Mechanism of the Ring Contraction Step in Vitamin B_{12} Biosynthesis: The Origin and Subsequent Fate of the Oxygen Functionalities in Precorrin-3x

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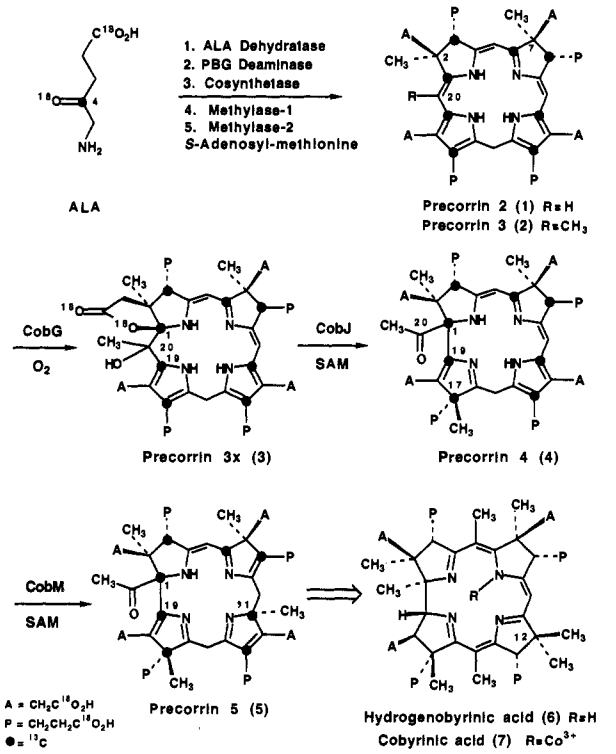
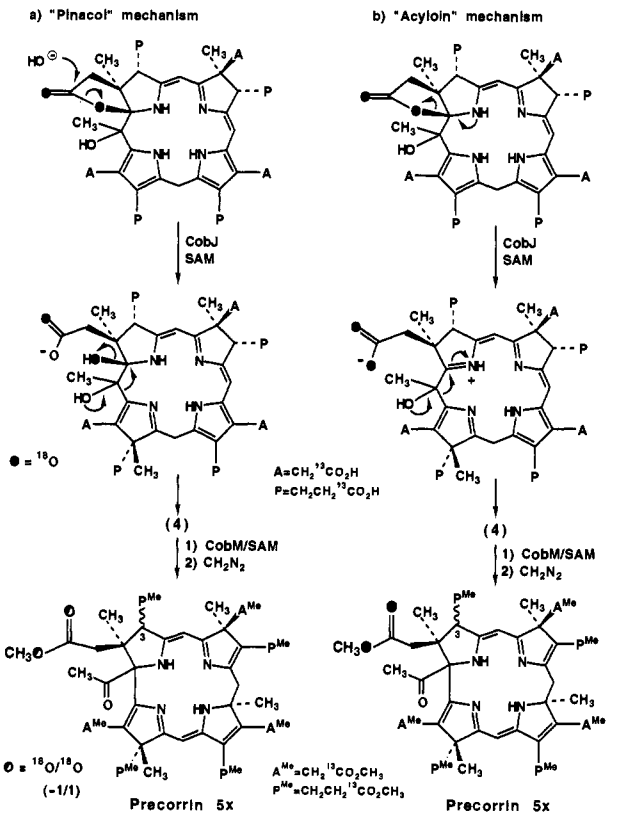
The cloning and overexpression of the entire repertoire of vitamin B_{12} biosynthetic *cob* genes from *Pseudomonas denitrificans* has facilitated the study of the formation of each intermediate of the pathway in detail.[1] A particularly fascinating stage in this aerobic biosynthesis of the corrin structure is the ring contraction process which converts precorrin-3 (**2**) to precorrin-4[2,3] (**4**) (Scheme 1). Precorrin-3 is first prepared for ring contraction by hydroxylation at C-20 with concomitant participation of the ring A acetate carboxyl to form the γ-lactone, precorrin-3x (**3**), a process, catalyzed by CobG, which utilizes molecular oxygen to effect the hydroxylation step.[4] The next enzyme of the pathway, CobJ, not only carries out the methylation of precorrin-3x at C-17 but, remarkably, mediates the rearrangement which results in the ring-contracted product, precorrin-4. The mechanism of this transposition could occur either by a true pinacol rearrangement preceded by hydrolysis of the lactone to a diol intermediate (Scheme 2a) or by direct elimination of the lactone followed by an acyloin-like rearrangement[5] (Scheme 2b). While the former mechanism would feature exchange with external solvent of one of the oxygen atoms of the regenerated ring A acetate, as has already been demonstrated in the anaerobic biosynthesis of B_{12} in *Propionibacterium shermanii*,[6,7] no exchange should occur in the latter scenario. In order to differentiate between these mechanisms in the aerobic pathway we have followed the fate of the carboxylic acid functions labeled with ^{13}C and ^{18}O during the processing of 5-aminolevulinic acid (ALA) through these intermediates.

First, in order to ascertain the origin of the oxygen atoms of the γ-lactone in (**3**), [4-^{13}C,1,1,4-$^{18}O_3$]ALA was prepared[8] and converted to precorrin-3x with the set of six enzymes as before.[2] This isotopomeric version of ALA places a ^{13}C label (●) at C-1 of (**3**), thus allowing detection of ^{18}O in the lactone terminating at this carbon. The ^{18}O-induced shift (3.9 Hz) on the C-1 resonance of the methyl ester clearly demonstrates that the lactone is formed at C-1 without exchange (Figure 1A), ruling out both a mechanism that proceeds via insertion of a hydroxyl group at C-1 from the medium with subsequent lactone formation, and one in which a δ-lactone is first established at C-20 which then undergoes translactonization to C-1.[9] The primary event in

Scheme 1



Scheme 2



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(2) Scott, A. I.; Roessner, C. A.; Stolowich, N. J.; Spencer, J. B.; Min, C.; Ozaki, S.-I. *FEBS Lett.* **1993**, *331*, 105. Precorrins-3 and -3x have been renamed Precorrin 3A and 3B, respectively, in an independent work.[3a,b] Pending agreement on nomenclature we retain the original names precorrin-3 and precorrin-3x in this communication.

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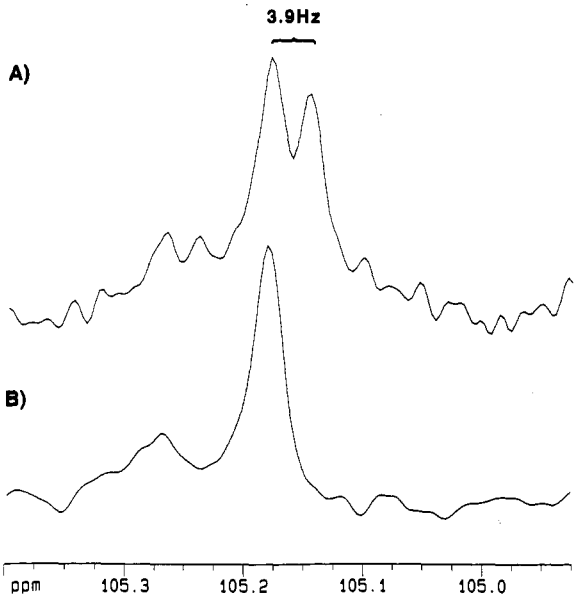

Figure 1. Region of 125-MHz ^{13}C NMR spectrum ($C_6{}^2H_6$) for the C-1 resonance of precorrin-3x methyl ester generated (A) from a mixture of equal amounts of [4-^{13}C,1,1,4-$^{18}O_3$]ALA (99 atom % ^{13}C, 90 atom % ^{18}O) and [4-^{13}C]ALA and (B) from [4-^{13}C]ALA. The upfield 3.9-Hz (0.031 ppm) shift observed in spectrum A is in agreement with a single-bonded ^{18}O atom on the ^{13}C center.[16]

precorrin-3x biosynthesis is probably hydroxylation with molecular oxygen at C-20 followed by addition of the ring A acetate carboxyl to the resultant imino function at C-1. A second possibility which cannot yet be excluded is that an epoxide is first formed and subsequently opened by the acetate carboxyl. In both mechanisms the adjacent nitrogen stabilizes the developing positive charge at C-1.

Having established that no exchange of an oxygen atom takes place during formation of the lactone in precorrin-3x, we next investigated how the ring A carboxylate is re-formed after ring contraction. Precorrin-3 was synthesized from [1-^{13}C,1,1,4-$^{18}O_3$]-ALA in a five-enzyme one-pot incubation as previously described.[2] NMR analysis showed that no exchange of oxygen had occurred from any of the eight carboxyl groups. This isotopomer was converted to precorrin-5 (**5**) (using CobG, CobJ, and CobM[10]), which was isolated as its methyl ester (5x)[11] using diazomethane rather than the normal method of derivatization (H_2SO_4/methanol) to avoid any exchange of the carboxyl oxygens. Comparison of the NMR spectrum (Figure 2A) with that of 5x synthesized from [1-^{13}C]ALA (Figure 2B) unenriched with ^{18}O clearly shows that no exchange has occurred in any of the carboxyl groups. This result eliminates a mechanism where lactone hydrolysis forms the diol (Scheme 2a) followed by a formal pinacol rearrangement,[9] which would require loss of half of the original ^{18}O label to external solvent. Thus the mechanism of ring contraction in *P. denitrificans* involves *cleavage of the carbon-oxygen bond between C-1 and the lactone terminus.* This process is probably assisted by the ring A nitrogen to form an imino function (*vide supra*) followed by migration of ring D, a process

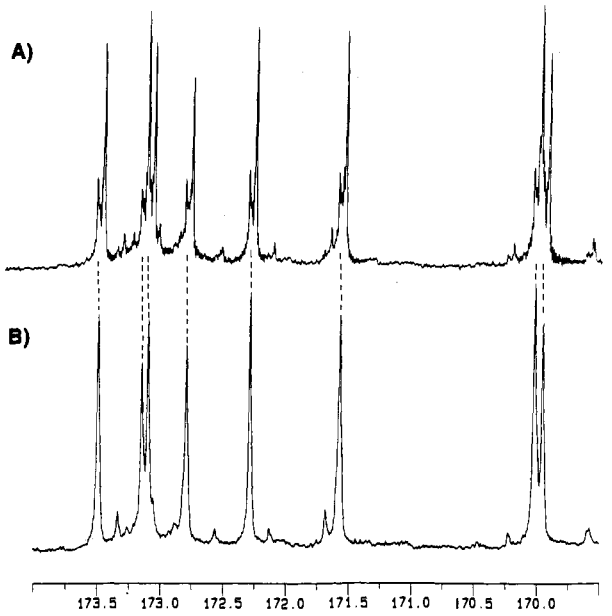

Figure 2. ^{13}C NMR spectrum (125 MHz) ($C_6{}^2H_6$) of the major epimer of precorrin-5x methyl ester,[9] prepared (A) from [1-^{13}C,1,1,4-$^{18}O_3$]ALA (99 atom % ^{13}C, 90 atom % ^{18}O) containing 20% [1-^{13}C]ALA not enriched in ^{18}O as internal chemical shift standard and (B) from [1-^{13}C]ALA. The major signals in spectrum A originate from a two-atom ^{18}O perturbation (6.5 Hz, 0.052 ppm) on the ^{13}C carbonyl and clearly demonstrate that ^{18}O is fully retained in all eight carboxy esters. In addition, two single ^{18}O-induced shifts [$^{13}C(=^{16}O)-^{18}O$ Me and $^{13}C(=^{18}O)-^{16}O$ Me species] are visible as shoulders inside the $^{13}C^{18}O_2$Me and $^{13}C^{16}O_2$Me signals, resulting from the 10% ^{16}O originally present in the ALA.

which requires prior methylation at C-17 (Scheme 2b).[12] The closest analogy for the ring contraction is an acyloin mechanism similar to that found in the biosynthesis of the branched-chain α-amino acids, valine and isoleucine, involving acyloin rearrangement of the corresponding α-hydroxy β-keto acid to the isomeric β-hydroxy α-keto acid.[13]

The finding that both oxygens of the ring A acetate carboxyl are retained in precorrin-5 invokes an interesting comparison with the reported exchange with the medium of one of these oxygens somewhere between precorrin-2 (**1**) and cobyrinic acid[14] (**7**) (Scheme 1) in cell free extracts of *P. shermanii*[6] and in the almost complete and regiospecific loss of this same label in the acetamido function of vitamin B_{12} formed in *P. shermanii* whole cells.[7] In light of these results we suggest that the ring contraction process operates by quite different mechanisms in the two pathways to B_{12}, on the one hand anaerobically (*P. shermanii*), involving loss of ^{18}O by hydrolysis of a γ-lactone (or δ-lactone function in a cobalt complex[15] corresponding to precorrin-3x which cannot involve O_2 in its formation, and on the other hand aerobically (*P. denitrificans*) in the metal-free series in which all of the original carboxyl oxygens are retained at least up to precorrin-5 and probably as far as hydrogenobyrinic acid (**6**).

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(11) Precorrin-5x methyl ester was utilized for analysis because all eight carboxylic ester resonances are more clearly resolved from one another than in precorrin-4. Precorrin-5x methyl ester is a tautomer of precorrin-5 formed by migration of the double bonds during esterification with diazomethane. In addition, both precorrin-5 and precorrin-5x methyl ester exist as epimers about C-3, and the latter can be separated by HPLC on a C-18 column (Alltech 10 × 250 mm) eluting with methanol/H_2O (80:20) at 4 mL min^{-1}.

(12) When SAM is omitted from the incubation with CobJ, ring contraction does not occur, thus providing strong circumstantial evidence that the new methyl group is introduced at C-17 *before* ring contraction, a mechanistic enigma which is under investigation.

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(14) This intermediate is equivalent to the metal free hydrogenobyrinic acid in the *P. denitrificans* pathway.

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