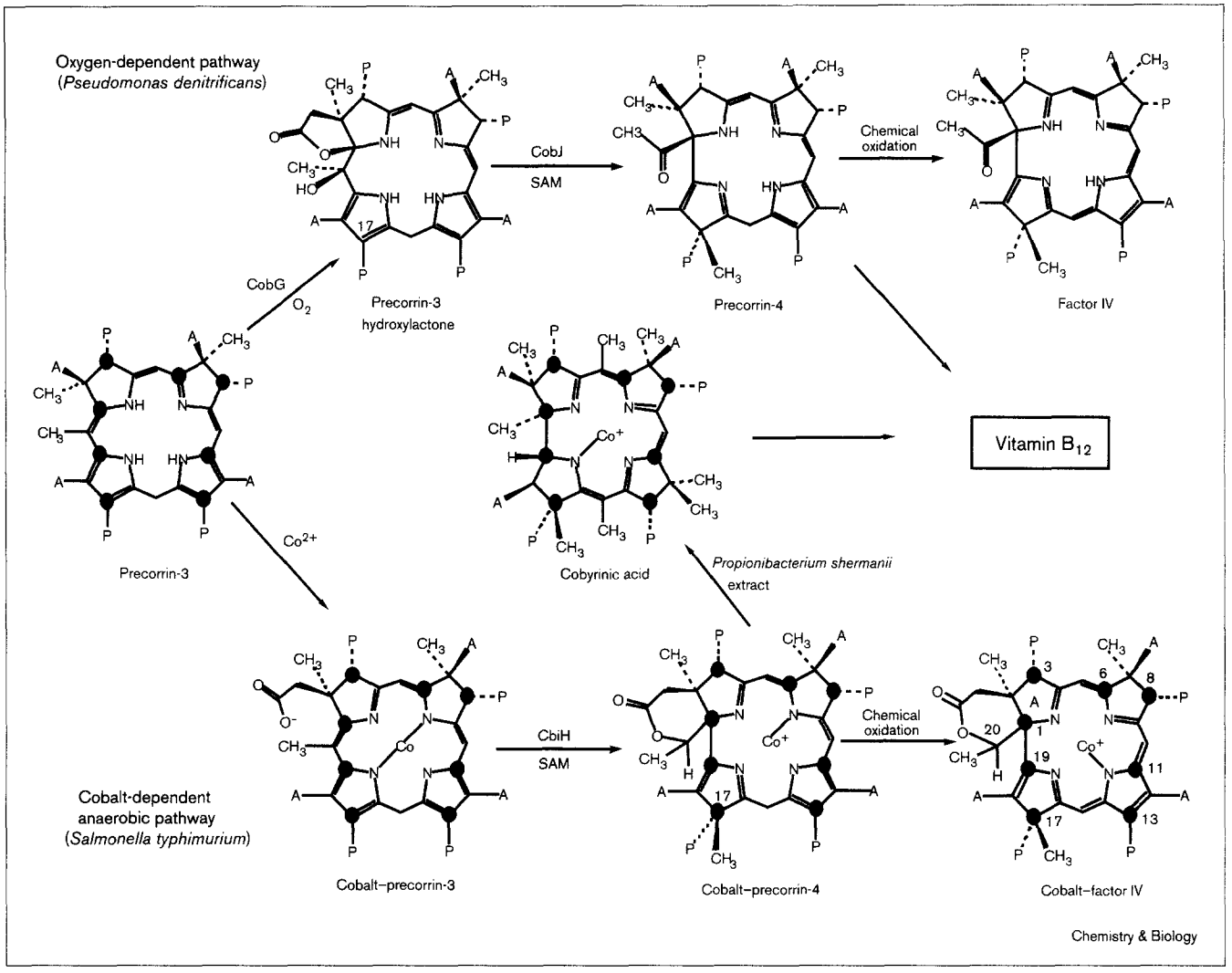
An important clue towards resolving the paradox of ring contraction in the absence of oxygen was provided by studies performed with *P. shermanii* cell extracts [12,13], which showed that cobalt insertion into the macrocycle occurs before ring contraction. This is in contrast to the oxygen-dependent pathway of *P. denitrificans* in which cobalt is inserted into the late intermediate hydrogenobyrinic acid *a,c*-diamide (for labeling, see Figure 1), the formation of which requires eight distinct reactions catalyzed by six different enzymes after contraction of the macrocycle [1,2]. This result suggested that formation of a cobalt complex of an early intermediate such as precorrin-2 or precorrin-3 is required to provide the proper substrate for the enzyme that catalyzes ring contraction. Analysis of the homology between CobJ, which triggers ring contraction by the addition of a methyl group at C-17 of precorrin-3 hydroxylactone (Figure 2), and the predicted methyltransferases of *S. typhimurium* [9] suggested that the enzyme CbiH is the most likely candidate for setting the stage for ring contraction in this organism. In this report, we show that CbiH from *S. typhimurium* not only catalyzes C-17 methylation, but also mediates formation of a δ-lactone and contraction of the precorrin macrocycle in the presence of cobalt. We thereby provide experimental evidence for the *modus operandi* of the pivotal ring-contraction mechanism for vitamin B_{12} biosynthesis in anaerobes, and unequivocal proof for the existence of two distinct pathways to vitamin B_{12}, for which only circumstantial evidence has been available hitherto.

Results and discussion

Methylation and ring contraction

When *E. coli* cell lysates containing CbiH from *S. typhimurium* [14], *S*-adenosyl-L-methionine (SAM), cobalt chloride and precorrin-3 were incubated in the absence of oxygen, a new green compound was produced, which, on esterification and separation by thin layer chromatography from unconverted precorrin-3 and several other porphyrinoids, exhibited the same physical properties (R_f value and ultra-violet/visible spectrum) as 8-epi factor IV, a cobalt-containing corrinoid isolated from *P. shermanii* ([15] and G. Müller, personal communication). To determine whether the macrocycle had undergone ring contraction, as suggested by the physical characteristics of the green compound, precorrin-3 was prepared from [4-^{13}C]-ALA (ALA: 5-aminolevulinic acid) to provide the isotopomer labeled as shown in Figure 2. With this pattern of eight labeled carbons, contraction of the macrocycle would result in the direct connection between C-1 and C-19, the signals from which should then appear in the nuclear magnetic resonance (NMR) spectrum as doublets with the same coupling constant. The isotopomer of precorrin-3 was incubated with cobalt chloride, SAM and CbiH, and the purified product was analyzed by ^{13}C NMR (Figure 3). The spectrum was found to have two doublets with chemical shifts of 83.3 ppm and 152.2 ppm, demonstrating coupling between C-1 (J = 55 Hz) and C-19 (J = 55 Hz), respectively, and thereby proving that CbiH had, indeed, induced contraction of the macrocycle. In the *P. denitrificans* oxygen-dependent pathway, CobJ triggers ring contraction by addition of a methyl group at C-17 of the hydroxylactone of precorrin-3. Similarly, CbiH catalyzed addition of a methyl group, as demonstrated by the appearance of a new resonance (sp^3 carbon) assigned to a propionate terminus in the product. The chemical shift

Figure 2

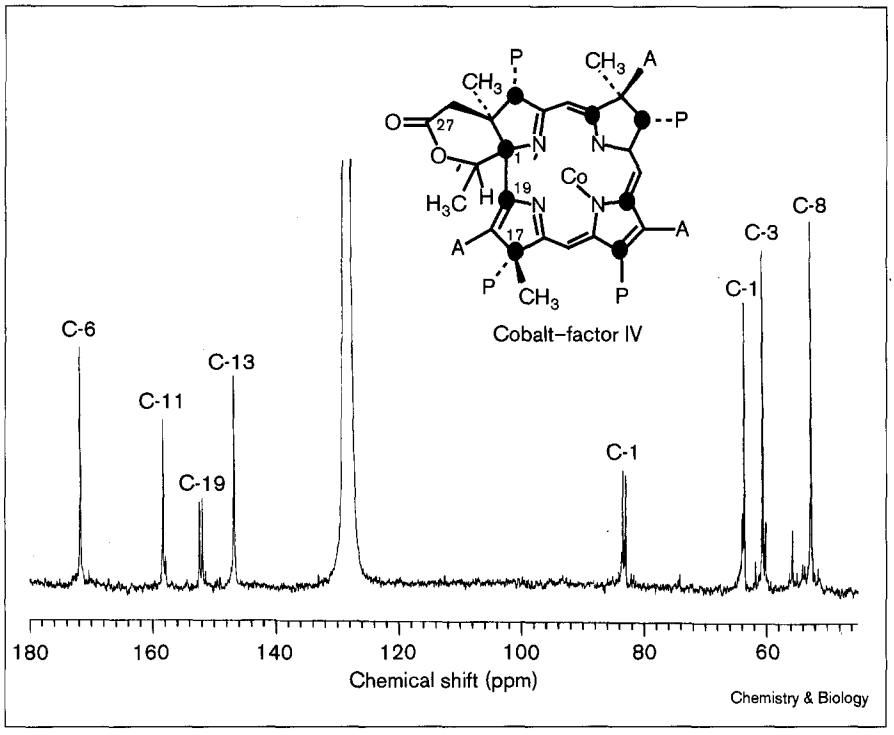


The two pathways for the biosynthesis of vitamin B_{12}. Upper scheme: the oxygen-dependent route from precorrin-3 to the ring-contracted precorrin-4 via the intermediate precorrin-3 hydroxylactone, during vitamin B_{12} biosynthesis in *Pseudomonas denitrificans*. Lower scheme: the proposed cobalt-dependent route to corrinoids during vitamin B_{12} biosynthesis in anaerobic organisms. The labeled carbons of the isotopomers derived from [4-^{13}C]-ALA (ALA: 5-aminolevulinic acid) are indicated by a filled circle. A, CH_2CO_2H; P, $CH_2CH_2CO_2H$; CobG, monooxygenase; CobJ, methyltransferase/ring contractase; SAM, *S*-adenosyl-L-methionine; CbiH, the single enzyme responsible for ring contraction during anaerobic vitamin B_{12} biosynthesis.

of this signal (63.7 ppm) is consistent with methylation at C-17 [15]. When the incubation was performed with [$^{13}CH_3$]-SAM, the signal appeared as a doublet with a corresponding doublet in the methyl region (not shown), again demonstrating that the new sp^3 center is a result of C-methylation.

The CbiH product, as isolated for analysis, was esterified and became oxidized during the aerobic workup prior to NMR analysis. In accordance with the convention of naming vitamin B_{12} intermediates according to their oxidation level and number of methyl groups, the isolated product was designated factor IV and its reduced form was designated precorrin-4. The related, but dissimilar, CobJ product of *P. denitrificans* (Figure 2) has, however, already been referred to as precorrin-4 and its oxidized form as factor IV. Further confusing the nomenclature, a cobalt-containing, tetramethylated, ring-contracted compound recently isolated from cell-free incubations with *P. shermanii* extracts was also referred to as factor IV [15]. The actual CbiH product, prior to aerobic workup, is assumed to be in the reduced form, named cobalt–precorrin-4, one possible structure for which is shown in Figure 2 (other tautomeric forms may exist), and in this paper will hereafter be called cobalt–precorrin-4, which, when oxidized, becomes cobalt–factor IV (Figure 2).

Figure 3



^{13}C nuclear magnetic resonance spectrum of the product isolated from the incubation of ^{13}C-precorrin-3 with cobalt, SAM, and CbiH (abbreviations as in Figure 2). The eight labeled carbons derived from [4-^{13}C]-ALA are indicated by filled circles.

CysG may be required for conversion of precorrin-3 to cobalt–precorrin-4 *in vivo*, but not *in vitro*

In *S. typhimurium*, CysG has been implicated as the enzyme responsible not only for the synthesis of precorrin-2 and siroheme (via NAD(P)-dependent oxidation of precorrin-2 and iron chelation), but also for the chelation of cobalt by precorrin-2 during vitamin B_{12} biosynthesis [16,17]. In this scenario, the resultant cobalt–precorrin-2 would then be converted to cobalt–precorrin-3 by methylation of C-20 by CbiL [9,14]. In most of our experiments, the cell-free lysates that provided the CbiH enzyme also contained low amounts of endogenous CysG (provided by the *cysG* gene of the *E. coli* host), which could have been involved in the oxidation or reduction of precorrin-3 or cobalt insertion during the synthesis of cobalt–precorrin-4. However, the corrinoid was still obtained when CbiH was produced in *E. coli* strain 302Δa, in which the *cysG* gene is deleted [18]. In addition, control experiments using cell lysates derived from the *cysG*$^+$ *E. coli* host without CbiH did not synthesize the corrinoid. These results indicate that, in our anaerobic cell-free system, the precorrin-3 is not oxidized to factor III prior to cobalt insertion and then reduced back to cobalt–precorrin-3, and that cobalt chelation into precorrin-3 occurs spontaneously in the presence of excess cobalt ions. Although we have previously reported [14] methylase activity for the *S. typhimurium* CbiL (low activity) and CbiF (high activity) methyltransferases, we have never observed methylation of precorrin-3 by CbiH in the absence of cobalt; this confirms the requirement for cobalt in the substrate for CbiH. The metal insertion can be achieved chemically (as described in the present work) or enzymatically, as in the presence of CysG.

Cobalt–precorrin-4 is produced in cell-free extracts of *P. shermanii*

Assignments of the remaining five carbons of cobalt–factor IV derived from [4-^{13}C]-ALA and of the eight carbons of the isotopomer derived from [5-^{13}C]-ALA (Table 1), fortified by ultra-violet/visible and mass spectral comparisons, demonstrate that cobalt–factor IV prepared as above is virtually identical to the factor IV recently isolated after incubation of the C-8 β-propionate epimer of precorrin-3 — (8-*epi*)-precorrin-3 — and cobalt in cell-free lysates of *P. shermanii* [15]. In this experiment, the use of 'unnatural' (8-*epi*)-precorrin-3 as substrate was designed to derail the pathway to permit accumulation of the product. This epimeric center also accounts for the only significant difference between the NMR spectra of the two compounds (Table 1), namely the chemical shift of C-8. Thus, cobalt–*epi*-factor IV, isolated in the *P. shermanii* experiment, was probably formed by C-17 methylation of cobalt–(8-*epi*)-precorrin-3 (by the CbiH methyltransferase present in the total cell-free extract), which formed cobalt–*epi* precorrin-4 and was then oxidized during workup and could re-enter the pathway only after reduction of the macrocycle and epimerization at C-8 [15].

Table 1

^{13}C NMR assignments (in benzene $^{2}H_6$) of the heptamethyl ester of the CbiH product and comparison with the assignments of the same carbons of the factor IV heptamethyl ester.

Chemical shifts of ester from [4-^{13}C]-ALA (ppm)			Chemical shifts of ester from [5-^{13}C]-ALA (ppm)		
Carbon	CbiH product	Factor IV	Carbon	CbiH product	Factor IV
C-1	83.5	83.4	C-4	178.4	178.0
C-3	60.2	60.1	C-5	95.1	95.2
C-6	171.8	171.8	C-9	173.1	173.0
C-8	52.8	55.7	C-10	95.1	96.6
C-11	158.4	157.9	C-14	151.5	151.8
C-13	146.8	146.7	C-15	98.8	99.8
C-17	63.7	64.0	C-16	178.4	177.5
C-19	151.9	151.4	C-20	82.0	81.9

Cobalt–precorrin-4 is an intermediate in anaerobic vitamin B_{12} biosynthesis

Using ^{13}C-labeled precursors we have also shown that cobalt–precorrin-4 is efficiently converted to cobyrinic acid in a cell-free *P. shermanii* extract, thereby providing compelling evidence that it is a true intermediate in the anaerobic vitamin B_{12} pathway. To monitor conversion of cobalt–precorrin-4 to cobyrinic acid, cobalt–precorrin-4 was first synthesized in an incubation containing precorrin-3 (derived from [4-^{13}C]-ALA), cobalt, CbiH, and [^{13}C]-SAM, so that only eight ring carbons and the C-17 methyl group were labeled. The incubation products, including cobalt–precorrin-4 (not isolated) and unconverted cobalt–precorrin-3, were then separated from nonincorporated [^{13}C]-SAM and incubated in a cell-free extract of *P. shermanii* in the presence of unlabeled SAM. The ^{13}C-labeled cobyrinic acid, derived from both cobalt–precorrin-3 and cobalt–precorrin-4, was isolated as its heptamethyl ester (cobester) and analyzed by NMR (Figure 4). The analysis revealed the presence of only one ^{13}C-enriched methyl group at 18.1 ppm, a value previously assigned as the chemical shift for the methyl group at C-17 in cobester [19]. The signal was a doublet (J = 34 Hz) and, on close inspection, the corresponding doublet (J = 34 Hz) could be resolved at the base of the signal previously assigned to the chemical shift of C-17 in cobester [19], a result that not only confirms the biointermediacy of the CbiH product, but securely places the fourth methyl of cobalt–precorrin-4 at C-17.

We can conclude that, in the anaerobic pathway of vitamin B_{12} biosynthesis, CbiH catalyzes the methylation of the C-17 of cobalt–precorrin-3 (Figure 2, lower scheme), and that this methylation triggers a cascade of events leading to δ-lactone formation and contraction of the macrocycle to give a product that is an intermediate in the pathway to vitamin B_{12}. Thus, in the anaerobic pathway, only one enzyme and the presence of cobalt are needed, compared with the two enzymes and oxygen required in the aerobic route to vitamin B_{12}.

Figure 4

The upfield region of the ^{13}C NMR spectrum of the heptamethyl ester of cobyrinic acid (cobester) showing the propionate terminal carbons and the methyl region. The cobester was synthesized in a cell-free *P. shermanii* extract from a mixture of cobalt–precorrin-3 (major peaks), labeled from [4-^{13}C]-ALA, and cobalt–precorrin-4, doubly labeled from [4-^{13}C]-ALA and [^{13}C]-SAM, as described in the text. The doublet signals derived from the ^{13}C-labeled methyl group, the ^{13}C-labeled methyl group, and the carbon to which it is attached are highlighted.

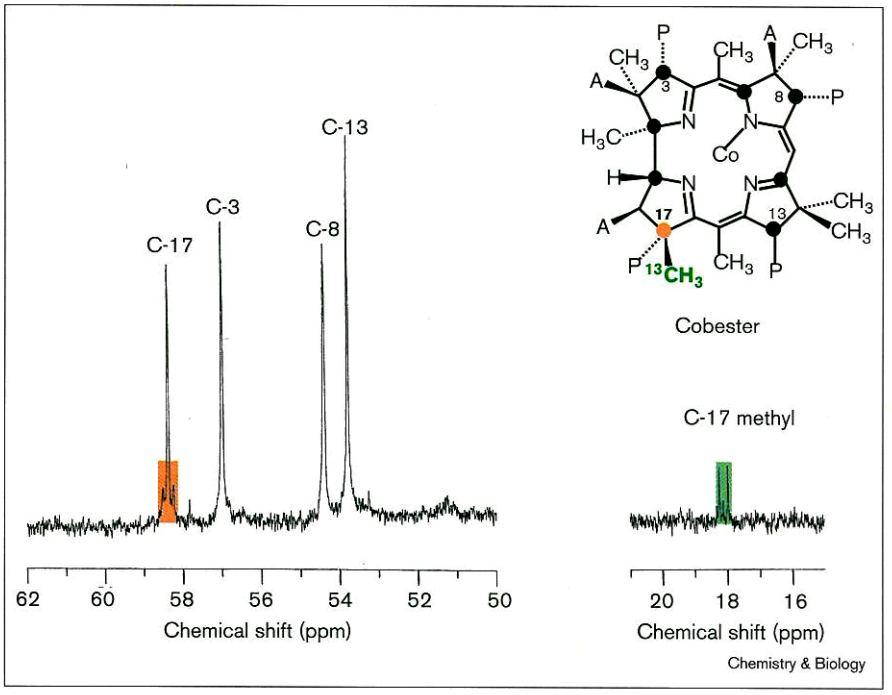
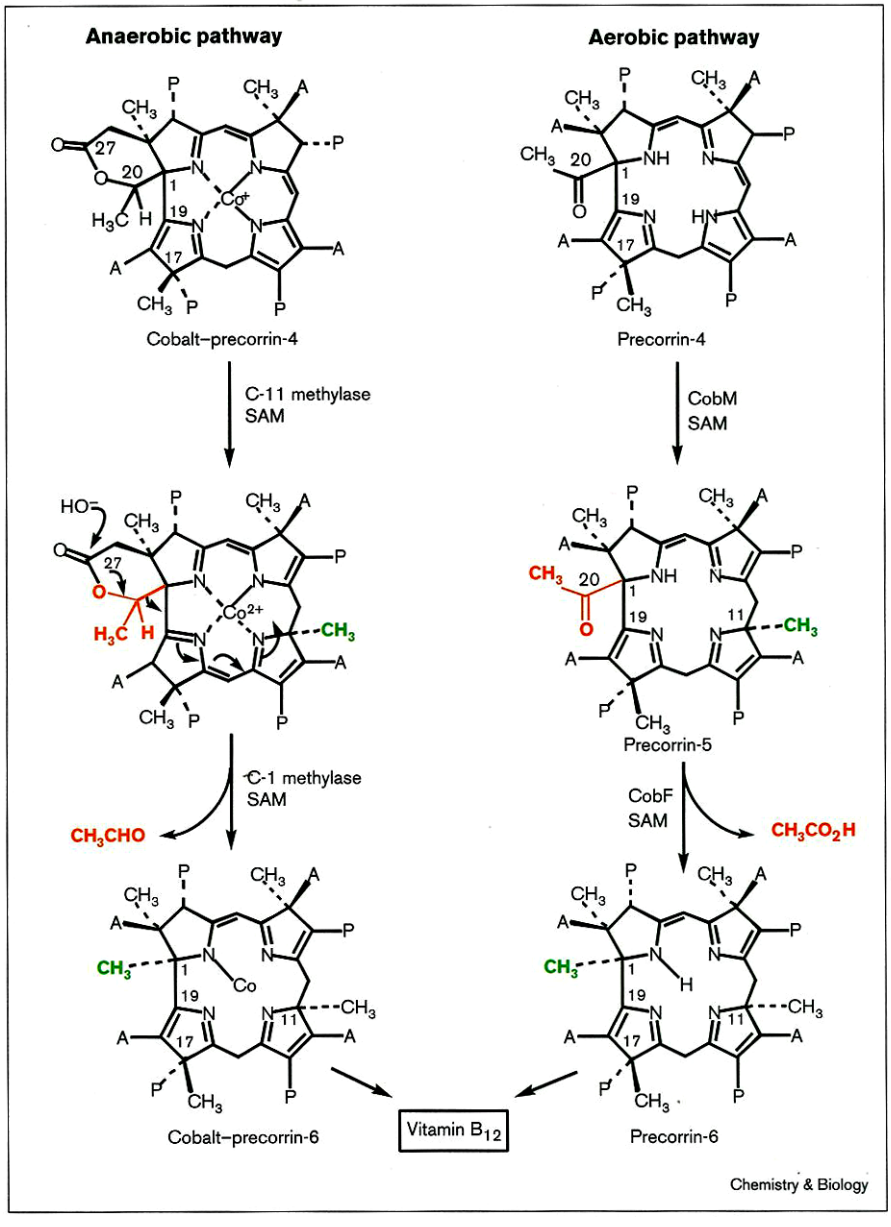


Figure 5



A comparison of the steps proposed for the conversion of cobalt–precorrin-4 to cobalt–precorrin-6 in the anaerobic pathway (left-hand scheme) to the parallel pathway for the conversion of precorrin-4 to precorrin-6 in the aerobic pathway (right-hand scheme). The new methyl group in each step is highlighted in green and the origin of acetaldehyde in the anaerobic pathway and the origin of acetic acid in the aerobic pathway are highlighted in red. Abbreviations as in Figure 2.

Other differences between the two pathways

The evolution of the oxygen-dependent pathway has conserved many aspects of the anaerobic pathway, but has also resulted in significant differences. For example, the carboxyl of the ring A acetate (Figure 5, left-hand scheme, C-27) undergoes extensive exchange with the water from the medium in the anaerobic pathway, but not in the oxygen-dependent pathway [20,21]. In addition, in the anaerobic pathway, C-20 and its attached methyl group are extruded as acetaldehyde [22], whose oxygen is derived from the carboxyl of the ring A acetate (Figure 5, left-hand scheme; [23]), whereas the oxygen-dependent route produces acetic acid directly with no involvement of the ring A carboxyl (Figure 5, right-hand scheme). These differences are probably a reflection of the dissimilarity in the steps immediately following ring contraction that are required to convert precorrin-4 to precorrin-6 in the aerobic pathway and cobalt–precorrin-4 to cobalt–precorrin-6 in the anaerobic pathway (Figure 5). The conversion in the anaerobic pathway requires opening of the δ-lactone ring, C-11 methylation, extrusion of acetaldehyde, and C-1 methylation. In this scenario, exchange with water by the carboxyl of the ring A acetate results from hydration during opening of the δ-lactone, and the oxygen of the acetaldehyde is concomitantly derived from the ring A acetate during this process. The enzyme catalyzing this

event remains to be identified, but as no function has been assigned to the indispensable *S. typhimurium* CbiD or CbiG proteins, one or both of these may be involved. By analogy to the aerobic pathway, cobalt–precorrin-5 is plausibly formed by C-11 methylation, which could occur either before or after hydrolysis of the lactone. This step is most likely to be catalyzed by CbiF, which has C-11 methylase activity [14] and is similar to CobM, which performs the analogous methylation in the aerobic series, but there are no enzymes within the known anaerobic repertoire for C-1 methylation and acetaldehyde-yielding hydrolysis that would convert cobalt–precorrin-5 to the next putative intermediate, cobalt–precorrin-6. Curiously, CobF, responsible for the similar process (deacylation, C-1 methylation) in *P. denitrificans* (Figure 5) has no counterpart in *S. typhimurium*, suggesting that one of the previously identified methyltransferases of the latter organism, perhaps CysG or CbiF, returns to play a second role in the production of vitamin B_{12}. In addition, because it has been shown that precorrin-6 of the oxygen-dependent series can be converted to cobyrinic acid in *P. shermanii* extracts [24], the remainder of the anaerobic route, from cobalt–precorrin-6 onwards, appears to parallel that of the oxygen-dependent pathway, with the exception that the intermediates are all cobalt complexes. Confirmatory evidence for the biosynthetic sequence suggested above is being sought by identification of the functions of the remaining Cbi enzymes from *S. typhimurium* and *P. shermanii*.

Significance

The biosynthesis of vitamin B_{12} by the aerobic bacterium *Pseudomonas denitrificans* requires the monooxygenase CobG and the bifunctional enzyme CobJ to convert precorrin-3 to the ring-contracted intermediate, precorrin-4. In vitamin B_{12} producing anaerobic bacteria and members of the ancient Archaea, molecular oxygen is not available. A different mechanism must, therefore, occur in these microbes to enable them to accomplish the key ring-contraction process.

Identification of the enzyme, CbiH, responsible for C-17 methylation, ring contraction, and δ-lactone formation in the anaerobic pathway of vitamin B_{12} biosynthesis has revealed the major difference between the ring-contraction mechanism in anaerobic organisms and the oxygen-dependent process used by aerobes. The operation of two parallel, but distinct, routes to vitamin B_{12} in nature has been rigorously proved by the discovery of a novel mechanism that requires the presence of cobalt coordinated to the ligand of precorrin-3. To the best of our knowledge, this biosynthesis of a major cofactor by two different pathways is a very rare occurrence in nature and can be compared with the duality of the biosynthesis of 4,5-dimethylbenzimidazole (DBI), which in aerobes is formed by oxidative degradation of riboflavin, but in anaerobes is constructed *de novo* from glycine, erythrose phosphate and *S*-adenosyl-L-methionine [25]. Both aerobes and anaerobes use DBI as the axial ligand to cobalt in vitamin B_{12} as part of the nucleotide loop (see Figure 1).

Materials and methods

Biosynthesis and isolation of cobalt–precorrin-4 (as cobalt–factor IV)

Cobalt–precorrin-4 was formed in lysates containing CbiH prepared in 50 mM potassium phosphate buffer, pH 7.6, to which was added 0.5 mM SAM, 0.15 M KCl, 0.8 mM $CoCl_2.6H_2O$, and 0.0375 mM ^{13}C-labeled precorrin-3. The KCl, cobalt, and precorrin-3 were preincubated at room temperature at a tenfold higher concentration for 6 h prior to addition to the lysate. The precorrin-3 was enzymatically prepared from ^{13}C-labeled aminolevulinic acid (ALA) in a multienzyme reaction containing ALA dehydratase, porphobilinogen deaminase, uroporphyrinogen (urogen) III synthase, urogen III methyltransferase (CobA), and precorrin-2 methyltransferase (CobI) as previously described [26] with the exception that a lysate of a new recombinant strain of *E. coli* (CR462) that simultaneously overexpresses the two methyltransferases, (Roessner C.A. and Holderman M.T., unpublished observations), was used for the source of these two enzymes. After a 16 h incubation at 30°C, the tetrapyrrolic products were adsorbed to DEAE-Sephadex, esterified anaerobically in MeOH:H_2SO_4 (95:5), separated by thin layer chromatography on silica plates (CH_2Cl_2:MeOH, 95:5), and the oxidized product, cobalt-factor IV heptamethyl ester, examined by NMR as previously described [15]. The mass spectra and ultra-violet/visible spectra for the esterified labeled product are: m/z 1054 (MALDI) and λ_{max} (750, 680, 630, 387, 340).

Conversion of cobalt–precorrin-4 to cobyrinic acid in a P. shermanii *cell-free extract*

P. shermanii cell-free extracts were prepared as previously described [15]. For preparation of cobalt precorrin-4 so that both C-17 and its attached methyl group were labeled, precorrin-3 was prepared from [4-^{13}C]-ALA and then incubated with cobalt, CbiH, and ^{13}C-SAM such that eight ring carbons were labeled as shown in Figure 2 and the only labeled methyl group was the one at C-17. The incubation products, a mixture of cobalt–precorrin-4 (not isolated) and unconverted cobalt–precorrin-3, were then separated from nonincorporated ^{13}C-SAM and incubated without esterification/oxidation, in a cell-free extract of *P. shermanii* in the presence of unlabeled SAM. The tetrapyrroles were isolated and esterified and the cobester was isolated by thin layer chromatography.

Acknowledgements

We thank NIH for financial support, Mario Jimenez for technical assistance, Jeff Cole for supplying *E. coli* strain 302Δa, Jianji Wang for synthesis of [4-^{13}C]-ALA, Lloyd Sumner for mass spectrometric analysis, and Jeong-Ho Park for synthesis of [$^{13}CH_3$]-SAM.

References

1. Blanche, F., *et al.*, & Battersby, A.R. (1995). Vitamin B_{12}: how the problem of its biosynthesis was solved. *Angew. Chem. Int. Ed. Engl.* **34**, 383-411.
2. Roessner, C.A., *et al.*, & Scott, A.I. (1994). Genetically engineered synthesis of precorrin 6-x and the complete corrinoid, hydrogenobyrinic acid, an advanced precursor of vitamin B_{12}. *Chem. Biol.* **1**, 119-124.
3. Scott, A.I., Roessner, C.A., Stolowich, N.J., Spencer, J.B., Min, C. & Ozaki, S. (1993). Biosynthesis of vitamin B_{12}: discovery of the enzymes for oxidative ring contraction and insertion of the fourth methyl group. *FEBS Lett.* **331**, 105-108.
4. Spencer, J.B., Stolowich, N.J., Roessner, C.A., Min, C. & Scott, A.I. (1993). Biosynthesis of vitamin B_{12}: ring contraction is preceded by incorporation of molecular oxygen into precorrin 3. *J. Am. Chem. Soc.* **115**, 11610-11611.
5. Scott, A.I. (1993). How nature synthesizes vitamin B_{12}. A survey of the last four billion years. *Angew. Chem. Int. Ed. Engl.* **32**, 1223-1243.

6. Jeter, R., Olivera, B.M. & Roth, J.R. (1984). *Salmonella typhimurium* synthesizes cobalamin (vitamin B_{12}) de novo under anaerobic growth conditions. *J. Bacteriol.* **159**, 206-216.
7. Bult, C.J., *et al.*, & Venter, J.C. (1996). Complete genomic sequence of the methanogenic archaeon, *Methanococcus jannaschii. Science* **273**, 1058-1073.
8. Roessner, C.A., Huang K. & Scott, A.I. (1997). Isolation and characterization of genes required for vitamin B_{12} biosynthesis in *Propionibacterium freudenreichii* (*shermanii*). In *Proceedings of the 4th European Symposium on Vitamin B_{12} and B_{12} Proteins*, VCH, Weinheim, Germany, in press.
9. Roth, J.R., Lawrence, J.G., Rubenfield, M., Kieffer-Higgens, S. & Church, G.M. (1993). Characterization of the cobalamin (vitamin B_{12}) biosynthetic genes of *Salmonella typhimurium. J. Bacteriol.* **175**, 3303-3316.
10. Raux, E., *et al.*, & Thermes, C. (1996). *S. typhimurium* cobalamin (vitamin B_{12}) biosynthetic genes; functional studies in *S. typhimurium* and *E. coli. J. Bacteriol.* **178**, 753-767.
11. Raux, E., Warren, M.J., Lanois, A., Rambach, A. & Thermes, C. (1997). Identification and characterisation of a *Bacillus megaterium cob* operon: an obligate aerobe with an anaerobic cobalamin biosynthetic pathway. In *Proceedings of the 4th European Symposium on Vitamin B_{12} and B_{12} Proteins*, VCH, Weinheim, Germany, in press.
12. Müller, G., *et al.*, & Thibaut, D. (1991). Timing of cobalt insertion in vitamin B_{12} biosynthesis. *J. Am. Chem. Soc.* **113**, 9891-9893.
13. Balchandran, S., *et al.*, & Battersby, A.R. (1994). Biosynthesis of porphyrins and related macrocycles. Pulse labeling experiments concerning the timing of cobalt insertion during vitamin B_{12} biosynthesis. *J. Chem. Soc. Perkin Trans.* **1**, 487-491.
14. Roessner, C.A., *et al.*, & Scott, A.I. (1992). Expression of 9 *Salmonella typhimurium* enzymes for cobinamide biosynthesis: identification of the 11-methyl and 20-methyl transferases of corrin biosynthesis. *FEBS Lett.* **301**, 73-78.
15. Scott, A.I., Stolowich, N.J., Wang, J., Gawatz, O., Fridrich, E., & Müller, G. (1996). Biosynthesis of vitamin B_{12}: factor IV, a new intermediate in the anaerobic pathway. *Proc. Natl Acad. Sci. USA* **93**, 14316-14319.
16. Fazzio, T.G. & Roth, J.R. (1996). Evidence that the CysG protein catalyzes the first reaction specific to B_{12} synthesis in *Salmonella typhimurium*, insertion of cobalt. *J. Bacteriol.* **178**, 6952-6959.
17. Raux, E., Beck, R., Levillayer, F., Rambach, A., Thermes, C. & Warren, M.J. (1997). A role for *Salmonella typhimurium cbiK* in cobalamin (vitamin B_{12}) and siroheme biosynthesis. *J. Bacteriol.* **179**, 3202-3212.
18. Griffiths, L.A., & Cole, J.A. (1987). Lack of redox control of the anaerobically-induced *nirB*$^+$ gene of *Escherichia coli* K12. *Arch. Microbiol.* **147**, 364-369.
19. Battersby, A.R., Edington, C., Fookes, C.J.R. & Hook, J.M. (1982). Biosynthesis of vitamin B_{12}: analysis of the ^1H and ^{13}C n. m. r. spectra of heptamethyl dicyanocobyrinate (cobester). *J. Chem. Soc. Perkin Trans.* **1**, 2265-2277.
20. Scott, A.I., *et al.*, & Okazaki, T. (1991). Timing and mechanistic implications of regiospecific carbonyl oxygen isotope exchange during vitamin B_{12} synthesis. *J. Am. Chem. Soc.* **113**, 9893-9895.
21. Vishwakarma, R., *et al.*, & Battersby, A.R. (1993). Biosynthesis of porphyrins and related macrocycles. Fate of oxygen atoms as precorrin-2 carrying eight labeled carboxyl groups ($^{13}C^{18}O_2H$) is enzymatically converted into cobyrinic acid. *J. Chem. Soc. Perkin Trans.* **1**, 2893-2899.
22. Wang, J., Stolowich, N.J., Santander, P.J., Park, J-H., & Scott, A.I. (1996). Biosynthesis of vitamin B_{12}: concerning the identity of the two-carbon fragment eliminated during anaerobic formation of cobyrinic acid. *Proc. Natl Acad. Sci. USA* **93**, 14320-14322.
23. Arigoni, D. (1994). The biosynthesis of the tetrapyrrole pigments. *CIBA Found. Symp.* **180**, 280.
24. Blanche, F., Thibaut, D., Debussche, L., Hertle, R., Zipfel, F. & Müller, G. (1993). Parallels and decisive differences in vitamin B_{12} biosyntheses. *Angew. Chem. Int. Ed. Engl.* **32**, 1651-1653.
25. Renz, P. (1997). Recent investigations on the biosynthesis of the 5,6-dimethylbenzimidazole moiety of vitamin B_{12}. In *Proceedings of the 4th European Symposium on Vitamin B_{12} and B_{12} Proteins*, VCH, Weinheim, Germany in press.
26. Roessner, C.A., *et al.*, & Scott, A.I. (1995). Overexpression in *Escherichia coli* of twelve vitamin B_{12} biosynthetic enzymes. *Protein Expr. Purif.* **6**, 155-163.