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Vitamin B_{12}. Recent discoveries cast new light on an ancient structure

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Abstract: Recent discoveries in the biochemistry and biosynthesis of vitamin B_{12} are reviewed. The mechanism of methionine synthase involves a remarkable interchange of the DMBI moiety with a histidine group of the enzyme. It has been found that there are two distinct pathways to vitamin B_{12} in nature, depending on whether the B_{12} producing organism is aerobic or anaerobic. The multi-enzyme synthesis of the corrin, hydrogenobyrinic acid, has been accomplished.

An upsurge of research activity has uncovered some remarkable features of the mechanism of action and of the biochemical origin of vitamin B_{12}, a molecule which has been aptly described as "Nature's most beautiful cofactor" (1). The basis of this renaissance is undoubtedly the acquisition, by cloning, sequencing and expression, of the genes required for the biosynthetic pathway and for several coenzyme B_{12} - mediated processes.

HOW METHIONINE SYNTHASE BINDS B_{12}; SURPRISES ON BOTH FACES

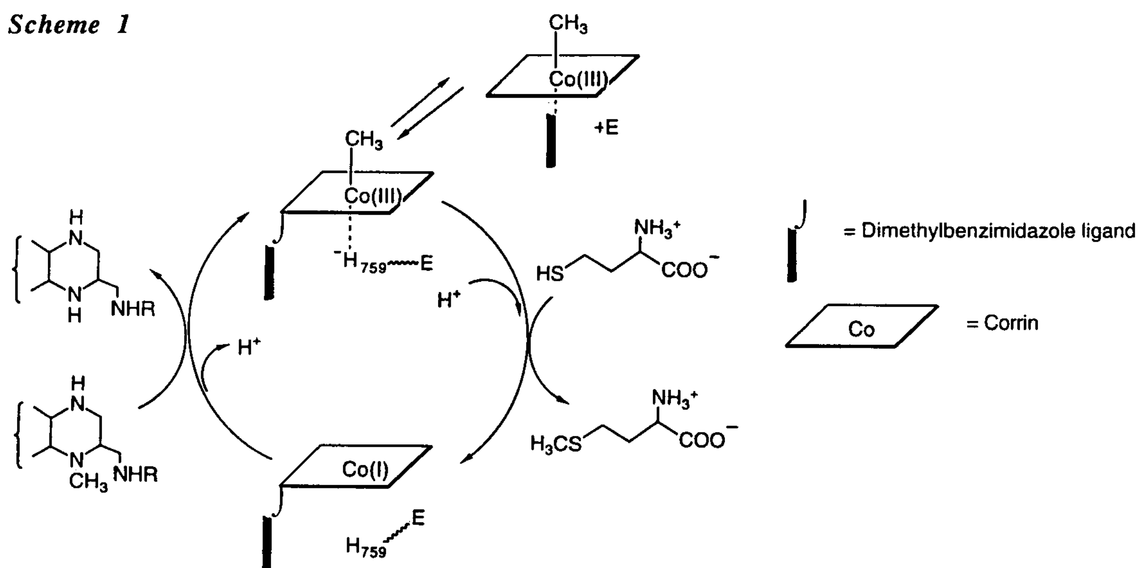
In contradistinction to the mutases which depend on the homolytic fission of the cobalt-carbon bond of adenosyl cobalamin, methionine synthase catalyzes a cycle of methyl transfers initiated by formal heterolytic cleavage at the cobalt (I) - carbon bond of the cofactor. The resultant methyl cobalamin transfers its methyl to homocysteine to form methionine and is then regenerated with methyl group transfer from tetrahydrofolate (Scheme 1).

Central to the mechanistic dichotomy between methionine synthase and the other coenzyme mediated reactions (e.g. methyl malonyl-succinyl CoA mutase) is the method whereby fission of the cobalt carbon bond is directed to favor homolysis in the mutases and heterolysis in methionine synthase.

Drennan, Huang, Drummond, Matthews and Ludwig (2) have determined the structure of methyl cobalamin bound to a 27-rD fragment of methionine synthase at 3.0Å resolution which provides a remarkable view of how this control might be achieved. The DMBI moiety, normally occupying the lower axial position, has swung away from the underside and becomes buried in a hydrophobic pocket. Its place has been taken by His ^{759} from the enzyme, suggesting a protonation-deprotonation mechanistic shuttle as depicted in Scheme 1.

Something very interesting can also be seen on the top face where the methyl ligand is situated within a hydrophobic domain thereby shielding it from direct attack by the incoming homocysteine. Presumably this protective ensemble moves away when the substrates homocysteine and methyl tetrahydrofolate (CH_3 N-THF) successively approach the upper face during the catalytic cycle.

Scheme 1



A CATALYTIC QUARTET?

The unexpected replacement of dimethyl benzimidazole by His^{759} has been invoked to explain how the protein, by replacing the normal ligand, may modulate the necessary valency changes on cobalt via protonation - deprotonation of Asp^{757} and Ser^{810} (adjacent to His^{759}) providing a pathway for proton transfer to the buried His^{759}. Thus the Ser-Asp-His triad coupled to the cobalt can be seen to modulate the carbon-cobalt bond strengths in the various intermediates, since it is well known that the carbon-cobalt bond is stabilized against homolylic cleavage to form Cob(II) alamin, and against heterolytic cleavage to form Cob(I) alamin by *basic* ligands on the lower face, which increase the electron density on cobalt. Thus partial deprotonation of His^{759} would increase the ligand basicity, thereby stabilizing the methyl cobalamin bound form. This intriguing idea of tuning the basicity of the lower ligand by H-bonding networking finds a counterpart in the B_{12} - requiring mutases, all of which appear to work by homolytic Co-C cleavage, which in turn could be controlled entirely from the lower face ligand, especially since sequence alignment suggests a common His^{759} bonding motif, at least for the methyl malonyl CoA and glutamate mutases. There is both circumstantial and direct evidence for radical intermediates in the reactions catalyzed by these mutases and by B_{12} mediated ribonucleotide reductase (3).

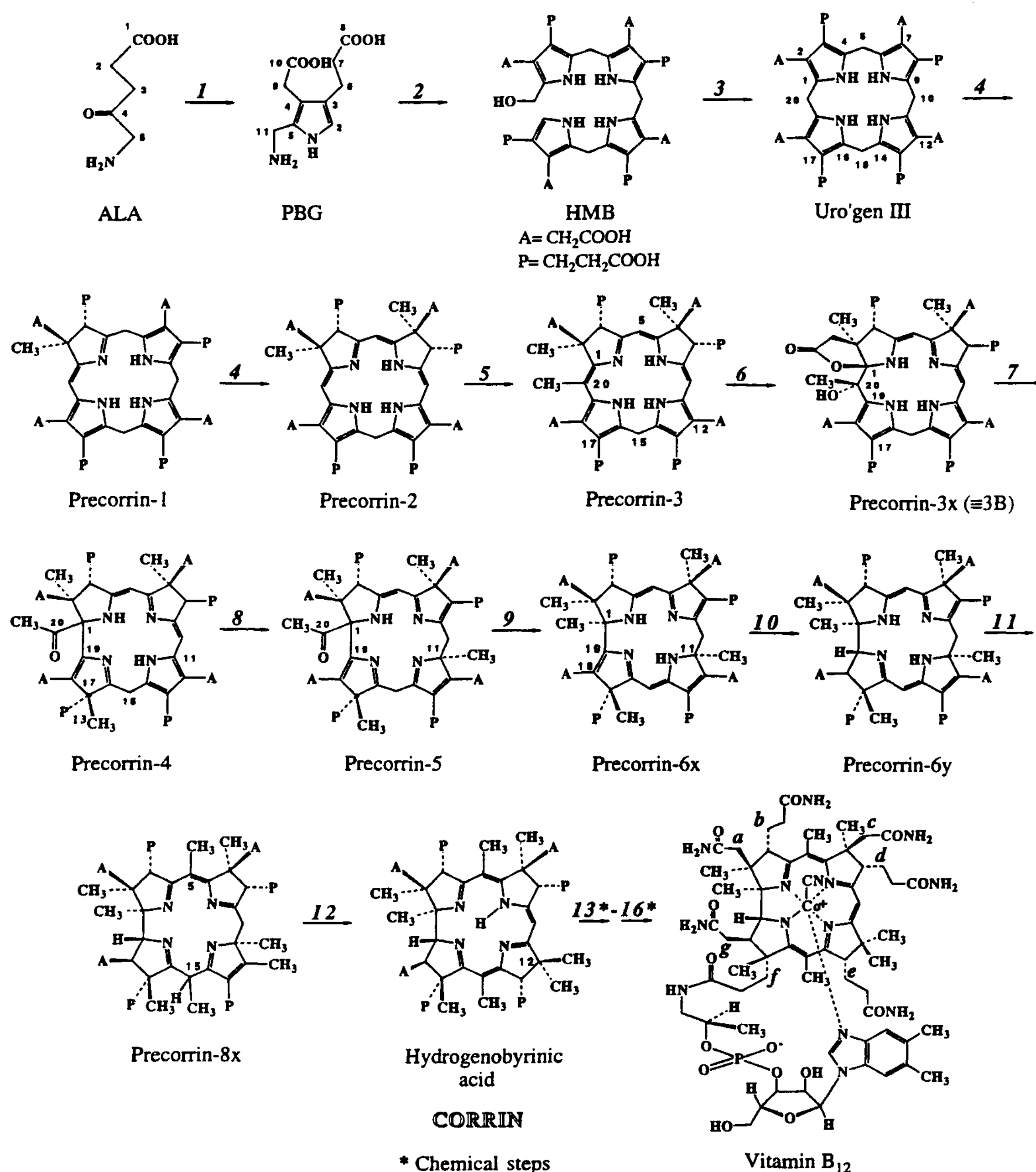
It will obviously be of great relevance to compare these two classes of enzymes, a possibility now on the horizon, since the crystallization of methyl malonyl CoA mutase has been described, and an X-ray structure appears imminent (4).

BIOSYNTHESIS OF VITAMIN B_{12}: REVELATION OF DUAL PATHWAYS TO CORRINS

In spite of Herculean efforts since the 1960s' in several laboratories engaged in the study of how B_{12} is biosynthesized, the intricate details of how the porphyrinoid template is modified by C-methylation and ring contraction into the finished molecule, cyanocobalamin (Scheme 2) remained tantalizingly obscure until the late 1980's when molecular biology came to the rescue.

Following the discovery in 1972 that Urogen III is the precursor of vitamin B_{12} (5), the search began for the intermediates corresponding to C-methylation and by 1980, precorrins -1, 2 & 3 had been found in their oxidized forms (6). Since no further intermediates were identified over the next decade,

Scheme 2



1. ALA dehydratase (*hemB*); 2. PBG deaminase (*hemC*); 3. Uro'gen III synthase [cosynthase] (*hemD*); 4. Uro'gen III methylase [M-1] (*cysG/cobA*); 5. M-2 (*cobI, cbiL*); 6. Precorrin-3x (3B) synthase (*cobG*); 7. Ring contractase/17 methyl transferase [M-3] (*cobJ*); 8. M-4 (*cobM*); 9. M-5 (*cobF*); 10. Reductase (*cobK*); 11. Precorrin-8x synthase (M-6/decarboxylase) (*cobL*); 12. [1,5]-sigmatropic shiftase [hydrogenobyrinic acid synthase] (*cobH*); 13. Insert Co; 14. Esterify; 15. Add nucleotide; 16. Ammonolysis

an intensive effort in Paris and Texas was launched in order to uncover the enzymes responsible for C-methylation and ring contraction. Cloning and overexpression of the first methyl transferase converting Urogen III to precorrin-1 and 2 was described independently by the two groups. Whereas S-adenosyl methionine Urogen III methyl transferase (SUMT) (7) from the aerobe, *Pseudomonas denitrificans* seems to be specific for B_{12} production, the corresponding gene product of cysG, ubiquitous as part of the machinery for cysteine biosynthesis, is multifunctional, being required for

anaerobic B_{12} synthesis and for siroheme synthesis in *Salmonella* where oxidation with NAD and insertion of iron are also part of its catalytic manifold (8), (9). Recently, it was found (10) that, in *Propionibacterium shermanii*, the gene product of *CobA* carries out the synthesis of precorrin 2 destined for B_{12}, but like SUMT, shows no multifunctional activity. The next enzyme, precorrin 3 synthase (The C-20 methylase), is encoded by *CobI* in *Ps. denitrificans* and by *CbiL* in *Salmonella* . With the route clearly established at the enzyme level from ALA to precorrin-3, the multi-enzyme synthesis (11) using the five necessary enzymes (Hem B, C, D, CobA, CobI) in the presence of ALA and SAM allowed the preparation of substantial amounts of precorrin-3, labelled with ^{13}C, which served as the starting point for the journey into the unknown terriority.

At this juncture, the main research efforts of the Texas and Paris-Cambridge teams took different, but parallel paths in the search for the intermediates between precorrin-3 and B_{12}. In Paris, the acquisition of recombinant strains of *Ps. denitrifricans* harboring selected combinations from the repertoire of eight genes (12) necessary for the formation of the cobalt-free hydrogenobyrinic and (HBA) allowed the accumulation of a new intermediate. Thus when NADPH was withdrawn from the cell free extract, precorrin-6x accumulated, and the structure determined by NMR spectroscopy at Cambridge, *via* ^{13}C-enrichment of sets of carbons derived from ^{13}C-isotopomers of ALA (Scheme 2).

By the use of mutants deficient in each of the biosynthetic genes and making full use of NMR spectroscopy, the intermediates between precorrin-6x and HBA were established as precorrin-6y and precorrin-8x[12] (Scheme 2). Application of the same mutant approach allowed the French workers to identify precorrin-3B (independently found in Texas and named 3x), as well as oxidized versions of precorrin-4 and a deacetylated compound, Factor V which was not an intermediate. The complete pathway downstream from HBA was also established at Rhone-Poulenc. A full account of the work of the French and English groups has recently appeared (13).

THE TEXAS APPROACH: MULTI-ENZYME SYNTHESIS

Since high production, recombinant strains and their engineered mutants were unavailable, the strategy in College Station had to be different. Starting with the gene sequences of the B_{12} pathway in *Salmonella* and *Ps. denitrificans*, the Texas group set about the cloning and overexpression of the complete repertoire of individual biosynthetic gene products from both organisms (14). Then by adding each purified enzyme to ^{13}C-enriched precorrin-3, obtained in multi-milligram amounts by the 5-enzyme synthesis described above, the changes in the ^{13}C-NMR spectrum were followed directly in the NMR tube when the incubation containing substrate and $^{13}CH_3$-SAM was challenged with each enzyme in turn. The results of this step-by-step approach highlighted below and summarized in Scheme 2, can be described as both remarkable and unexpected, for they revealed the mechanism whereby the template is prepared for ring-contraction.

A MAJOR SURPRISE: MOLECULAR OXYGEN IS THE MISSING COFACTOR REQUIRED FOR THE SYNTHESIS OF PRECORRIN-3X

The French group had found that the enzyme CobG, a non-heme, iron-sulfur protein, turned over precorrin 3A to 3B(=3x) in the presence of an oxygen-consuming coupled enzyme system but the process was found to be stoichiometric rather than catalytic (12). The poor performance of CobG, which was were also observed independently in Texas, was attributed to the absence of a vital cofactor. To everyone's surprise the catalytic power of CobG was restored when the incubation was performed in the presence of molecular oxygen (15) and, by using $^{18}O_2$ the fate of the oxygen was

found to reside in the C-20 hydroxyl group of precorrin-3x, whose novel functionality prepares the template for ring contraction - but only when the next enzyme, CobJ (another methyltransferase) is brought into play. Moving forward, one enzyme at a time, the structures of the next intermediates, precorrins -4 and -5 were determined, again by on-line NMR followed by rigorous purification and detailed spectroscopic assignment (16). These true intermediates corresponded to the factors 4 and 5 found in Paris but the former had undergone oxidation and the latter deacetylation, and so are not, in fact, the actual biochemical intermediates. Having filled in the gap between precorrins-3 and 6x and with all the remaining enzymes in hand, the stage was set to initiate the most ambitious multi-enzyme synthesis to date viz.the reconstitution of the complete sequence from ALA to HBA (Scheme 2).

MULTI-ENZYME SYNTHESIS OF HYDROGENOBYRINIC ACID

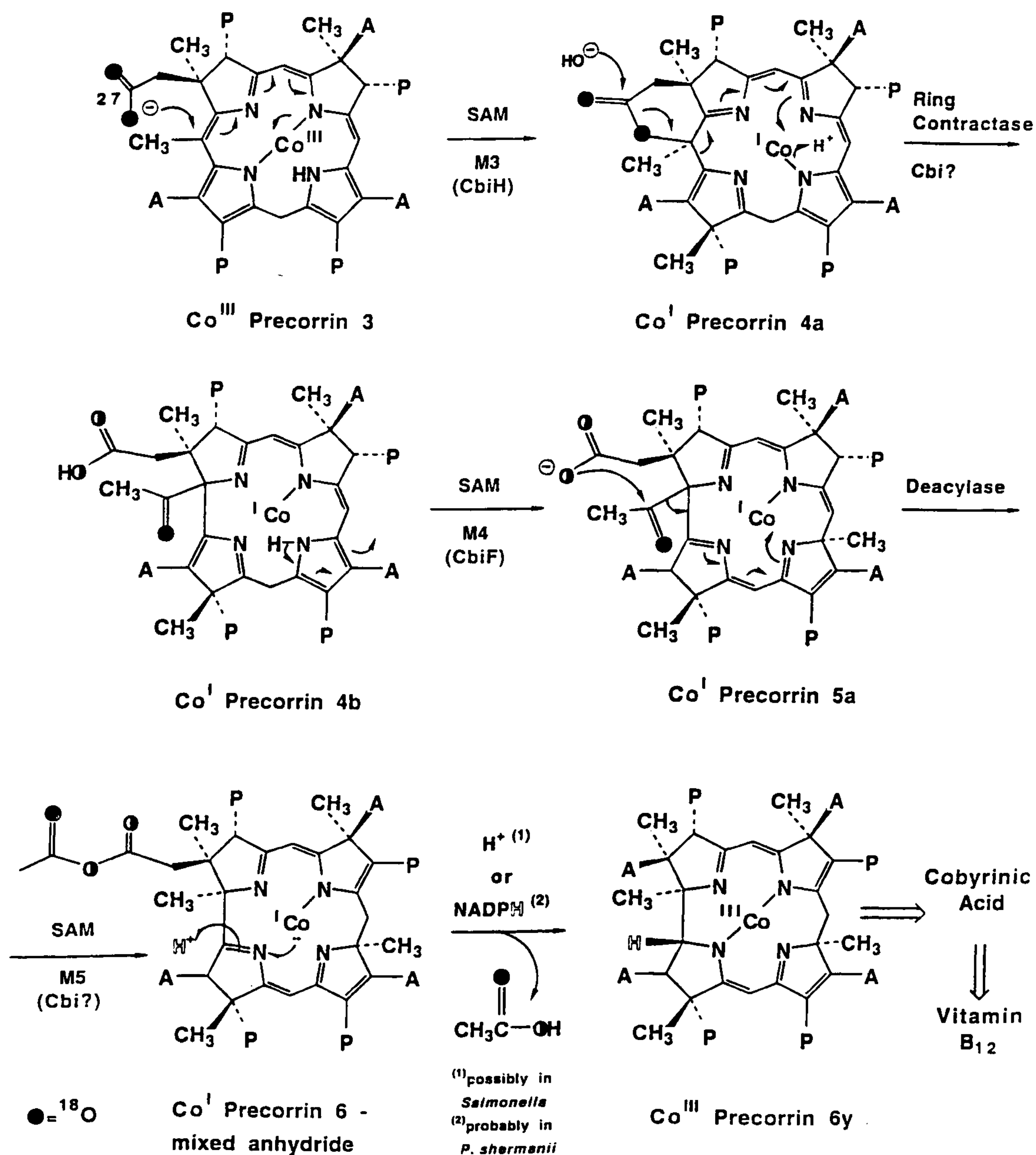
In spite of their optimism about a favorable outcome, the Texas group was already aware that when the French researchers mixed the four pure enzymes required to convert precorrin-3 to precorrin 6x, low amounts (<10%) of the latter were formed in a "poorly reproducible manner (12)." Some improvement was gained when an oxygen consuming system was added to the incubation resulting in a 50% conversion of precorrin-3 to -6x.

In the event, it was found that all twelve enzymes could be used simultaneously and an overnight incubation in presence of ALA, SAM, NADPH and O_2 afforded the target corrin in 20-25% yield (17). The *average* yield is >90% for each of the 17 steps, necessary to convert ALA to HBA as depicted in Scheme 2. Fortunately, the last enzyme CobH, catalyzing the rearrangement of precorrin -8x to HBA, has such high affinity for the product, that almost pure HBA can be isolated from the protein, after washing out all other small molecules. Perhaps this fact, in combination with the rapid transfer of labile substrates (e.g. precorrin -5) in the multi-enzyme preparation, is responsible for the exceptionally high conversion rates. These can be improved even further by adding excess of the final enzyme, CobH. When combined with the known chemistry (Eschenmoser) which converts HBA to cyanocobalamin (Scheme 2), the total chemo-enzymatic synthesis of B_{12} had been reached in a process which now can be carried out in a matter of days, using the battery of purified enzymes or more conveniently, the lysates from each of the 12 engineered strains of *E. coli*. As the first step towards a continuous batch system for B_{12} synthesis, the first three enzymes have recently been immobilized and a continuous process for producing gram quantities of Urogen III is now available (18).

With the establishment of the aerobic pathway in *Ps. denitrificans*, it might be imagined that the saga of B_{12} biosynthesis is at an end (13). However, nature has been making B_{12} for ~four billion years in completely anaerobic archaebacteria and it is now abundantly clear that there are, remarkably, two separate pathways to corrins running parallel at times, but profoundly different at certain vital points (14), (16). In *Pr. shermanii* it was found that cobalt is inserted early, at the stage of precorrin -2, and furthermore that in view of the presence of the B_{12} pathway in obligate anaerobes such as the methanobacteria, oxygen cannot be used as a triggering device for ring contraction. The ancient, anaerobic route plausibly uses valency changes in cobalt (19) and H_2O as the equivalent reagents for this process, before the appearance of the oxidative route, which has existed for about 1.8 billion years and may use a mechanism such as the hypothetical one (19) shown in Scheme 3. On the other hand the methanogens, which harbor the anaerobic pathway, can be dated to about 3.9 x 10^9 years. So a new and perhaps even more formidable challenge awaits the gene hunters in the academic world.

Work has now begun (10) with a genomic library of *Pr. shermanii* which has already provided the CobA (M-1) gene necessary for precorrin -2 synthesis and is expected to reveal the "anaerobic" route to B_{12} in the near future. Based on what has been discovered in the last few years, it is clear that many surprises are still in store - especially the way in which the ring contraction mechanism is achieved in *Pr. Shermanii.*

Scheme 3



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