

## Biosynthesis of Vitamin B<sub>12</sub>: Ring Contraction Is Preceded by Incorporation of Molecular Oxygen into Precorrin-3

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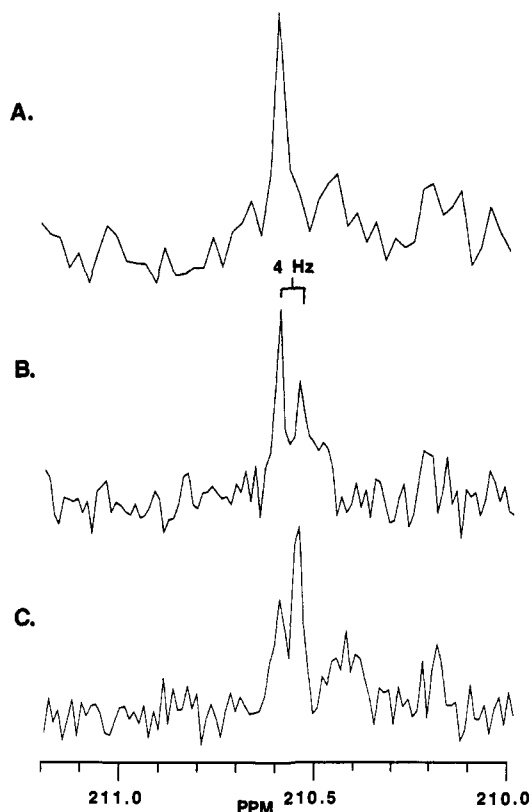
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Recently it has been demonstrated that precorrin-3 (1) is converted to precorrin-3x (2), a hydroxy  $\gamma$ -lactone, by the enzyme CobG from *Pseudomonas denitrificans*.<sup>1,2</sup> Precorrin-3x then becomes the substrate for CobJ, an enzyme which, remarkably, performs both *S*-adenosylmethionine (SAM) dependent C-methylation at C-17 and ring contraction to furnish precorrin-4<sup>3</sup> (3) (Scheme I). Precorrin-4 in turn is methylated at C-11 by CobM to form precorrin-5 (4),<sup>4</sup> which is deacetylated at C-1 and subsequently methylated by CobF at this position to afford precorrin-6x (5), a well characterized intermediate on the B<sub>12</sub> pathway<sup>5</sup> in the aerobic *P. denitrificans* which, unlike anaerobic B<sub>12</sub> producers, inserts cobalt into the corrin nucleus at a very late stage, i.e. after hydrogenobyrinic acid (6).

The acquisition of CobG, an iron containing enzyme,<sup>2</sup> by cloning and overexpression<sup>1</sup> has made possible a detailed study of its mechanism, and we now report on the role of dioxygen in the conversion of 1 to 2, a reaction which prepares the precorrinoid template for ring contraction by introduction of a hydroxyl function at C-20, accompanied by  $\gamma$ -lactone formation of the ring A acetate terminating at C-1.

First it was established that catalytic turnover of 1 to 2 is dependent on the presence of CobG and O<sub>2</sub>. Next, in order to trace the origin of the oxygen introduced at C-20, <sup>18</sup>O labeling was used to follow the conversion of precorrin-3 (1) to precorrin-4 (3), which was examined for evidence of an isotopic shift of <sup>18</sup>O on the <sup>13</sup>C chemical shift of C-20.<sup>6,7</sup>

The <sup>13</sup>C-NMR spectrum of precorrin-4 (3; ●) derived from [5-<sup>13</sup>C]-5-aminolevulinic acid shows a signal at 210.6 ppm assigned to C-20 (Figure 1A). When the same <sup>13</sup>C-isotopomer of precorrin-4 is formed from precorrin-3 in the presence of CobG and CobJ under an atmosphere enriched with <sup>18</sup>O<sub>2</sub> (<sup>18</sup>O/<sup>16</sup>O ratio 40/60), this signal is shifted upfield by 4 Hz<sup>6</sup> (Figure 1B) thereby revealing the presence of <sup>18</sup>O bonded directly to carbon. By varying the ratio of <sup>16</sup>O/<sup>18</sup>O it was clearly demonstrated by NMR (Figure 1C) that oxygen is quantitatively incorporated at C-20 in precorrin-4. CobG must therefore utilize molecular oxygen to effect the hydroxylation of 1 at C-20 in the synthesis of 2. A plausible mechanism for the genesis of precorrin-3x, shown in



**Figure 1.** Region of the 75.47-MHz <sup>13</sup>C-NMR spectrum for the C-20 acetyl carbonyl resonance of precorrin-4 (3), generated from 1 by the combined action of CobG and CobJ in the presence of (A) <sup>16</sup>O<sub>2</sub>, (B) 60% <sup>16</sup>O<sub>2</sub> and 40% <sup>18</sup>O<sub>2</sub>, and (C) 30% <sup>16</sup>O<sub>2</sub> and 70% <sup>18</sup>O<sub>2</sub>. Precorrin-4 was synthesized as described previously<sup>1</sup> except that the solution was degassed and an atmosphere of 20% oxygen (containing a mixture of <sup>16</sup>O<sub>2</sub>/<sup>18</sup>O<sub>2</sub>) and 80% nitrogen reintroduced before the reaction was initiated with the addition of 10 mg of precorrin-3. The precise ratio of <sup>16</sup>O<sub>2</sub>/<sup>18</sup>O<sub>2</sub> present in the atmosphere of the incubation was determined by mass spectrometry. After incubation for 4 h at 30 °C the product precorrin-4 was isolated anaerobically by adsorption to DEAE-Sephadex and prepared for NMR analysis. Each spectrum represents the direct Fourier transformation of the 32K data point FID without further resolution enhancement.

Scheme II, features the formation of the tertiary alcohol function at C-20 followed by addition of the ring A acetate carboxyl to the resultant imino function at C-1. The masked pinacol system in 2 then provides the requisite functionality for ring contraction to 3, mediated by CobJ, which is also a 17-methyltransferase.

Interestingly, the hydroxy-lactone structure of precorrin 3x is also found at the C<sub>5</sub>-C<sub>6</sub> position in the xanthocorrinoids which are found chemically from vitamin B<sub>12</sub> derivatives using either *Udenfriend's* reagent (Fe<sup>2+</sup>, ascorbic acid/O<sub>2</sub>)<sup>8</sup> or simply O<sub>2</sub> in the presence of a reducing agent, the coordination of H<sub>2</sub>O<sub>2</sub> to cobalt being proposed as the first step under the latter conditions.<sup>9</sup> On the basis of this model we suggest (Scheme II) that CobG may generate an iron-oxo species (Fe<sup>III</sup>-O<sup>•</sup> ↔ <sup>••</sup>Fe<sup>IV</sup>=O) which hydroxylates C-20 of precorrin-3 followed by formation of the  $\gamma$ -lactone at C-1.<sup>8b</sup> Future experiments in which O<sub>2</sub> is replaced with H<sub>2</sub>O<sub>2</sub> should establish whether the biochemical reaction mechanism utilizes a peroxy intermediate.

The finding that one atom of dioxygen is incorporated into precorrin-3x at C-20 is apparently contradictory to previous statements<sup>5a,10</sup> that NADPH-deficient incubations of *P. deni-*

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(3) The structures of precorrin-3x (2) and precorrin-4 (3) at physiological pH are shown in Scheme I. Recently the French group<sup>2</sup> reported different chromophores in 2 and 3 in which the 9,10 double bond has moved to the 8,9 position in ring B presumably due to the acidic conditions used for isolation and spectroscopy. Also 2 was named precorrin-3B in ref 2.

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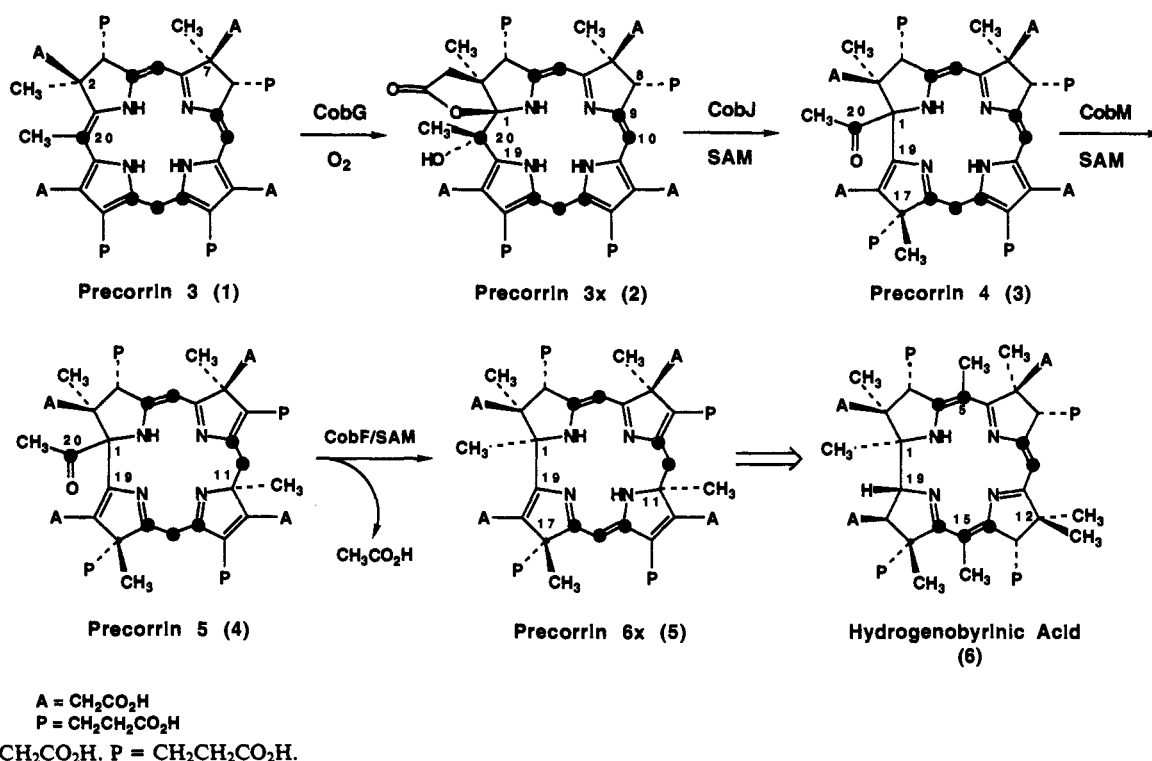
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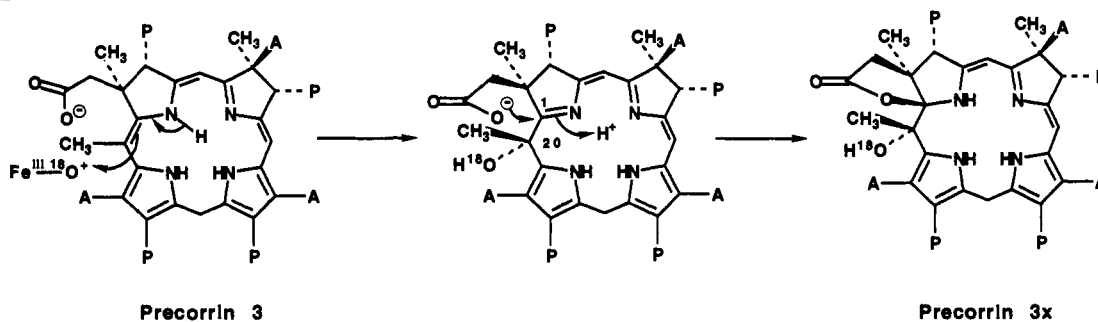
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Scheme I<sup>a</sup>

## Scheme II



*trificans* amplified with an 8-gene cluster (containing *cobG*) leading from precorrin-3 to precorrin-6x (and hence involving the intermediacy of precorrin-3x) require anaerobic conditions. Thus the "anaerobic" incubations must have contained sufficient O<sub>2</sub> to allow stoichiometric turnover. With this new understanding of the oxidative process, catalyzed by CobG/O<sub>2</sub>, which sets the machinery in place for the subsequent methylative ring contraction to precorrin-4, it now remains to identify the reducing cofactor associated with CobG and establish the temporal resolution of ring contraction and 17-methylation events in the conversion of 2 to 3.

The active participation of molecular oxygen in *P. denitrificans* is particularly fascinating since vitamin B<sub>12</sub> is biosynthesized anaerobically in *Propionibacterium shermanii*<sup>11</sup> and *Salmonella typhimurium*.<sup>12</sup> If oxygen is truly absent in the latter organisms, a different mechanism must operate for the synthesis of precorrin-3x, via the formation of alternative, as yet unidentified, cobalt complexes. The biosynthetic genes responsible for the synthesis of B<sub>12</sub> in *S. typhimurium* have been identified<sup>13</sup> and nine of them cloned and overexpressed,<sup>14</sup> and a substantial number of these

proteins have high homology with their counterparts from *P. denitrificans*.<sup>15</sup> Significantly CobG, however, has no homolog in *S. typhimurium*,<sup>16</sup> thus strengthening the case for major differences in the vitamin B<sub>12</sub> pathway in aerobic and anaerobic organisms.

It is attractive to speculate that the anaerobes use the redox chemistry of cobalt to effect the equivalent of oxidative ring contraction, since it is known that *P. shermanii* uses the cobalt complex of precorrin-3 as a precursor for cobyrinic acid.<sup>19</sup>

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