New light on vitamin B_{12}: The adenosylcobalamin-dependent photoreceptor protein CarH

AUTHOR:
Susan M. Chemaly[1]

AFFILIATION:
[1]School of Chemistry, University of the Witwatersrand, Johannesburg, South Africa

CORRESPONDENCE TO:
Susan Chemaly

EMAIL:
susan.chemaly@wits.ac.za

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Adenosylcobalamin (AdoCbl), or coenzyme B_{12}, is a cofactor for enzymes important in metabolism in humans (and other mammals) and bacteria. AdoCbl contains a Co-C bond and is extremely light sensitive, but, until recently, this light sensitivity appeared to have no physiological function. Recently, AdoCbl has been found to act as cofactor for a photoreceptor protein (CarH) that controls the expression of DNA coding for transcription of the proteins needed for synthesis of carotenes in certain non-photosynthetic bacteria. In 2015, the X-ray crystal structures of two dark states of the photoreceptor protein from the bacterium *Thermus thermophilus* were determined: CarH bound to AdoCbl and CarH bound to a large portion of the cognate DNA operator (and AdoCbl); a light state was also determined in which CarH was bound to cobalamin in which the Co-C bond had been broken. The breaking of the Co-C bond of Ado-Cbl acts as a trigger for the regulatory switch that allows the transcription of DNA. In the two dark states AdoCbl is bound to a conserved histidine from CarH, which displaces the lower 5,6-dimethylbenzimidazole ligand of AdoCbl. In the light state the 5'-deoxyadenosyl group of AdoCbl is replaced by a second histidine from CarH, giving a bis-histidine cobalamin and 4',5'-anhydroadenosine. Genes for B_{12}-dependent photoreceptors are widespread in bacteria. Control of DNA transcription may represent an evolutionarily ancient function of AdoCbl, possibly pre-dating its function as a protein cofactor.

Significance:

- A new function for adenosylcobalamin, a light-sensitive form of vitamin B_{12} with a Co-C bond, has been discovered in bacteria
- Some non-photosynthetic bacteria use adenosylcobalamin as a cofactor for the protein CarH, which controls DNA transcription
- Three X-ray crystal structures of CarH have been determined: bound to adenosylcobalamin, DNA and after light exposure
- A mechanism of action for CarH, based on its structure and on model reactions of vitamin B_{12}, is proposed

Introduction

It is appropriate that in 2015, the UNESCO year of light, the X-ray crystal structure of a novel coenzyme B_{12}-dependent photoreceptor protein was reported.[1] Vitamin B_{12} was first discovered as cyanocobalamin (CNCbl), an inactive form of the vitamin, as an artefact of the isolation procedure, which utilised cyanide. (For reviews on vitamin B_{12}, see [2–5]). The coenzyme forms of vitamin B_{12} – adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl) (Figure 1) – are extremely unstable to light and were converted to CNCbl by photolysis during the work-up procedure of their original isolation. This sensitivity to light is considered to be a great nuisance by researchers because all experiments involving coenzymes must be performed in the dark or under dim red light. However, until recently, the photosensitivity of B_{12} coenzymes appeared to have no physiological function.[1,6,7]

Humans have only two vitamin B_{12}-dependent enzymes: methylmalonyl-coenzyme A (CoA) mutase which utilises AdoCbl as its coenzyme and methionine synthase which utilises MeCbl. Methylmalonyl-CoA mutase, an example of an isomerase enzyme, converts methylmalonyl-CoA to succinyl-CoA in the oxidation of odd-chain fatty acids from the degradation of isoleucine and valine. Methionine synthase, an example of a methyl transfer enzyme, converts homocysteine to methionine. Humans (and other mammals) are unable to synthesise cobalamins and rely on dietary sources for this essential vitamin. Bacteria are much more versatile and some can synthesise cobalamins *de novo* as well as use them in a much wider variety of enzyme reactions. Bacteria utilise MeCbl in the fixation of carbon dioxide through the acetyl-CoA pathway, methanogenesis and in a variety of methylation reactions, including some involved in the synthesis of the corrinoid precursors of cobalamins. AdoCbl-requiring enzymes in bacteria include several isomerases, amino mutases, diol dehydratase, ethanolamine ammonia lyase and a ribonucleotide reductase.

The mechanism of action of AdoCbl-requiring enzymes involves homolysis of the Co-C bond of AdoCbl to give cobalamin in the Co(II) oxidation state and the 5'-deoxyadenosyl free radical. This reaction is similar to that taking place when free AdoCbl is photolysed. For AdoCbl-requiring enzymes, the 5'-deoxyadenosyl free radical takes part in rearrangement of substrate to product. For photolysis of free AdoCbl in the absence of oxygen, the 5'-deoxyadenosyl radical cyclises to give 5',8-cycloadenosine[8] and in the presence of oxygen it gives adenosine 5'-aldehyde and 5'-peroxyadenosine (Scheme 1)[9-11].

Dorothy Hodgkin determined the X-ray crystal structure of vitamin B_{12} in 1955.[12] This structure was part of the research for which she was awarded a Nobel prize in chemistry. The structures of a large number of cobalamins, including AdoCbl and MeCbl, were subsequently determined. This determination was followed, from the mid-1990s, by the protein X-ray crystal structures of first the MeCbl-binding subunit of methionine synthase[13] and then a large variety of other cobalamin-dependent enzymes, including methylmalonyl-CoA mutase[14].

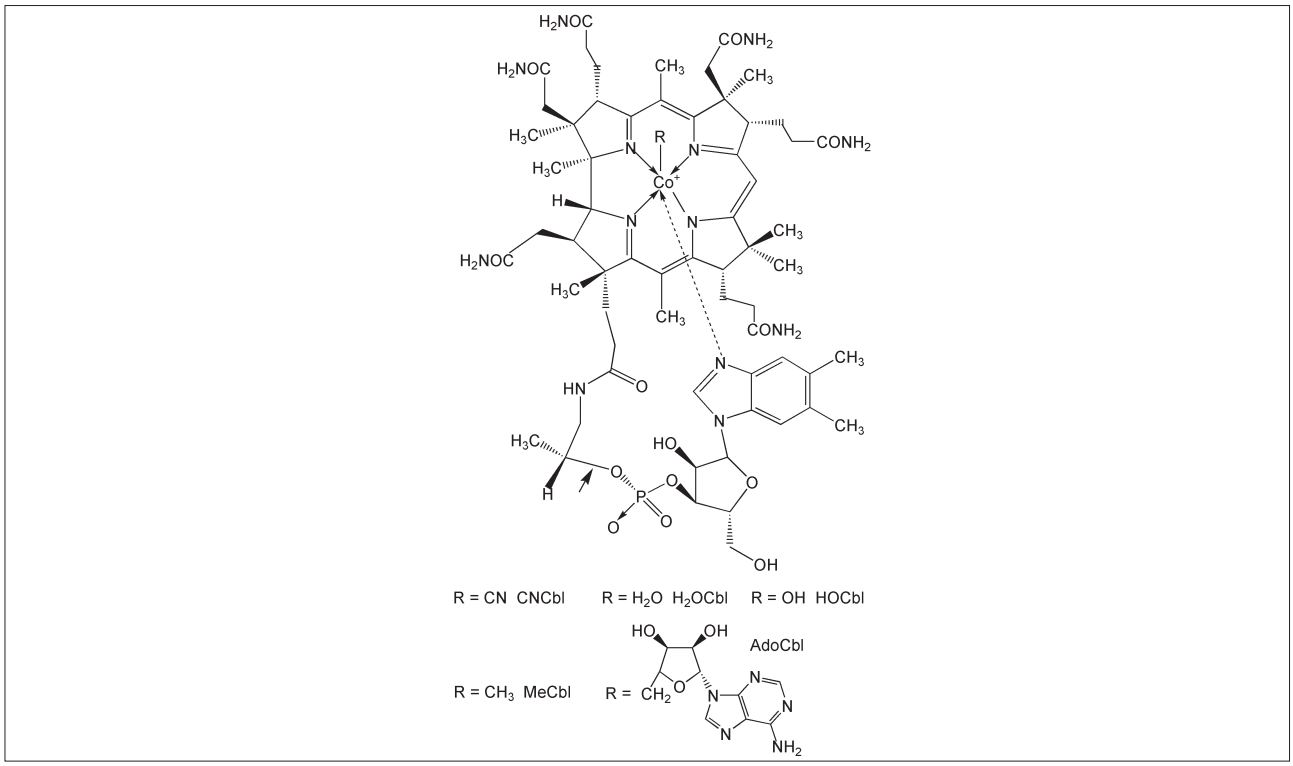
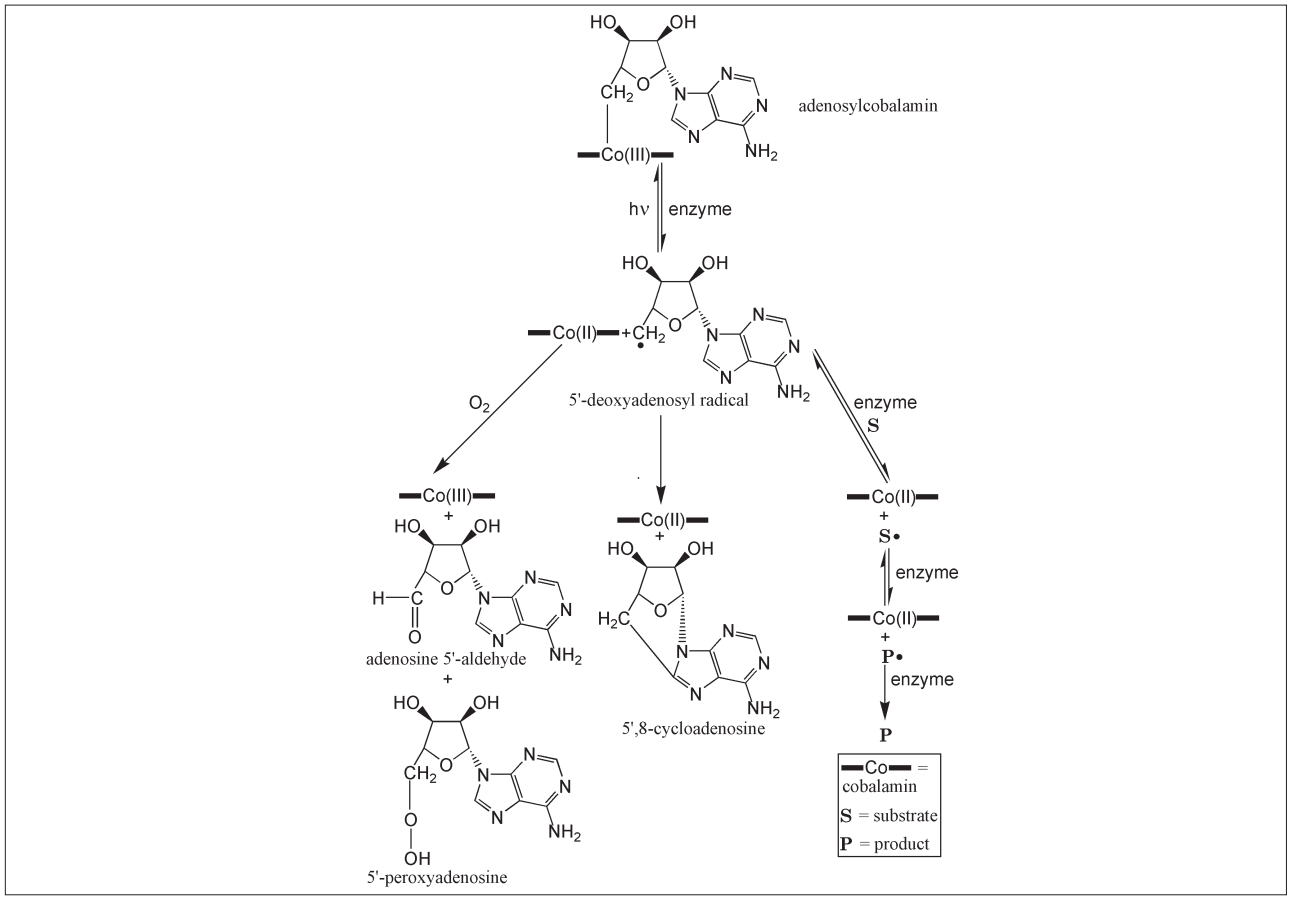


Figure 1: Structures of cobalamins. Vitamin B_{12} or cyanocobalamin (R=CN, CNCbl), aquocobalamin (R=H_2O, H_2OCbl), hydroxocobalamin (R=OH, HOCbl), methylcobalamin (R=CH_3, MeCbl) and adenosylcobalamin, also known as coenzyme B_{12} (R=5'-deoxyadenosyl, AdoCbl). Cobalamins are shown in the 'base-on' form in which the 5,6-dimethylbenzimidazole base (dbzm) is coordinated to Co, but they can also exist in the 'base-off' form in which dbzm is not coordinated to Co and may be replaced by another ligand. H_2OCbl and HOCbl are found in an acid/base-dependent equilibrium and both are present at physiological pH. Hydrolysis of the bond at → gives the corresponding cobinamide.



Scheme 1: Homolysis of the Co-C bond of adenosylcobalamin (AdoCbl).

X-ray crystallography has shown that B_{12} enzymes can bind AdoCbl in two ways: in the 'base-off' form whereby a histidine from the protein displaces the 5,6-dimethylbenzimidazole (dbzm) base, as in methionine synthase[13] and methylmalonylCoA mutase[14] and in a 'base-on' form as in diol dehydratase and ethanolamine ammonia lyase[5]. X-ray crystallography has been invaluable in providing insight into the structures and reaction mechanisms of B_{12} enzymes.

AdoCbl is also known to interact directly with messenger RNA in riboswitches. (A riboswitch is a mRNA which interacts directly and selectively with a small molecule.[15]) One of the first riboswitches to be discovered was the *E. coli btuB* mRNA,[16] which interacts selectively with AdoCbl in order to control the synthesis of the BtuB transmembrane protein, which transports corrinoids across the outer membrane of the bacteria. At low AdoCbl concentrations the riboswitch is 'on', and at high concentrations the switch is 'off'. In the 'off' position, AdoCbl binds to the mRNA, changing its three-dimensional structure, preventing the association of mRNA with the ribosome and stopping the synthesis of BtuB. AdoCbl riboswitches are widespread in bacteria and are involved in the synthesis and transport of cobalamins and the transport of metal ions.[16,17]

Adenosylcobalamin-dependent photoreceptor proteins in bacteria

Recently, a new group of photoreceptor proteins that uses AdoCbl to sense light was discovered in certain non-photosynthetic bacteria.[6,18-27] These bacteria produce carotenoids – yellow, orange or red pigments – which protect them from photo-oxidative damage by quenching the singlet oxygen free radicals produced by absorption of energy from light.[19] The photoreceptor proteins were first discovered in the bacterium *Myxococcus xanthus*,[6,18-20] which turns red in the presence of light, as a result of the synthesis of carotenoids, but is pale yellow in the dark. However, carotenoids have also been found in a variety of other bacteria, including *Streptomyces coelicolor*[21-23], *Thermus thermophilus*[24-26] and *Bacillus megaterium*[27]. Homologous sequences to the photoreceptor protein are found in the genomes of many bacteria and these proteins are probably widely distributed in non-photosynthetic bacteria.[6,17,19,22,25,26] The photoreceptor proteins were named CarH in *M. xanthus*[20], TtCarH[25] or LitR[24] in *T. thermophilus* and LitR[27] in *B. megaterium*, but, for simplicity, and because the photoreceptor proteins are all very similar, in this review I shall use the designation CarH for the protein and *carH* for the gene.

The non-photosynthetic bacteria in which CarH has been identified vary widely in their habitat and metabolism. *M. xanthus* is a rod-shaped Gram-negative predatory bacterium. It is unable to synthesise B_{12} but can obtain B_{12} from its food and convert it to AdoCbl, presumably using an ATP:corrinoid adenosyltransferase enzyme because it has a gene encoding this enzyme.[6] *T. thermophilus* is a Gram-negative, extremely thermophilic bacterium (extremophile).[28,29] *S. coelicolor* is a Gram-positive filamentous bacterium.[21,23] *B. megaterium* is a Gram-positive endospore-forming soil bacterium.[27] *T. thermophilus*[28,29], *S. coelicolor*[21,23] and *B. megaterium*[30] are all capable of synthesising cobalamins *de novo*. *M. xanthus* and *T. thermophilus* are the bacteria that have been studied most intensively. In *M. xanthus,* the *carH* gene is found on the chromosome[19] but in *T. thermophilus* it is found, together with genes for the later stages of B_{12} biosynthesis, on the megaplasmid, which is a large extrachromosomal element.[24]

The monomer of the photoreceptor protein CarH was found, by analysis of its primary structure, to consist of two regions joined by a short flexible linker chain: a C-terminal (carboxy) region, which can bind to cobalamins, and an N-terminal (amino) region, which can bind to DNA. The C-terminal end detects light and the N-terminal end allows gene expression for the production of carotenoids. The amino acid sequence at the N-terminal end of CarH is very similar to that of the DNA-binding domain of the N-termini of a family of transcriptional activators, known as the MerR-like proteins, which mediate responses to stress arising from exposure to toxic compounds or organic free radicals.[20] The amino acid sequence of the carboxyl region of CarH is very similar to that of the MeCbl-binding domain of methionine synthase and shows a typical cobalamin-binding fingerprint sequence.[6,13,18,19,31,32] However, the prosthetic group of CarH is AdoCbl, rather than MeCbl, as in methionine synthase.[6] AdoCbl is bound to CarH through displacement of dbzm by the imidazole group of a conserved histidine (His193 in *M. xanthus* and His177 in *T. thermophilus*), similarly to the binding of MeCbl in methionine synthase.[6]

The active form of the CarH receptor protein is a tetramer containing four molecules of AdoCbl (Figure 2). CarH monomers oligomerise to form the tetramer only when AdoCbl is present. In the dark, the CarH tetramer binds to the DNA operator controlling carotenogenesis and prevents the action of RNA polymerase, thus blocking transcription of the proteins needed for synthesis of carotenes. In the presence of light at wavelengths at which AdoCbl absorbs light (360 nm, 438 nm or 540 nm), AdoCbl is photolysed, the tetramer dissociates into monomers and the gene repression is released. This process occurs only if the conserved histidine from the cobalamin-binding fingerprint sequence is present; mutants lacking this histidine, when histidine is replaced by alanine, do not show AdoCbl-dependent oligomerisation.[6]

It was later discovered that vitamin B_{12} also has a gene regulation function in the photosynthetic purple bacteria *Rhodobacter capsulatus*.[33] In the presence of light but the absence of oxygen, *R. capsulatus* produces large amounts of bacteriochlorophyll, and photosynthesis takes place through an anaerobic pathway. However, *R. capsulatus* can also grow aerobically in the dark.[34] In the presence of sufficient oxygen, when the anaerobic pathway is not needed, the pathway is repressed because it also produces destructive singlet oxygen.[17,33] The protein CrtJ represses the synthesis of bacteriochlorophyll and is linked to the protein AerR, which is also an aerobic repressor of photosynthesis genes.[35] The AerR protein uses AdoCbl or MeCbl as its cofactor but does not bind to either.[17,33] It was proposed (Figure 3) that AdoCbl or MeCbl is converted to hydroxocobalamin (HOCbl) in the presence of light, which is followed by binding of HOCbl to AerR, then binding of HOCbl/AerR to the gene repressor CrtJ, and finally release of the repression of the genes for synthesis of bacteriochlorophyll so that anaerobic photosynthesis can take place. AerR does not bind to AdoCbl or MeCbl but does bind to their photolysis product, HOCbl, and can therefore also be considered as a light-sensing protein.[17,33]

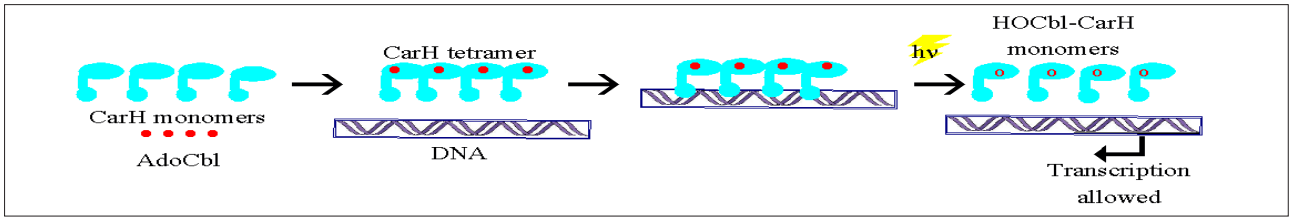


Figure 2: Model for the response to light of the AdoCbl regulatory switch in *Myxococcus xanthus*.[6] In the dark, AdoCbl binds to CarH monomers to give the CarH tetramer and the CarH tetramer binds to DNA, preventing transcription. In the presence of light, AdoCbl is photolysed and CarH dissociates, allowing transcription.

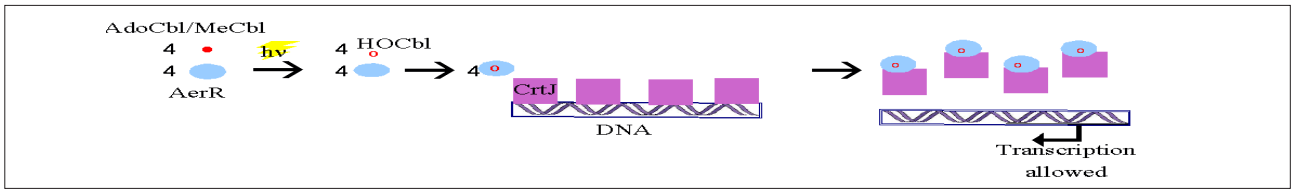


Figure 3: Model for the response to light of the B_{12}-dependent regulatory switch in *Rhodobacter capsulatus*.[33] In the dark, the CrtJ repressor binds to DNA, preventing transcription. In the presence of light, AdoCbl or MeCbl is photolysed to give HOCbl which binds to AerR. The AerR–cobalamin complex binds to CrtJ and the CrtJ dissociates, allowing transcription.

The AerR protein is similar to CarH in that it is homologous to CarH and methionine synthase, has a typical cobalamin binding fingerprint sequence and contains a histidine (His145) which binds to the lower position of B_{12}, displacing dbzm. It is different in that it binds only HOCbl (rather than AdoCbl or MeCbl) and does not contain any DNA-binding region, but instead interacts with the protein CrtJ. AerR binds to HOCbl very strongly, much more strongly than expected from an ionic interaction between cobalamin and a single histidine on AerR. It was proposed that the upper position of the cobalamin is occupied by a second histidine (His10) from AerR and that this bond is stronger than that to the lower histidine.[33]

X-ray crystal structure of the CarH photoreceptor protein

In 2015, Jost, Drennan and coworkers[1] determined the X-ray crystal structure of the photoreceptor CarH from *T. Thermophilus* in three different states: two dark states, in one of which CarH is free and in the other is attached to DNA and a state in which CarH has dissociated from DNA after exposure to visible light (Figure 4).

In the free dark state, CarH is a tetramer with one molecule of AdoCbl bound to each monomer.[1] Each monomer has one domain that attaches to DNA at the N-terminal end and a second domain that binds the light-sensing AdoCbl molecule at the C-terminal end. The N-terminal domain consists of a DNA-recognition helix and a β-hairpin wing. The C-terminal domain consists of a four-helix bundle and a Rossman fold cobalamin-binding region. AdoCbl, in which the bond length of the Co-C bond is 2.0 Å, similarly to that of free AdoCbl (2.030 Å)[36], is located between the four-helix bundle and the Rossman fold[1]. The upper ligand, 5'-deoxyadenosyl, is orientated towards the four-helix bundle, close to a tryptophan residue, Trp131, and the lower ligand dbzm has been displaced by a histidine ligand (His177) from the Rossman fold. The C-terminal domain is rigid but the N-terminal domain is more flexible. The tetramer assembles itself from the monomers only if AdoCbl is present. The tetramer can be considered as a dimer of dimers, in which each dimer has its monomers in a head-to-tail orientation with respect to the AdoCbl-binding domain. The monomers within the dimer are held together by many hydrogen bonds and electrostatic interactions from a variety of side-chains and from the 5'-deoxyadenosyl ligand. The dimers are joined through their AdoCbl-binding domains in a staggered fashion to give the tetramer, involving Gly160 and Gly192 on each dimer. The C-terminal domains of the monomers form the centre of the tetramer with the N-terminal domains on the exterior.[1]

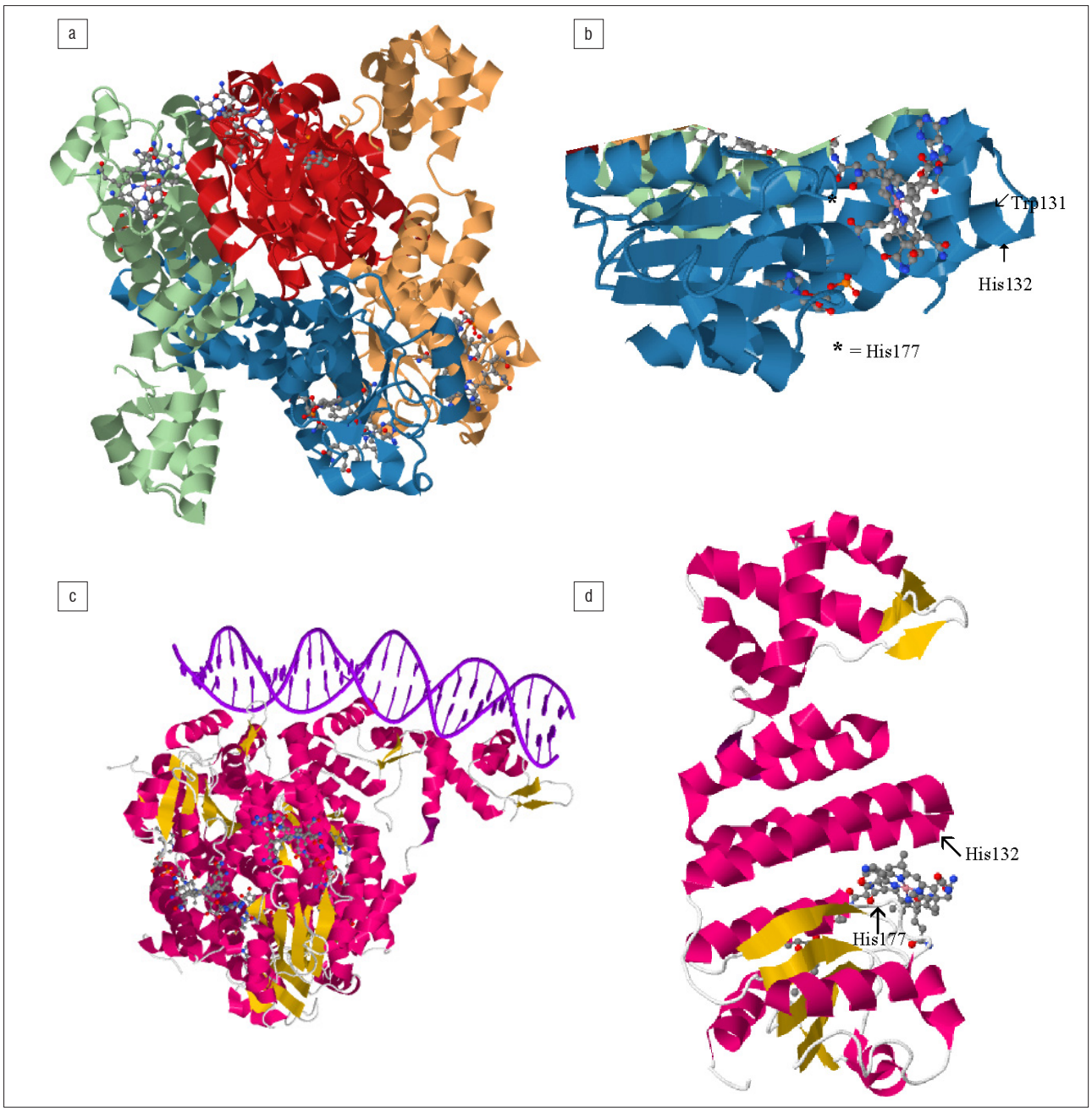
It is interesting that the primary structure of the AdoCbl-binding domain of CarH is similar to that of the MeCbl-binding domain of methionine synthase, rather than to any of the enzymes using AdoCbl as a prosthetic group, especially as the adenosyl group is much larger than the methyl group.[1,6,13,18,19,31,32] However, the cobalamin binding pocket in CarH is bigger than that in methionine synthase because the four-helix bundle is situated 2.5 Å further away from the cobalamin and the leucine in methionine synthase is replaced by a smaller valine (Val138) residue. The pocket also provides more opportunities for hydrogen bonding by replacing a valine with glutamic acid (Glu141) and for polar interactions by replacing a valine with histidine (His142) and phenylalanine with tryptophan (Trp131).[1]

In the model for the AdoCbl-based regulatory switch (Figure 2), the CarH tetramer binds to its cognate DNA operator, a 30-bp (base pair) portion of the region of DNA between the gene encoding CarH and the gene encoding CrtB (the operator controlling carotenogenesis). In the crystal structure of the second dark state, the CarH tetramer is bound to a large piece (26 bp) of the cognate DNA operator.[1] Three of the four DNA-binding domains of the CarH tetramer bind to DNA, with the fourth domain being disordered and not visible in the crystal structure. The three detectable DNA-binding domains all face in the same direction but otherwise the structure is similar to that of the free dark state. All three visible DNA-binding domains are important in binding CarH to DNA, forming a variety of hydrogen bonds and electrostatic interactions between the protein and the phosphate backbone of the DNA. In addition, the recognition helix of each DNA domain on CarH inserts into the DNA major groove and a histidine from the β-hairpin wing fits into the DNA minor groove. The recognition helix of the middle DNA domain covers the promoter -35 element for the gene corresponding to the σ^A-associated bacterial RNA polymerase, in which σ^A is the protein subunit necessary for initiation of RNA synthesis[37], and thus blocks access of the RNA polymerase to the promoter. (The promoter -35 element is so called because it is 35 nucleotides upstream, or counting backwards, from the transcription start site of σ^A.[38])

In contrast to the dark-state tetramer, the light-state CarH protein is monomeric and contains cobalamin, but has lost the 5'-deoxyadenosyl group.[1] When comparing the light-state CarH monomer with the corresponding dark-state monomer, it can be seen that the Co-C bond of AdoCbl has been broken and the 5'-deoxyadenosyl group is absent. The helical bundle, including Trp131, has moved into the vacant space left by the 5'-deoxyadenosyl group and is positioned very much closer to cobalamin. Very importantly, a histidine, His132, on the helical bundle has shifted into a suitable position to coordinate with Co(III) on the cobalamin through its imidazole side-chain.

Mechanism of action of the CarH regulatory switch

The X-ray crystal structures of the three CarH proteins are consistent with the AdoCbl-based regulatory switch proposed in Figure 2, but give a much more detailed insight into its mechanism. In the dark state, the lower ligand (dbzm) of AdoCbl is displaced by the imidazole group of His177 and is thus bound through this histidine to CarH. In the His177 $\rightarrow$ Ala mutant, this step is blocked and the regulatory switch cannot function.[1,6] In the dark, when carotenogenesis is not needed, the CarH tetramer binds to the DNA operator, preventing the transcription of carotenogenesis proteins. The X-ray crystal structures show that the DNA-binding domains of CarH are conveniently located on the outside of the tetramer, whereas the AdoCbl-binding domains are each buried in a deep pocket. In the presence of visible light, when carotenogenesis is of advantage to the bacteria, the Co-C bond of the bound AdoCbl is broken and the 5'-deoxyadenosyl moiety drifts away. The change in conformation on the breaking of the Co-C bond causes the movement of the helical bundle into the pocket, bringing His132 into position to bind to Co(III) through its imidazole side-chain. The change of conformation disrupts the head-to-tail dimer interaction and disassembles the tetramer, causing CarH to dissociate from the DNA promoter and relieving the blockage of access of RNA polymerase to the promoter so that transcription is allowed.[1]



Source: Images from the RCSB PDB (www.rcsb.org)*; PDB IDs: 5C8D, 5C8A, 5C8E and 5C8F.*[1]

Figure 4: Structures of CarH. (a) CarH dark-state tetramer, showing the four identical subunits in four colours, and four molecules of AdoCbl (Co, coral; C, grey; N, blue; O, red; P, orange), one bound to each subunit (PDB ID: 5C8D). (b) CarH dark-state monomer, showing one of the four identical subunits (blue) and one molecule of AdoCbl (colours as in (a)). The truncated form of CarH (PDB ID: 5C8A), which consists of only the light-sensitive C terminal domain, is depicted in order to show the binding of AdoCbl more clearly. Trp131 and His132 are indicated by arrows and His177 by an asterisk. (c) CarH dark-state tetramer bound to 26 base pairs of the cognate DNA operator (DNA, purple; α helix, pink; β sheet, yellow; all other structures, white; colours of the AdoCbl molecules are as in (a)). The three DNA-binding domains, all facing in the same direction, can be seen at the bottom of the figure (PDB ID: 5C8E). (d) CarH light-state monomer (colours as in (c)). His132 and His177 are indicated by arrows and Trp 131 is behind His132 (PDB ID: 5C8F)

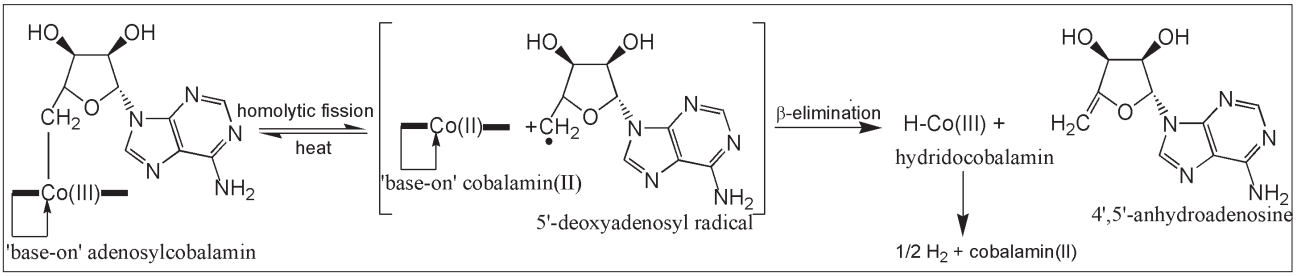
In conjunction with X-ray crystallography, Jost et al.[1,7] have used other techniques to answer questions about the mechanism of photolysis of CarH-bound AdoCbl. Two of these questions are:

1. What are the products of photolysis of CarH-bound AdoCbl and how exactly is the Co-C bond in CarH-bound AdoCbl broken?
2. How is the Co(III) in the light-state CarH protein bonded to the second histidine (His 132) on the protein?

Both questions have already generated controversy and will be considered further here.

CarH-bound AdoCbl: Products of photolysis and the Co-C bond

The UV-visible spectrum of the CarH free dark state is very similar to that of free AdoCbl and is completely consistent with the imidazole group of histidine being the lower ligand.[1]



Scheme 2: Homolytic fission followed by β-elimination for the thermolysis of adenosylcobalamin in glycerol.

After photolysis in the presence of oxygen the CarH-bound cobalamin is in the Co(III) oxidation state[1,7] but in the absence of oxygen the cobalamin is in the Co(II) oxidation state, as shown by UV-visible spectroscopy and electron spin resonance spectroscopy.[7] Exposure of the CarH-bound cobalamin in the Co(II) oxidation state to oxygen, after anaerobic photolysis, gives the same Co(III) product as does aerobic photolysis. However, the organic product of photolysis of CarH-bound AdoCbl was completely unexpected. Jost, Drennan and coworkers[7] have shown unequivocally by liquid chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy that the organic photolysis product of AdoCbl-bound CarH under both anaerobic and aerobic conditions is solely 4',5'-anhydroadenosine. The product 4',5'-anhydroadenosine had never been observed previously in the photolysis of AdoCbl (see above). However 4',5'-anhydroadenosine has been observed in the thermolysis of AdoCbl in glycerol[39-41], which could provide a model for the photolysis of CarH-bound AdoCbl.

Garr and Finke[39,41] found that, in the highly viscous solvent glycerol, thermolysis of AdoCbl at 110 °C gives rise to 5% 4',5'-anhydroadenosine (in addition to 5',8-cycloadenosine and 5'-deoxyadenosine), but in the less viscous solvent, ethylene glycol, no 4',5'-anhydroadenosine is seen. Thermolysis of adenosylcobinamide (the 5-coordinate analogue of AdoCbl in which the nucleotide base has been removed) in ethylene glycol gives 4',5'-anhydroadenosine as a major product (33%).[40,41] For both AdoCbl and adenosylcobinamide, the corrinoid partner of 4',5'-anhydroadenosine is Co(II).[39-41] No 4',5'-anhydroadenosine is seen in thermolysis of AdoCbl[42] or adenosylcobinamide[43] in aqueous solution. A viscous solvent, which can act as a strong cage for Co(II) and the 5'-deoxadenosyl radical, appears to be necessary for the comparatively slow formation of 4',5'-anhydroadenosine. Garr and Finke[39-41] proposed that, while in close proximity in the cage, the Co(II) and the 5'-deoxyadenosyl radical (generated by homolytic fission) undergo a β-elimination reaction to give hydridocobalamin and 4',5'-anhydroadenosine. Hydridocobalamin then rapidly decomposes to Co(II) and hydrogen (Scheme 2).[39-41,44]

Based on the results of Garr and Finke[39-41], Jost and Drennan and coworkers[7] have proposed the following CarH photolysis mechanisms (Scheme 3, Paths 1 and 2). In Path 1 of Scheme 3, CarH-bound AdoCbl first undergoes photolysis to give Co(II) and the 5'-deoxyadenosyl radical by homolytic fission of the Co-C bond. Then, while in close proximity in the extra strong cage provided by the protein, β-elimination between Co(II) and the 5'-deoxyadenosyl radical takes place to give the Co(III) hydride (still bound to the protein) and 4',5'-anhydroadenosine. Clearly, the CarH protein would provide a much stronger cage than a viscous solvent, which would account for 4',5'-anhydroadenosine being the sole product of photolysis of CarH-bound AdoCbl.[7] There is a large amount of evidence in favour of Path 1 and this path is favoured by Jost and Drennan and colleagues[7]. Cage effects by proteins have been previously observed in the AdoCbl-dependent glutamate mutase[45,46] and in the MeCbl-binding domain of methionine synthase[47]. In addition to thermolysis of AdoCbl and adenosylcobinamide[39-41], homolytic fission followed by radical-mediated β-elimination is well documented in alkylcobalamins and alkylcobinamides[48-50]. The generation of 4',5'-anhydroadenosine (rather than more reactive species as in photolysis in vitro) as the organic partner of CarH photolysis may represent a safety mechanism in ensuring that the reactive 5'-deoxyadenosyl radical is not released.[1,7]

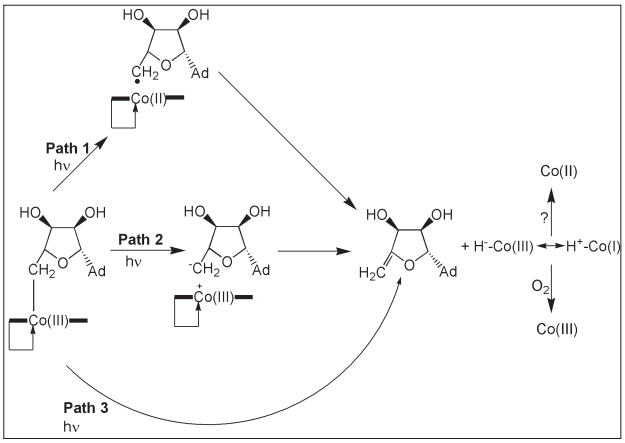
The intermediate hydridocobalamin, formulated

$$Co(I)\text{-}H^{+} \leftrightarrow Co(II)\text{-}H \leftrightarrow Co(III)\text{-}H^{-},$$

is an unstable species that rapidly disproportionates to give Co(II) and molecular hydrogen under anaerobic conditions[44] and is presumably oxidised to Co(III) under aerobic conditions. Hydridocobalamin is difficult to characterise because it is extremely unstable. However, from cyclic voltammetry[51], hydridocobalamin has $pK_a \approx 1$ and is protonated on the 5,6-dimethybenzimidazole base as well as on the Co[51-53]. Thus, hydridocobalamin can be considered as protonated Co(I) and can act as a Brönsted acid to protonate water:

$$Co(I)\text{-}H^{+} + H_2O \rightarrow Co(I) + H_3O^{+},$$

or possibly an amino acid side-chain on the protein.



Ad, adenine base

Scheme 3: Paths proposed for CarH photolysis.

Some doubt has been expressed about the structure of hydridocobalamin because 'hydridocobaloxime', which was proposed to have the same structure as hydridocobalamin[54], has been found to be a dimeric Co(II) compound with a long Co-Co bond[55]. Cobaloximes are not good models for cobalamins in this case because they are relatively flat and can dimerise more readily than cobalamins. Cobalamins are prevented from dimerising in the same way by steric hindrance from their side-chains preventing a close enough approach of the two Co atoms.

Path 2 in the scheme of Jost et al.[7] involves initial heterolytic cleavage of the Co-C bond to give Co(III) and the 5'-deoxyadenosyl anion, followed by β-elimination to give 4',5'-anhydroadenosine and hydridocobalamin. Kutta et al.[56] propose a variant of Path 2 in which heterolysis of the Co-C bond gives the 5'-deoxyadenosyl anion and either hydridocobalamin or a five-coordinate positively charged Co(III) as intermediates on the route to 4',5'-anhydroadenosine and cob(II)alamin. It would seem that Path 2 would be highly unlikely. The 5'-deoxyadenosyl anion species appears to be completely unprecedented; a search of SciFinder produced no references compared with 284 for the 5'-deoxyadenosyl radical.[57] An

anion such as the 5'-deoxyadenosyl anion would be expected to be extremely unstable and would immediately rearrange or decompose to give a more stable species. This expectation has been observed in at least one case. A model reaction for heterolytic cleavage of the Co-C bond in CarH-bound AdoCbl is provided by the thermolysis of AdoCbl in aqueous solution at pH 7.0 and 85 °C. This reaction proceeded by 10% heterolysis, in which the organic products of heterolysis were only adenine and 2,3-dihydroxypentenal, which are decomposition products of 5'-deoxyadenosyl.[42] The presence of exclusively 4',5'-anhydroadenosine (and no adenine) as the organic product of CarH photolysis strongly suggests that Path 2 is not operative in CarH photolysis.

Yet another path (Path 3) – concerted β-elimination by migration of a hydride ion (H^-) to give hydridocobalamin and 4',5'-anhydroadenosine directly (that is a reaction not proceeding through a radical pair intermediate) – is theoretically possible. This type of reaction seems unlikely because it generally requires a vacant coordination site on the metal[58] and has no precedent in organocobalamins.

How is Co(III) in the light-state CarH protein bonded to His 132?

After photolysis has taken place, the cobalamin moiety binds very strongly to light-state CarH, with the wild-type CarH forming a very stable complex with the cobalamin.[1] The cobalamin-dependent light-sensing protein AerR in *Rhodobacter capsulatus* similarly forms a very stable complex with cobalamin and it has been proposed that this complex contains cobalamin bound to two histidines from the protein at both the top and bottom positions.[17,33] However, this type of coordination of histidine is controversial because it has not been observed for free cobalamin (Marques et al.[59] and personal observations). It also means that it is not possible to directly compare the UV-visible spectrum of the light-state CarH protein with that of free bis-histidine cobalamin in aqueous solution. The equilibrium constant for coordination to Co(III) of the first histidine ($\log K_1$ 4.30) is favourable but coordination of the second histidine is much more difficult ($\log K_2 < -1$),[59] so that the spectrum of the bis-histidine complex is not observed.[59] However, the UV-visible spectra of cobalamin with histidine or imidazole in the upper position and dbzm in the lower position are almost indistinguishable,[59] and the UV-visible spectrum of (mainly) the bis-imidazole complex of cobalamin can be observed as $\log K_1 = 4.59$ and $\log K_2 = 0.6$.[59] The UV-visible spectrum of the light-state CarH protein is very similar to the UV-visible spectrum of the Co(III) cobalamin complex with two imidazole ligands binding through their N atoms in the upper and lower positions[1,59] and is consistent with the substitution of cobalamin by two histidines from the protein[1]. Presumably, the cobalamin is constrained by the protein so that substitution of a second histidine becomes possible. The extreme stability of light-state CarH[1] and AerR bound to cobalamin[33], both of which show a cobalamin adduct in mass spectrometry[1,33], suggests that the top histidine has a covalent bond to Co(III), rather than the ionic bond in the lower position[1,33]. It is possible that the top histidine is bound in the anion form, because imidazole bound as the anion has a larger logK than that for the neutral form (logK 4.60 rather than logK 4.30),[59] which might account for the great stability of the light-state CarH protein. If His132 in the light-state CarH protein is mutated to alanine so that only one histidine can bind, the UV-visible spectrum of the corresponding light-state CarH protein is very similar to the UV-visible spectrum of Co(III) cobalamin with only one imidazole[1,59] or histidine[59] ligand and it is less stable than the wild-type light CarH protein[1].

Photochemistry of CarH

In an attempt to determine a more detailed photochemical mechanism for CarH, Kutta et al.[56] performed photoexcitation experiments on the AdoCbl-bound CarH tetramer (ground-state CarH) and used transient UV-visible absorption spectroscopy on a femtosecond (10^{-15} s) to second timescale to follow the intermediates on the way to the light-state CarH product. They observed, in addition to Co(II) (and the presumed 5'-deoxyadenosyl radical), transient absorption spectra of at least eight intermediates and assigned these to possible structures, based on a model of the reaction pathway from ground-state CarH to light-state CarH. In their model, Intermediate A is the initial photoexcited state which, in their major pathway, immediately decays back to ground-state CarH either directly or through the Co(II)/5'-deoxyadenosyl radical pair, B. In their major pathway, the formation of the Co(II)/5'-deoxyadenosyl radical pair is unproductive, leading only to recombination to give AdoCbl. In their minor pathway, A converts to C, which has a spectrum that can be interpreted as a metal-to-ligand charge transfer complex, consisting of Co(III) with a partial positive charge and an incipient 5'-deoxyadenosyl anion. C then converts to D* (in the text) or C* (in their Figure 6),[56] which is interpreted as a five-coordinate, positively charged Co(III) together with a 5'-deoxyadenosyl anion, then to D, in which the 5'-deoxyadenosyl anion has moved away from Co and has been replaced by His132, and lastly to E, which is similar to light-state CarH.[56] Alternatively, they propose that Intermediate C corresponds to hydridocobalamin.[56] The UV-visible spectrum of C is very similar in shape to that proposed for base-off hydridocobalamin,[52] but is red-shifted relative to this spectrum. It is possible that C is the base-on form of hydridocobalamin but it is dangerous to speculate in this way (see below).

Further investigation is needed to elucidate the photochemical mechanism of CarH. Firstly, transient intermediate UV-visible spectra (300–700 nm) mainly tell us about the cobalamin partner and say very little about the 5'-deoxyadenosyl partner, which absorbs below 300 nm. It would perhaps be useful to extend the investigation into the UV region. Secondly, the identification of transient cobalamin intermediates in a mechanistic pathway, based only on their UV-visible spectra, is very difficult, unless suitable spectra of known species are available for comparison. For example, it is stated[56] that the spectrum of Intermediate C appears to be similar to the S_1 state seen in the photolysis of free MeCbl[601,61]. The S_1 state in MeCbl photolysis has now been characterised as a $d/\pi \rightarrow \pi^*$ metal-to-ligand charge-transfer state[61] (consistent with theoretical density functional theory calculations)[62], but was originally considered as 'a cob(III)alamin with a very weak axial ligand'[60]. The corresponding theoretical calculations for AdoCbl have not been completed to date but steady progress is being made with such calculations.[61]

Evolutionary implications

The discovery of the AdoCbl-dependent CarH photoreceptor and of the cobalamin-dependent AerR photoreceptor has many interesting implications. Genes for photoreceptors similar to CarH are found in a large number (>120) and variety of species of bacteria,[6] including bacteria that can biosynthesise cobalamins and those that acquire cobalamins from their food. Genes similar to that for AerR are found in several species of purple photosynthetic bacteria.[33] The presence of these genes suggests that B_{12}-dependent photoreceptors are widespread in both non-photosynthetic and photosynthetic bacteria. Together with adenosycobalamin-dependent riboswitches, CarH and AerR (in combination with its partner CrtJ) may represent an evolutionarily very old function for cobalamin: interaction with and control of nucleic acids. AdoCbl may have been co-opted by proteins only at a later date. AdoCbl, which is essential for B_{12} enzymes in humans (as well as other animals and bacteria), may have first evolved as a regulatory switch for DNA and RNA.

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