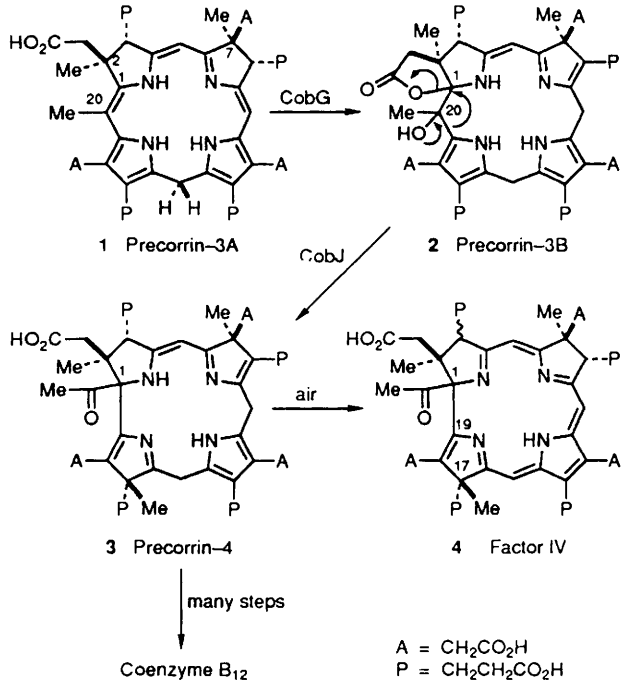
Biosynthesis of Vitamin B_{12}: Studies of the Oxidative and Lactone-forming Steps by ^{18}O-Labelling

Alex I. D. Alanine,[a] Yongfu Li,[a] N. Patrick J. Stamford,[a] Finian J. Leeper,[a] Francis Blanche,[b] Laurent Debussche[b] and Alan R. Battersby*[a]

[a] *University Chemical Laboratory, Lensfield Road, Cambridge, UK CB2 1EW*
[b] *Department Analyse, Centre de Recherche de Vitry Alfortville, Rhône-Poulenc Rorer, BP14, F-94403, Vitry-sur-Seine Cedex, France*

Experiments based on the use of $^{18}O_2$ and electrospray mass spectrometry show that during the conversion of precorrin-3A **1** into precorrin-3B **2** or precorrin-4 **3** just one atom of oxygen is incorporated.

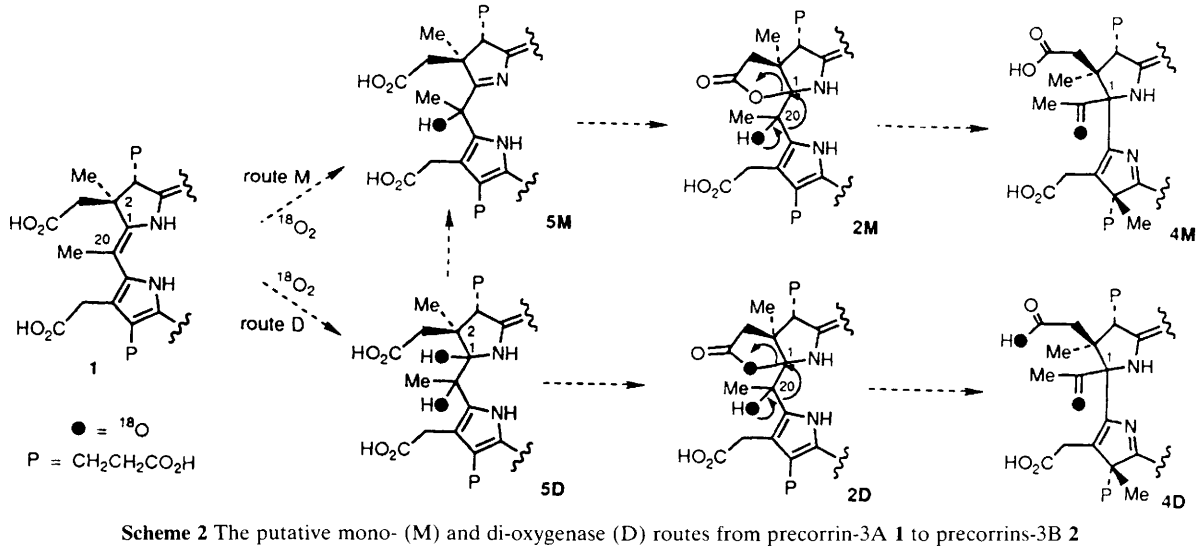
The structures of all the intermediates for B_{12}-biosynthesis in *Pseudomonas denitrificans* are now known for the entire pathway starting from primary metabolites.[1] This opens the way to mechanistic studies on several of the reactions involved; here we focus on the oxidative step which is now known[2,3] to occur in the early part of the pathway. Determination of the structure[4] of precorrin-4 **3** (Scheme 1) proved that the oxidation had already occurred by this tetramethylated stage. The substrate for the oxidative enzyme, CobG, was subsequently shown[3,5,6] to be precorrin-3A **1** and the structure of the product, precorrin-3B **2**, was established by multiple ^{13}C-labelling.[4] CobG is an iron–sulfur protein[5] which uses dioxygen.[6]



Scheme 1 Some early steps of coenzyme B_{12} biosynthesis

As shown in Scheme 2, CobG could be a mono-oxygenase (route M) or a dioxygenase (route D). Studies using ^{18}O-induced ^{13}C NMR shifts showed that, using enzymes from *Ps. denitrificans*, the carbonyl oxygen of the C-1 acetyl group of precorrin-4 **3** is derived from atmospheric oxygen.[7] However, because of the positioning of the ^{13}C atoms, this experiment could not give information as to whether a second oxygen atom is incorporated from O_2. Route M would result in the incorporation of only one ^{18}O atom, see **2M**, but the number of oxygen atoms remaining in precorrin-3B **2** from the putative diol **5D** on route D would depend on the mechanism of the lactonisation: this could involve (a) dehydration (**5D** → **5M**) followed by addition of the carboxylate anion onto C-1 to give **2M** or (b) direct lactonisation onto the C-1 hydroxy group yielding **2D** which has two ^{18}O atoms and has lost one of the original oxygen atoms of the C-2 acetate group. Since it is known that there is loss of at least one oxygen atom specifically from the C-2 acetate as B_{12}[8,9] or its precursor, cobyrinic acid,[10] are formed in *Propionibacterium shermanii*, possibility (b) above must be included even though (a) is mechanistically more attractive. Our approach to this problem has been to determine by mass spectrometry how many atoms of ^{18}O are incorporated from $^{18}O_2$.

Precorrin-3A **1** was prepared from 5-aminolaevulinic acid and *S*-adenosyl-L-methionine (SAM) using the necessary five overproduced enzymes.[11] After purification,† the precorrin-3A **1** was incubated in the presence of *ca.* 20% $^{18}O_2$: 80% N_2 with the enzyme preparation from an engineered strain of *Ps. denitrificans* [G3575 (pXL325)[5]] containing overproduced CobG enzyme. The initial enzyme preparation was not



Scheme 2 The putative mono- (M) and di-oxygenase (D) routes from precorrin-3A **1** to precorrins-3B **2**

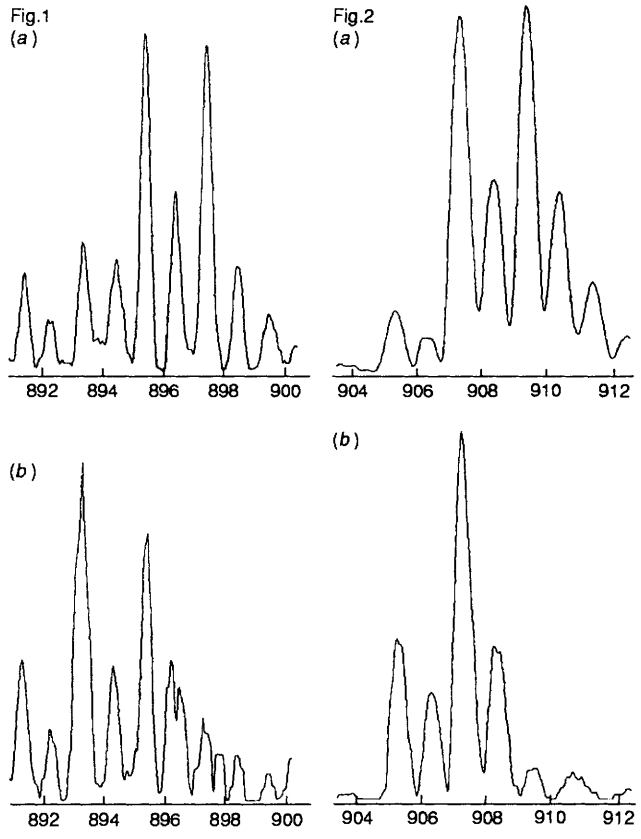


Fig. 1 Electrospray mass spectra of precorrin-3B **2** obtained using (*a*) $^{18}O_2$ and (*b*) $^{16}O_2$. The peak at 893.3 in (*b*) arises by aerial dehydrogenation of precorrin-3B to give its didehydro analogue, Factor IIIB.[5] Part of the intensity at 895.3 in (*a*) is probably due to similar dehydrogenation of the ^{18}O-labelled precorrin-3B at 897.3. Only the parent ion region for precorrin-3B is shown; the mass spectra of these unpurified samples also contained peaks corresponding to the starting material, didehydroprecorrin-3A, precorrin-3A and a small amount of Factor IV.

Fig. 2 Electrospray mass spectra of purified Factor IV **4** obtained using (*a*) $^{18}O_2$ and (*b*) $^{16}O_2$. Only the parent ion region is shown. The peak at 905.3 in (*b*) is probably due to a small amount of oxidation to give a monolactone of Factor IV.

degassed, allowing some $^{16}O_2$ to be retained and this provided an internal standard in the eventual mass spectrum. In addition, an identical experiment was carried out except that $^{18}O_2$ was replaced by $^{16}O_2$. Positive ion electrospray mass spectrometry on the resultant two samples of precorrin-3B gave the parent ion regions illustrated in Figs. 1(*a*) and 1(*b*). These show that use of $^{18}O_2$ causes a mass increase of 2 units, not 4 units, and hence just one oxygen atom from oxygen gas is retained in precorrin-3B, *i.e.* **2M** not **2D**. Accurate mass determination on the ^{18}O-shifted peak confirmed that it was due to the incorporation of ^{18}O (MH^+, 897.3274. $C_{43}H_{51}N_4{}^{16}O_{16}{}^{18}O$ requires 897.3292). Appropriate control experiments showed that the illustrated peaks from precorrin-3B **2** were obtained only in the presence of CobG enzyme and further that **2** was converted into precorrin-4 **3** when CobJ (the C-17 methyltransferase) and SAM were added.[1,5]

Next, the experiment was repeated but the precorrin-3B **2** was converted *in situ* into precorrin-4 **3** by having SAM and CobJ present. The two resultant samples of precorrin-4 from using $^{18}O_2$ and $^{16}O_2$, respectively, oxidised on handling in air to give Factor IV **4**, which was purified by HPLC. The mass spectra of **4** (Fig. 2) show that the $^{18}O_2$ experiment again resulted in a mass increase of 2 units, corresponding to **4M** not **4D**. (MH^+, 909.3204. $C_{44}H_{51}N_4{}^{16}O_{16}{}^{18}O$ requires 909.3292). This confirms the incorporation of just one oxygen atom from atmospheric oxygen during the oxidation of precorrin-3A **1** to precorrin-3B **2**.

This result, along with the earlier one,[7] allows the further conclusion that both oxygen atoms of the γ-lactone of precorrin-3B, see **2M**, are the original oxygen atoms of the C-2 acetate of precorrin-3A **1**; route D (**1** → **2D**, Scheme 1), which would cause loss of one of these oxygen atoms, has been excluded. This conclusion means that the results obtained with *Pr. shermanii* showing specific loss of oxygen from the C-2 acetate group during B_{12}-biosynthesis[8–10] and transfer of ^{18}O to C-20 from a labelled carboxyl group[12] (presumably the $CH_2C^{18}O_2H$ on C-2) remain to be explained. One explanation may be that the transfer of ^{18}O occurs during one of the subsequent steps leading to cobyrinic acid (possibly during the loss of the acetyl group). The other explanation is that a somewhat different mechanism for the oxidative step may be followed in *Pr. shermanii* compared to *Ps. denitrificans* (possibly involving generation of the δ-lactone from the C-2 acetate onto C-20).

Grateful acknowledgement is made to Zeneca, Roche Products, F. Hoffmann-La Roche, Rhône-Poulenc Rorer, the Leverhulme Trust and SERC for financial support. We also thank Dr J. Staunton for a gift of $^{18}O_2$, Dr C. Abell and D. Thibaut for helpful discussions, Mr P. Skelton for recording the mass spectra and J. Lunel, P. E. Bost and J.-C. Brunie for their support.

Received, 10th May 1994; Com. 4/02774D

Footnote

† Purification was carried out after aerial oxidation and esterification of precorrin-3A **1** to give its stable didehydro octamethyl ester. The pure ester was then hydrolysed and the octa-acid was added to the incubation mixture where it is known[2] to be enzymically reduced back to precorrin-3A.

References

1 Reviewed by A. R. Battersby, *Science*, 1994, **264**, 1551.
2 D. Thibaut, L. Debussche and F. Blanche, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 8795.
3 L. Debussche, D. Thibaut, M. Danzer, F. Debu, D. Frechet, F. Herman, F. Blanche and M. Vuilhorgne, *J. Chem. Soc., Chem. Commun.*, 1993, 1100.
4 D. Thibaut, L. Debussche, D. Frechet, F. Herman and M. Vuilhorgne, *J. Chem. Soc., Chem. Commun.*, 1993, 513.
5 L. Debussche, D. Thibaut, B. Cameron, J. Crouzet and F. Blanche, *J. Bacteriol.*, 1993, **175**, 7430.
6 *cf.* A. I. Scott, C. A. Roessner, N. J. Stolowich, J. B. Spencer, C. Min and S.-I. Ozaki, *FEBS Lett.*, 1993, **331**, 105.
7 J. B. Spencer, N. J. Stolowich, C. A. Roesner, C. Min and A. I. Scott, *J. Am. Chem. Soc.*, 1993, **115**, 11610.
8 K. Kurumaya, T. Okazaki and M. Kajiwara, *Chem. Pharm. Bull.*, 1989, **37**, 1151.
9 A. I. Scott, N. J. Stolowich, B. P. Atshaves, P. Karuso, M. J. Warren, M. Kajiwara, K. Kurumaya and T. Okazaki, *J. Am. Chem. Soc.*, 1991, **113**, 9891.
10 R. A. Vishwakarma, S. Balachandran, A. I. D. Alanine, N. P. J. Stamford, F. Kiuchi, F. J. Leeper and A. R. Battersby, *J. Chem. Soc., Perkin Trans. 1*, 1993, 2893.
11 N. P. J. Stamford, A. I. D. Alanine, A. R. Pitt, B. Cameron, J. Crouzet and A. R. Battersby, in preparation.
12 S. Broers, A. Berry and D. Arigoni, in *The Biosynthesis of the Tetrapyrrole Pigments*, Ciba Foundation Symposium, No. 180, Wiley, New York, 1994, p. 280.