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The Very Large Gorilla Sitting in the Room? Adenosylcobalamin is the Missing Link: its Radical and Tetrahydrobiopterin are the Principal *in vivo* Catalysts for Mammalian Nitric Oxide Synthases.

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Abstract

Mammalian nitric oxide synthases (NOS) are a source of the universal second messenger, and pivotal biochemical molecule, nitric oxide (·NO). NOS are assumed to function catalytically in a haem-centred manner, by analogy with cytochrome P450. Yet, they differ significantly. Cobalamin, vitamin B12, is believed to function almost solely as an ·NO scavenger and, latterly, as a direct, physiological inhibitor of the NOS. Yet, in pathology, associated to cobalamin deficiency, functional or otherwise, NOS over-produce superoxide, peroxynitrite ($ONOO^-$), and other reactive nitrite species, rather than ·NO (*Figure 7*). This paper offers a radical, new solution to the gaps and inconsistencies in the current understanding of the mechanism of haem-centred NOS catalysis, which also challenges the other existing paradigm of cobalamin as just an ·NO mop. Examination of a wide diversity of NOS and cobalamin-dependent enzyme structure-function studies, as well as data from the ·NO/cobalamin chemical, biochemical, immunological, genetic, and clinical literature, offers indications that cobalamin, specifically, in one of its active forms, adenosylcobalamin (AdoCbl), may have a third, eukaryotic coenzyme function as the principal cofactor of well-regulated NOS catalysis *in vivo*. The AdoCbl-centred NOS reaction is described in detail (*Figure 5*), and some existing evidence that, *in vitro*, without AdoCbl, NOS turnover activity is significantly slower than in *in vivo* AdoCbl-rich environments, is presented. AdoCbl, in conjunction with tetrahydrobiopterin, couples NOS oxygen binding/activation to L-arginine hydroxylation and ·NO synthesis much more effectively than does haem, overcoming NOS spatial and redox problems, leading to productive catalysis, decreased radical formation/escape, with a consequent increased ratio of ·NO to $ONOO^-$, and prevention of pathology (*Figures 5 & 7*). *In vivo*, haem-centred NOS catalysis may, in fact, be the back-up NOS reaction, and it's predominance in the absence of AdoCbl, with a consequent lowering of the ·NO/ONOO- ratio, is the real source of supposedly ·NO derived pathology.

Keywords: adenosylcobalamin, nitric oxide synthase catalysis, tetrahydrobiopterin, ZnS_2N_2 on/off switch, nitrosylcobalamin.

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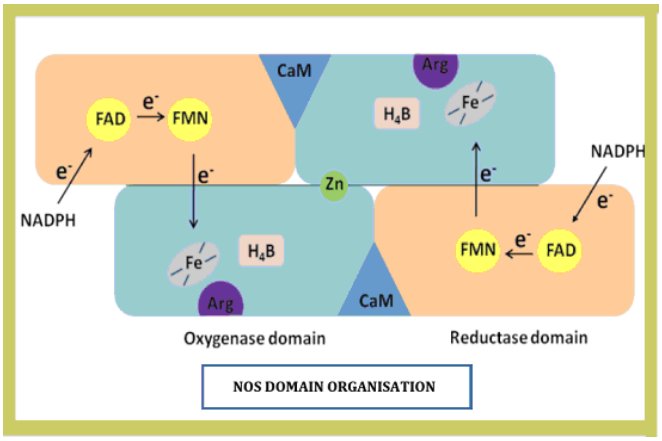
Introduction

The reactions catalysed by mammalian nitric oxide synthases (NOS) are central to life. Yet, when they become de-regulated, NOS are the source of serious inflammatory pathology, including the leading killers, cancer, heart disease and sepsis. NOS are believed to use L-arginine, 6*R*-5, 6, 7, 8-tetrahydrobiopterin (H_4B), NADPH, haem Fe[III] and O_2 to synthesize the radical, paramagnetic gas, nitric oxide. ·NO is the universal second messenger, involved in cell-signal transduction, gene expression, including epigenetic modifications, control of the cell cycle, apoptosis or growth and differentiation, respiration, neuro-transmission, muscle contraction, vaso-constriction, the coagulation cascade, co-ordination of the immune response and resolution of inflammation, pathogen cyto-toxicity, and host anti-oxidant actions.

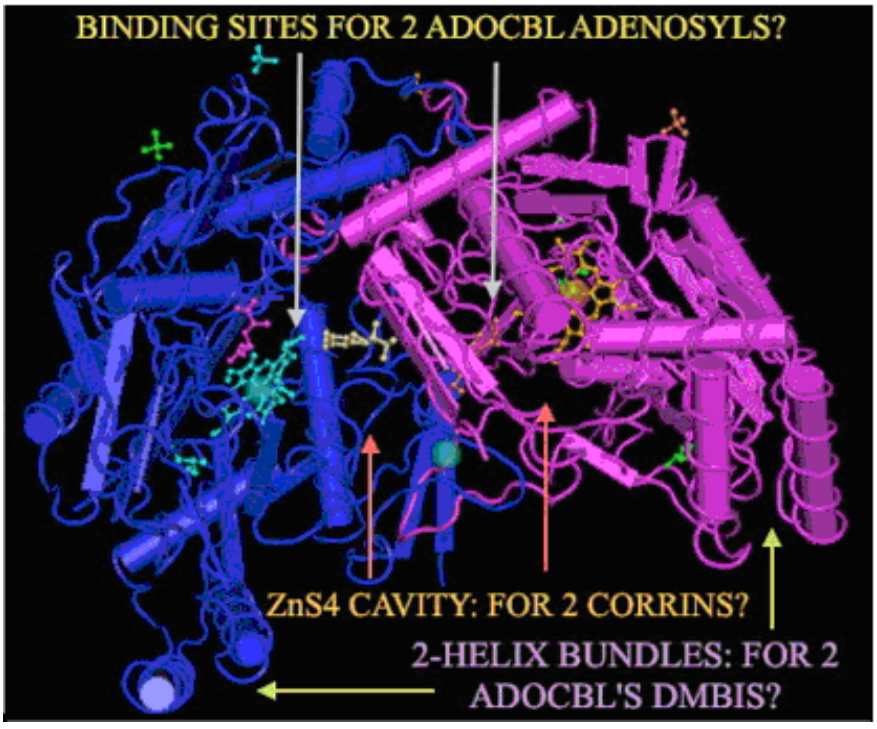
Figure 1: Pathway of electron flow crosses over between opposed subunits: the flavin domain of one half-dimer donating its electrons to the haem domain of the other.



NOS exist in three principal forms, two constitutive: endothelial and neuronal NOS (eNOS, nNOS), Ca^2+/calmodulin regulated, targeted to the caveolae: and the largely Ca^{2+}/calmodulin independent, inducible NOS (iNOS), active during growth and the immune response, at significantly higher expression levels than constitutive NOS, thus producing much higher amounts of ·NO, for which hepatic Küppfer cells, macrophages and polymorphonuclear neutrophils are a principal source. More recently a mitochondrial NOS has been added to the canon.

NOS function as homo-dimers, require a prosthetic haem to be bound for their assembly and are composed of discrete oxygenase and reductase domains linked by a calmodulin loop. The C-terminal reductase domain uses a chain of flavins, FAD/FMN, to transfer electrons from NADPH to the N-terminal oxygenase domain (*Figures 1 & 2).*

Figure 2: Human iNOS oxygenase domains, showing spatial arrangements of haems, (*turquoise/yellow* in *blue/pink* half-dimers) in relation to L-arginine (*pink*, clearly visible in *blue* half-dimer only) and H_4B ((*cream* in *blue* half-dimer) and the central zinc tetrathiolate hinge (*green*). Cylinders = α-helices, ribbons = β-sheet strands. PDB: 1NSI.



The reactions that yield ·NO and citrulline from L-arginine via the intermediate, N^{ω}-hydroxylated-L-arginine (NOHA) (*Figure 3*) are a two-step monoxygenation, currently believed to use 5 electrons.

However, after over a quarter century of research, there is no absolute or final consensus on many of the exact details of NOS catalysis, including the exact role of the cofactor H_4B. Various proposals for haem-based NOS catalysis have been espoused: a radical type auto-oxidation mechanism, [1, 2] or involvement of a nucleophilic peroxo-Fe[III] haem [3], or of the oxenoid species ($(Por^{+\cdot})$ Fe[IV](O) analogous to that of cP450 oxygenase [4, 5]. An example of one haem-based catalysis scenario, involving first a ferric haem-superoxo species, then a haem-peroxo species is illustrated in *Figure 4*. (For a detailed review of haem-based NOS catalysis competing theories and their problems see [6].)

The protein-bound haem Fe[III] is assumed to be the catalyst for the dual monoxygenation, largely an assumption from its presence, and by analogy with its role in other haem enzymes, such as cytochrome P450, and because, in studies with *purified* NOS, no other candidate has been observed.

Nevertheless, studies with purified NOS may not reflect what actually takes place *in vivo*.

Figure 3: Conversion of L-Arginine to ·NO/Citrulline, (NADPH stoichometry as currently accepted).

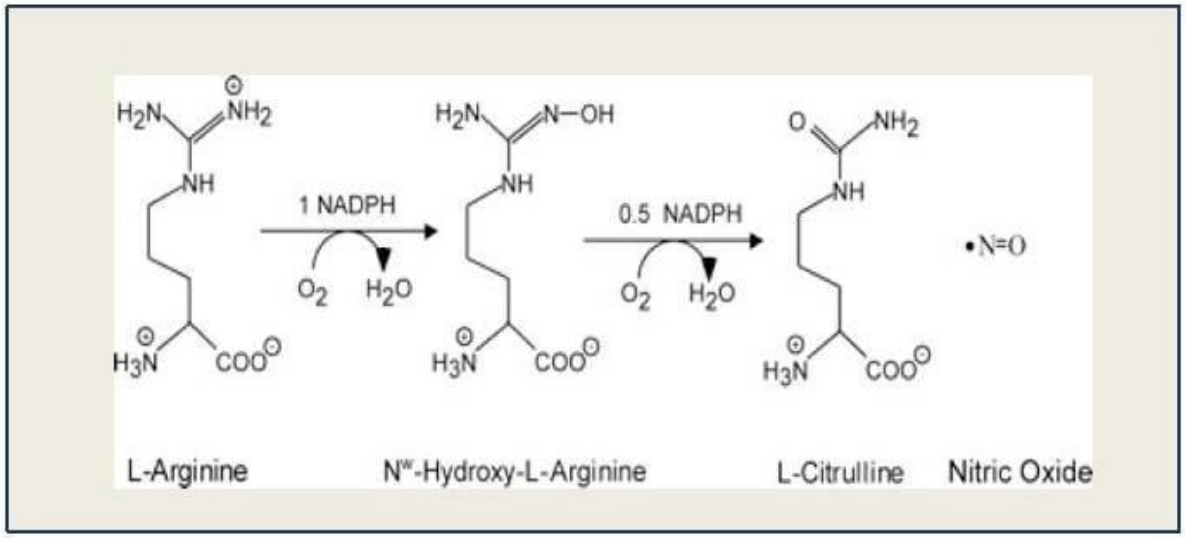


Figure 4: Haem-Based NOS Catalysis: a Difficult Balance. Final fast reduction of Fe[III]-·NO to Fe[II]-·NO/ slow dissociation of Fe[II]-·NO plus potential oxidation of Fe[II] to Fe[III] = nitrate not ·NO.

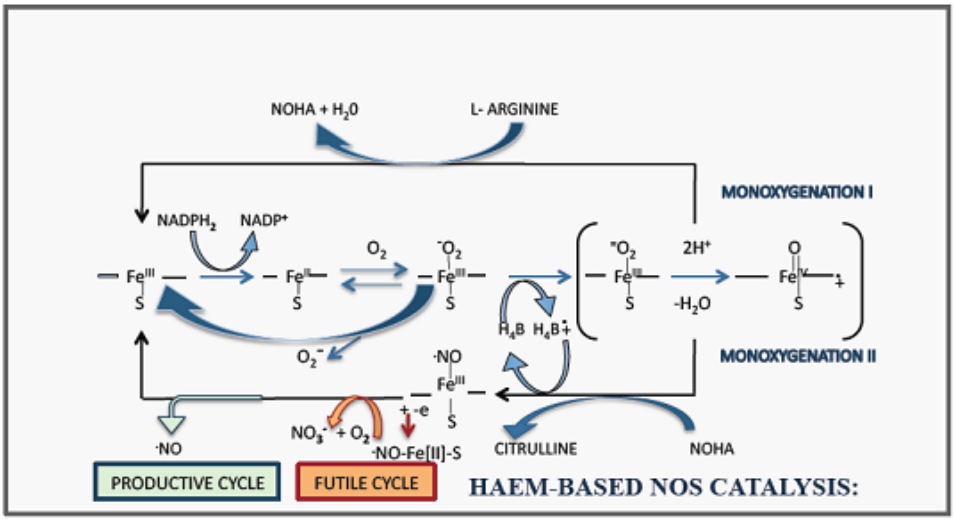


Figure 5: Adenosylcobalamin-Mediated NOS Catalysis.

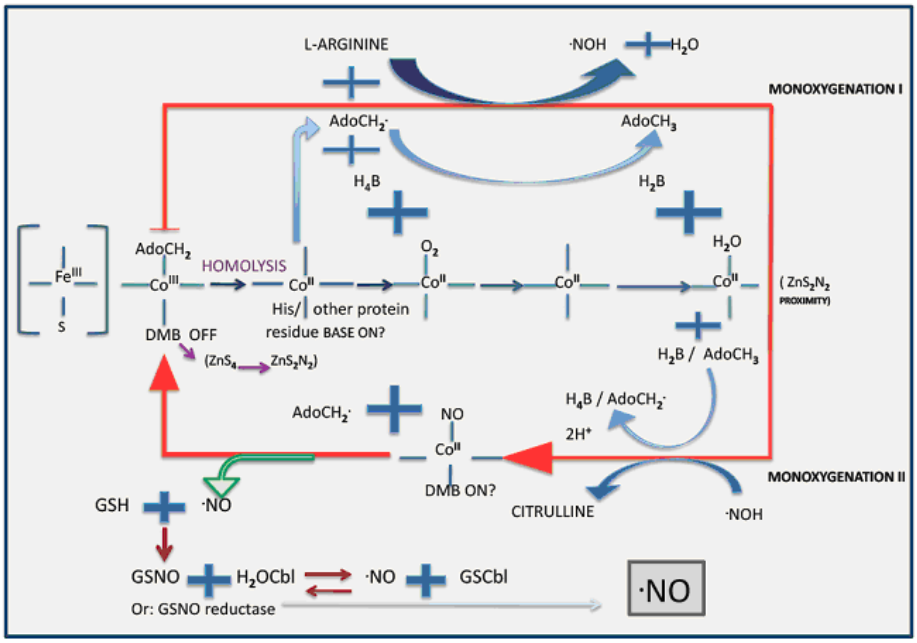
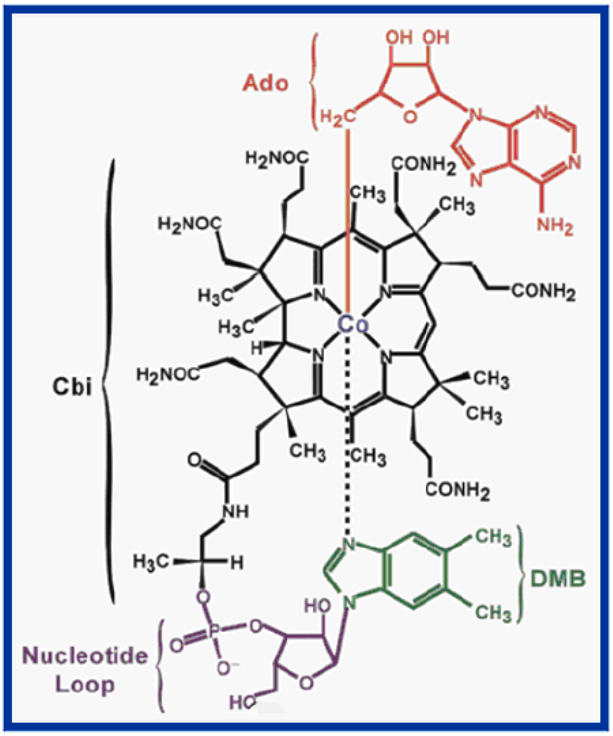


Figure 6: Structure of Adenosylcobalamin, showing upper β, adenosyl, and lower α dimethylbenzimidazole / DMB axial ligands.



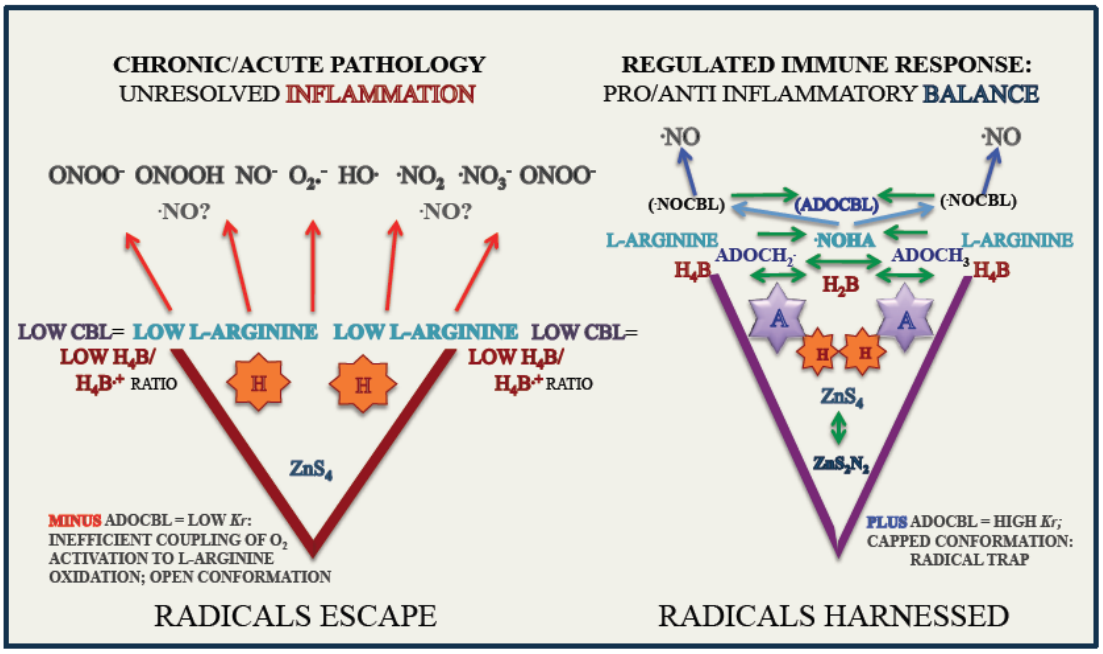
Cobalamin, the ·NO/ONOO⁻ ratio and pathology

Another unsolved mystery remains: what precisely makes NOS produce a regulated supply of ·NO, with predominantly beneficial cell signaling, / growth regulatory, / anti-oxidant effects in health, and yet, malfunction, with host detrimental consequences, in chronic and acute pathologies?

This hypothesis proposes that the critical factor behind this paradox is the ratio of ·NO to ONOO⁻

produced by NOS, high in health, low in pathology, and that a high NO/ONOO$^-$ ratio is determined by the bio-availability of cobalamin, in one of its principal active forms, AdoCbl *(Figure 6)*, as the primary participant in NOS catalysis (*(Figure 5)*. Haem-centred catalysis is the considerably less efficient, back-up reaction in the absence of AdoCbl (*Figure 4*), and thus the source of pathology when it becomes dominant (*Figure 7*). For the absence of AdoCbl from NOS is likely to reflect a general deficiency of Cbl, functional or otherwise, that will also result in decreased availability of H_4B [7], in turn associated to an increase in radical production by NOS [8, 9].

Figure 7: HAEM versus ADOCBL-Mediated NOS Catalysis.



The need for an alternative view of NOS catalysis involving AdoCbl

Apart from the fact that the problem of NOS-related pathology remains unsolved, there are several clues suggesting that the principal *in vivo* NOS reaction may differ from that observed *in vitro* in purified NOS, and that AdoCbl, (not, as previously proposed [10], glutathionylcobalamin/GSCbl), is a reasonable candidate:

1. Structural and kinetic features show NOS differ significantly from cP450s, and other haem-catalysed oxygenases. NOS share some features with AdoCbl-binding enzymes.

2. The NOS oxygenase domain structure is compatible with accommodation of AdoCbl in a position where its cobalt may be reduced in place of the haem.

3. Electron transfer to the L-arginine, bound as *anti*-stereoisomer to the haem, occurs at a distance from the haem. So an intermediary electron carrier is needed. Yet H_4B, a potential electron donor, is also bound at a distance from the haem and L-arginine. AdoCbl's adenosyl radical may bridge the gap.

4. Several studies suggest that *in vitro* the first product of haem-centred NOS catalysis may not be ·NO, but nitroxyl anion (NO$^-$), or other nitrite species. Furthermore, fast reduction of NOS haem Fe[III]-·NO to Fe[II]-NO, prior to release of ·NO, and slow dissociation of the latter, leads, in the presence of oxygen, to generation of Fe[III] plus nitrate, not ·NO (NOS futile cycle *Figure 4*) [11].

   Whereas, in AdoCbl-based NOS catalysis, rapid displacement of ·NO from the analogous ·NO-[CoII]Cbl by the adenosyl radical prevents the NOS futile, nitrate cycle, and excess radical generation (*Figures 5 & 7*).

5. Cobalamin is consistently associated with ·NO's positive effects in a variety of situations. Moreover, expressed *in vivo*, in AdoCbl-rich environments, nNOS yields significantly more protein [12, 13], and, in the L-arginine to citrulline NOS activity assay, is between 4.5 to 10-fold more productive than *in vitro* [12, 13, 14].

6. Unlike HOCbl/GSCbl, AdoCbl does not inhibit the NOS [15]. On the contrary, AdoCbl's characteristic dual role as both a generator of catalytic radicals and as the ultimate radical trap within enzymes [16] results in a dynamic chemistry, in known AdoCbl-dependent enzymatic reactions, which may also overcome the spatial, energetic and thermodynamic challenges of haem-based NOS catalysis.

These points will be illustrated briefly, followed by the detail of plausible AdoCbl-centred NOS catalysis.

NOS are structurally different to cP450s, more akin to AdoCbl-dependent enzymes

NOS and cP450s share a few common features apart from a haem presence: a FAD/FMN reductase C-terminal domain; substrate positioning over the haem; an enzymatic thiolate haem ligand [17]. However, the differences are more significant. Unlike NOS, cP450s can exist in monomeric or dimeric form, and their α helical architecture confers an extraordinary conformational flexibility enabling cP450s to continually remold the active site to fit a wide range of disparate ligands. By comparison, the NOS active site is relatively inflexible, being composed of complex β sheet structures extended in wing formation, with peripheral helices (*Figure 2*). This is distinct also from other haem enzymes: peroxidases, oxidases, catalases are all largely α helical, like cP450 [18]. Further unlike cP450s, NOS have an unusually open structure, "like a baseball catcher's mitt", with each haem bound in the β-sheet palm of the hand (*Figure 2*) [19].

It is unusual, moreover, to find a haem-binding site in a predominantly beta sheet structure [20]. But it is not unusual to find AdoCbl, and, indeed, MeCbl, bound in enzymes with a core 5-stranded β-sheet structure. Compare the topology of the NOS core 5-stranded β sheet haem-binding site (*Figure 8A*) with that of MeCbl in MS, and AdoCbl in MCM (*Figure 8 B & C*).

The NOS haem is also flipped 180^0 from its usual conformation in cP450 [19] facing a large exposed distal cavity (≈ 20 Å across by ≈11 Å deep [19]): *Figure 9*. 11 Å is, coincidentally, comparable to the size of AdoCbl, and its binding site cavity in at least one AdoCbl dependent enzyme, ethanolamine ammonia-lyase [21].

The NOS structure contains a Rossmann fold, a characteristic of nucleotide binding proteins, but, unusually, this has an α/β fold, and lacks the conserved water molecule of classical Rossmann fold containing proteins, such as cP450 [22]. A subdomain of the NOS oxygenase domain is made up of a two-helix bundle/α2-α1, which forms a hydrophobic core (*Figures 2 & 8A*).

AdoCbl characteristically binds within α/β Rossmann folds, in hydrophobic regions, containing α helix bundles, alongside five central β strands, in Class I and III enzymes [23] such as ornithine 4,5-aminomutase [24], glutamate mutase [25], lysine 5,6-aminomutase [26], methylmalonyl CoA mutase (*Figure 8C*) [27]. This is characteristic also of MeCbl in methionine synthase (*Figure 8B*) [28]. Thus the two α-helix, hydrophobic bundles in NOS are a potential location for two AdoCbls' "false" nucleotide dimethylbenzimidazoles (DMBs), which conceivably move into alignment with the 5 core β-strands in each half-dimer, once AdoCbl is bound above the haem.

Moreover, Rossmann fold proteins all have cofactors containing adenosine (adenine plus ribose, e.g. *Figure 10*), and all interact with the adenosine in virtually identical fashion, binding the adenosine ribose hydroxyls through hydrogen-bonding by an acidic amino acid side chain with conserved Asp/ Glu residues [29]. As seen in *Figure 9*, the NOS active site contains conserved Asp/Glu residues (Asn/Glu in eNOS) in an optimal position to H-bond the two hydroxyls of AdoCbl's adenosine ribose, right next to the substrate, L-arginine, itself H-bonded to Asp/Glu.

NOS are capable of accommodating AdoCbl

The NOS homodimer contains 4 cavities: apart from the two haem/L-arginine/H_4B binding pockets (≈4000 $Å^3$ each) a large, solvent-filled, inter-subunit cavity, (≈750 $Å^3$), surrounding a zinc tetrathiolate (ZnS_4) centre, capable of accommodating an as yet unidentified ligand [30, 31], and a smaller cavity at the dimer interface, adjacent to His 436/His 437 residue pair (*Figure 2*).

Figure 8: Structure of NOS and AdoCbl-binding enzymes. A) NOSox domain topology: blue arrows show the 5 β-strand haem-binding site (from Crane et al. *Science*, (1997), 278, 425-431). B. Methionine synthase dimer with 5-β strand(blue) / α-helices (red) TIM barrel: DMB within the α-β barrel, corrin of MeCbl (green ball and stick) above/below. Structure from PDB 1BMT. C. Methylmalonyl CoA mutase with 5 β-strand/α-helix TIM barrel (blue arrow): DMB within /corrin of AdoCbl; (grey ball and stick) below. PDB: 6REQ.

Figure 9: A: Human iNOSox haem binding site, with key residues (magenta/green) for the interface of half-dimers: short dotted arrow indicates the distal area above the haem (grey) where the corrin may stack up for reduction in place of haem: the longer arrow indicates the potential location of Asp382/Glu377 H-bonding of adenosine/release of the adenosyl radical, optimally poised for NOS catalysis between L-arginine (yellow) and H_4B (blue), as Asp382/Glu377 are also H-bonded (white dotted lines) to L-arginine. (Adapted from Alderton et al., *Biochem J*, (2001), 357, 593-615). B: Adenosyl.

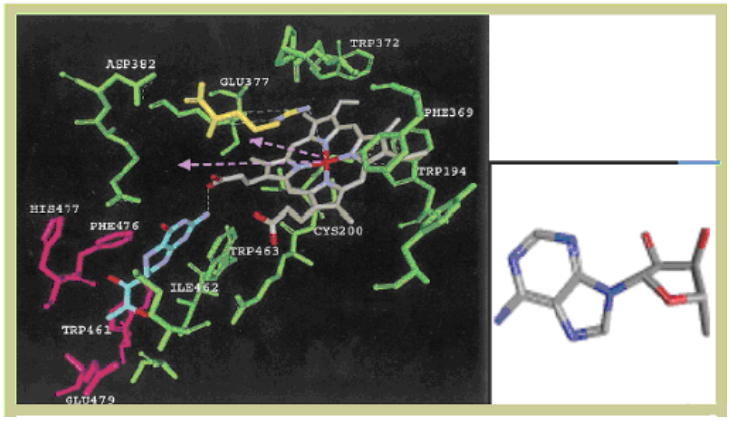
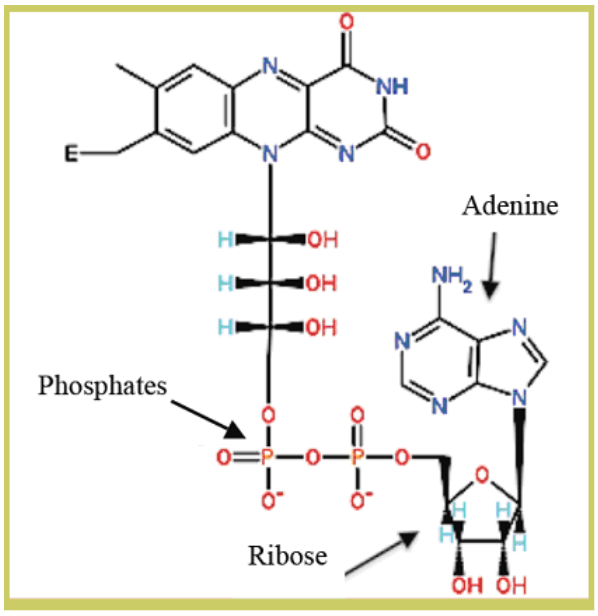


Figure 10: Structure of FAD, the flavin electron carrier from NOS reductase domain. Compare this with AdoCbl's DMB nucleotide and with the analogous adenine ribose portion of AdoCbl's adenosyl β ligand in Figure 7.



In the substrate pocket, Weinberg *et al.* observed that a very large interaction surface, ≈800 Å, is available for Cbl, and their molecular modeling studies, with manual computer-assisted, docking analysis, have now confirmed the prior imaginative observation of the author, who predicted that NOS may indeed be capable of Cbl accommodation in the base-off conformation, that is, with the bond broken between the cobalt and an imidazole nitrogen of cobalamin's lower axial DMB [10, 15] (*Figures 9 & 11)*. By coincidence, this is the conformation in which MeCbl is usually bound within mammalian methionine synthase (MS), AdoCbl in methylmalonyl CoA mutase (MU) [28, 27, 32], and in all other AdoCbl-dependent Class I and II enzymes [23].

Weinberg *et al.* found it possible to achieve at best an 80% overlap of the corrin with the haem (*Figure 11*). Thus the corrin macrocycle will be just above the core NOS 5 β-strand section, comparable to its position in known AdoCbl-binding enzymes (*Figure 8 B/C*). This is an optimal placement if the "ultimate free radical cage and trap" AdoCbl (plus enzyme) is

to act as a cap on the open NOS structure, and prevent free radical escape (*Figure 7* scheme).

Moreover, AdoCbl is not always buried in its dependent enzymes. In some, such as glutamate mutase [25], ornithine aminomutase [24] and bacterial ribonucleotide reductase [33], AdoCbl is initially bound relatively superficially. Compare the position of the NOS haem (*Figure 12*) and AdoCbl's position in *Figures 11* & *13.*

Figure 11: AdoCbl and Haem in iNOS (dark blue)/ nNOS (cyan) haem pockets. with active site residue chains: space-filling model close-up of AdoCbl (purple) "base-off", stacked against haem (red). PDB 1ZVL. (Adapted from Weinberg et al., *Free Radic Biol Med*, (2009), 46, 1626-1632.)

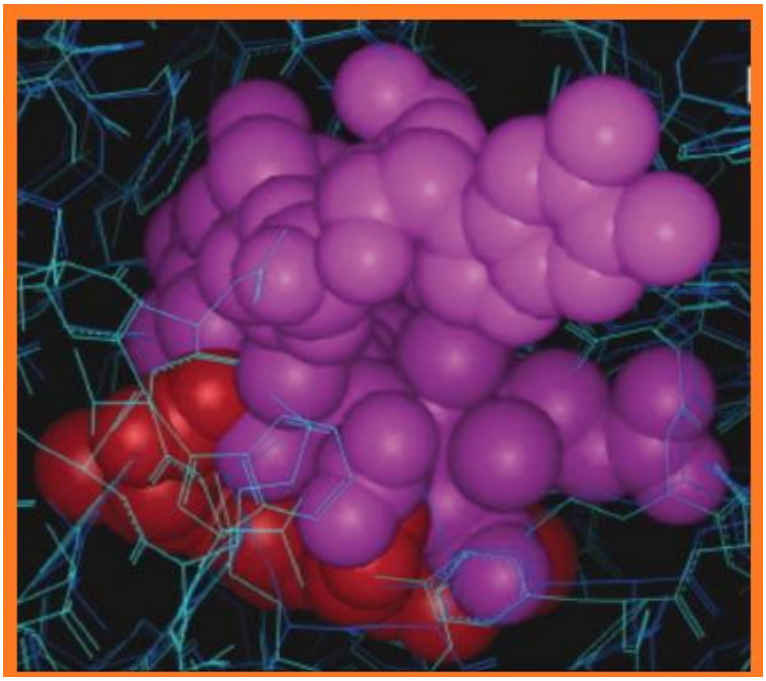


Figure 12: Space-filling model of iNOS dimer with one haem (gold) and inhibitor (blue) visible.

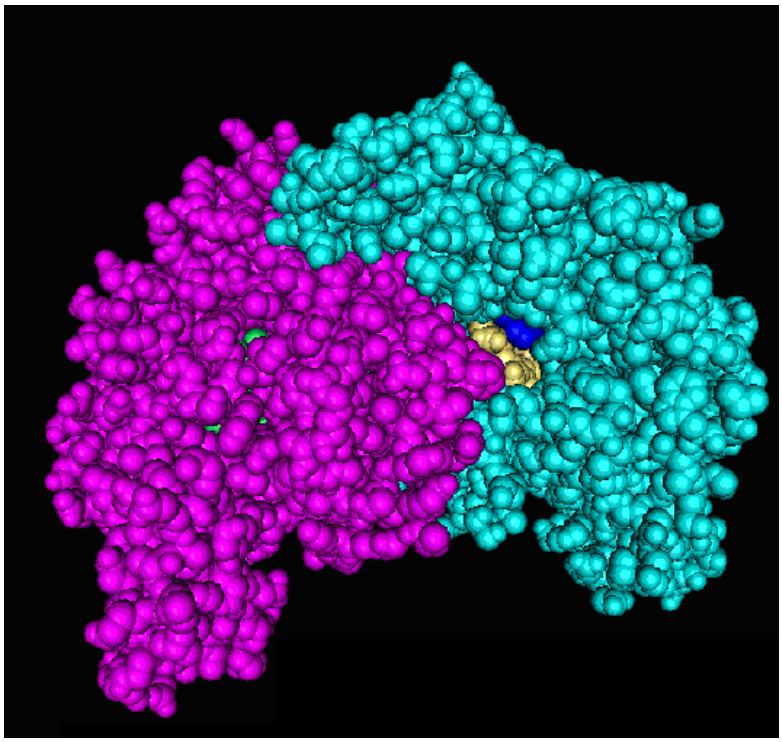
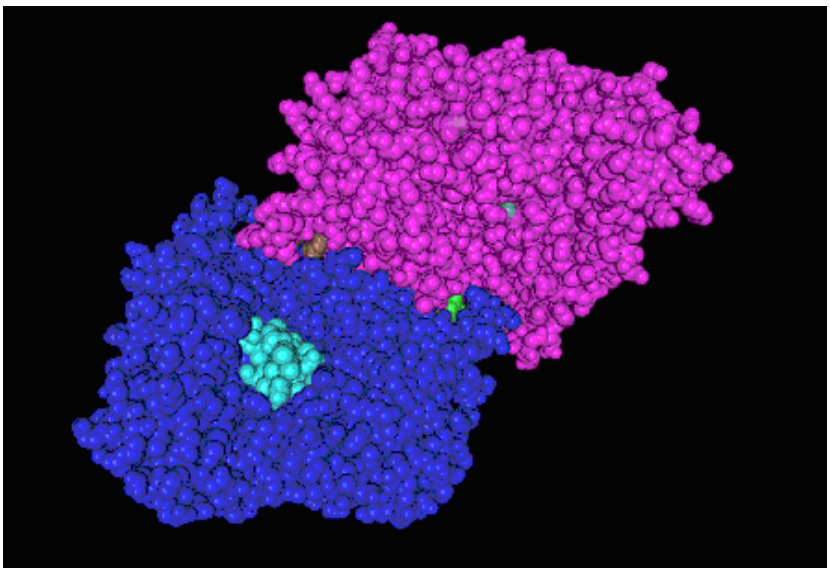


Figure 13: Space-filling model of bacterial ribonucleotide reductase dimer with one AdoCbl cofactor visible (turquoise). PDB 3O0O.



No change in the NOS open structure has been seen with diverse ligands. However, Rossmann folds are capable of some flexibility during catalysis: in AdoCbl-dependent lysine 4,5-aminomutase and ornithine 5,6-aminomutase there are significant active site conformational changes and large-scale domain dynamics, but these are entirely dependent on the presence of both substrate and, specifically, the AdoCbl ligand [26, 24].

In the structure of human eNOS, the region just beyond ZnS_4 (*Figure 14*), -only two residues away from the bilateral serine residues that H-bond directly to the hydroxyl side-chains of the two H_4Bs (≈12Å) [34], comprising amino acids 105 to 125 in eNOS-, shows potential for considerable protein flexibility, and has been dubbed "the flexible arm" [35]. One possibility is that the ZnS_4 cavity may be compressed to allow for further expansion of the haem cavity, or it may be an alternative docking space for two AdoCbl DMBs, that could displace two of the ZnS_4 thiol ligands with two imidazoles nitrogens, in a zinc finger motif. (The high ·NO producing NOS of (AdoCbl-rich) *Streptomyces turgidiscabies* has just such a ZnS_2N_2 [36].) Since, in each half-dimer, the flexible arm is linked directly to the 2-helix bundle, the latter may combine with the core 5 β-strands to form a hydrophobic sheath for DMB to thread through to the Zn^2+. This would effectively close the NOS enzyme, impeding haem access to the substrate (*Figures 14 & 15*), with formation of a TIM barrel, and release of the adenosyl radical in the active site, as in methylmalonyl CoA mutase [32].

Finally, AdoCbl's corrin, being considerably more reduced than the haem porphyrin ring, gives AdoCbl substantially more conformational freedom. This flexibility may also be relevant to how AdoCbl fits in the NOS *in vivo*.

NOS and cP450s: oxygen activation

Compared with cP450s, the NOS have quite a distinct response to oxygen, which participates in the catalytic two-step monoxygenations (*Figures 3 & 4*). According to Raman data, in NOS, haem-bound O_2 seems to point away from the substrate, L-arginine [37]. This is not inconsistent with the primary binding site for O_2 being the potentially better placed cobalt of AdoCbl (*Figure 9*). The possibility that AdoCbl participates in NOS catalysis would also make sense of the unusually slow ferric enzyme reduction (*Kr*) of the NOS, *in AdoCbl's absence*, in comparison not just to cP450, but other flavohaem enzymes: nNOS, 3-4 *Kr*; iNOS 0.9 – 1.5 *Kr*; eNOS 0.1 *Kr*; as opposed to: cP450BM3, 99*Kr* (15ºC); or flavohaemoglobin, 150 *Kr* (37ºC) [11].

This slow *Kr* makes it difficult for haem-based NOS to couple O_2 activation/haem reduction to substrate oxidation [38, 39].

NOS are thus much less tightly coupled than cP450, and consequently prone to generate excess free radicals (*Figure 7*).

Figure 14: NOS dimer flexibility. (Adapted from: Rafikov et al. *J Endocrinol*, (2011), 210, 271-284)

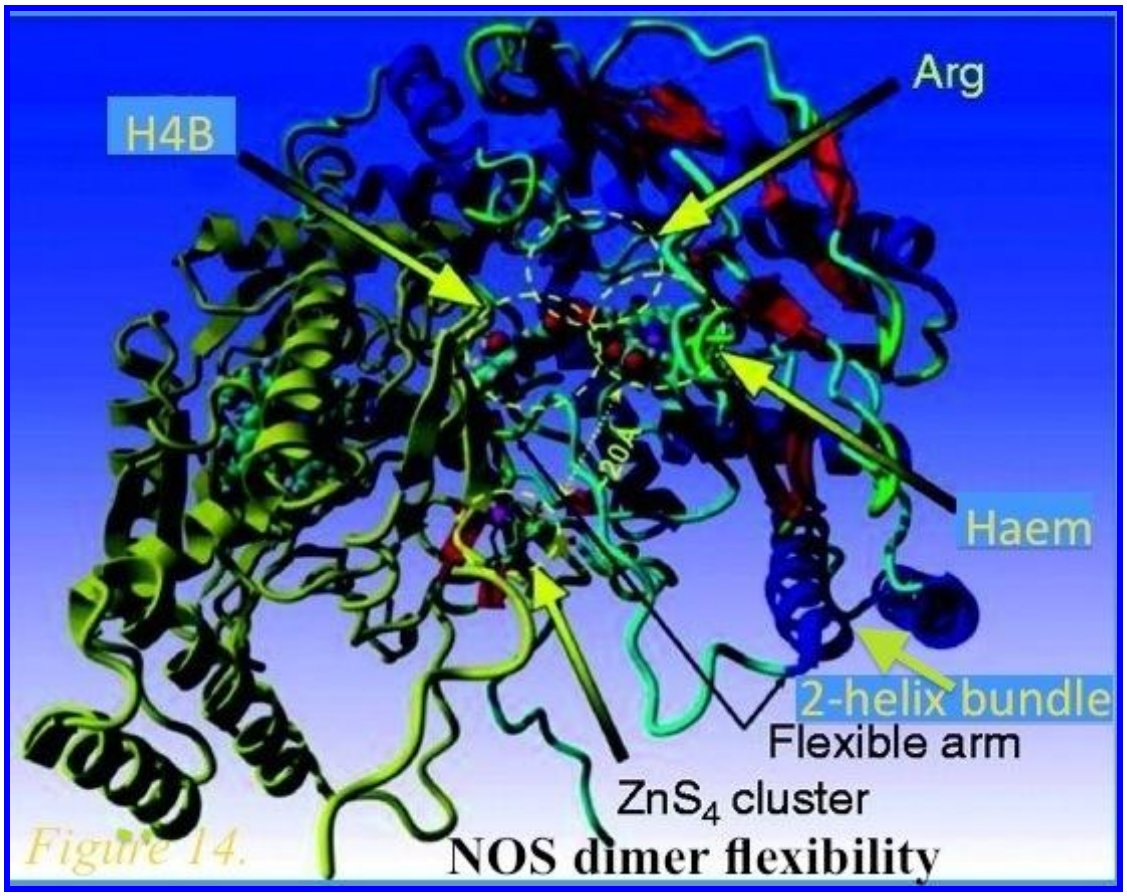
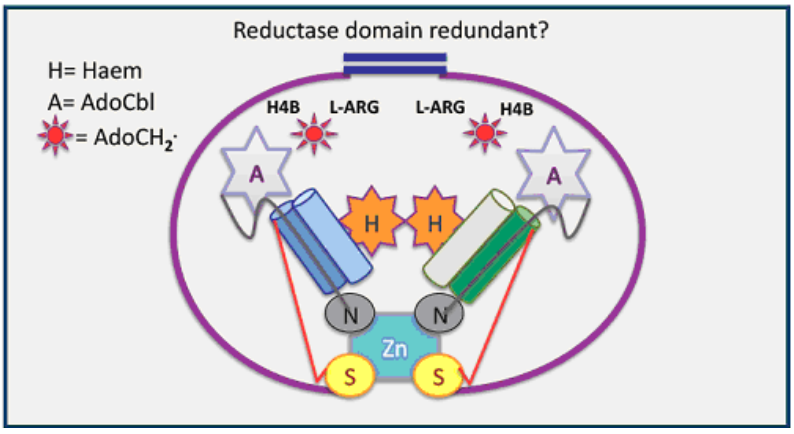


Figure 15: Schematic diagram of NOSox closed conformation with AdoCbl bound. ZnS_4 of open conformation becomes ZnS_2N_2 when 2 AdoCbl DMBs (purple) anchor. The protein flexible arms (red) position 2 α-helix bundles (blue-green) against 5 strand β-sheet core (not shown) to sheath 2 AdoCbl DMBs, and impede haem access to L-arginine substrate.



However, in spite of the slow *Kr*, the haem Fe[III] can be reduced in the absence of substrate L-arginine [40]. Compounding the problem, low H_4B, (a possible consequence of Cbl unavailability [7] increases NOS reduction of O_2 with formation of superoxide and hydrogen peroxide, at the expense of ·NO [31].

In addition to the unusually slow *Kr*, apparent *Km*O_2 values –that is, the concentration of O_2 at which the reaction rate is half of *Vmax*, for the NOS- not only differ between isoforms, but, in the case of nNOS (*350*) and iNOS (*130*), though not eNOS (*4*), are also considerably higher than those of other monoxygenases whose haem-based catalysis NOS are

thought to emulate [11]: cP450 and flavohaemoglobin are more obviously coupled to O_2 (*4-10* at 10º C and *60-90* at 20º C, respectively) [11].

Moreover, in the isolated iNOS dimer, before L-arginine and H_4B are bound, the haem is notably in a low spin state [41], unlike the haem in isolated cP450. This makes sense if the cobalt of AdoCbl is intended to take precedence as the primary target for reduction.

No ·NO from NOS? The NOS "futile cycle"

There is conflict of opinion as to whether ·NO is actually the end product of the NOS. Some have failed to observe ·NO, except in the presence of superoxide dismutase (SOD) [42, 43, 44].Thus it has been proposed that nitroxyl anion (NO^-), small amounts of other species from its decomposition, such as nitrous oxide and hydroxylamine [44, 45], and also $ONOO^-$, from superoxide and ·NO [46, 45], may be the primary NOS products.

Evidence shows the first observed product of NOS catalysis is a ferric haem-·NO complex, not free ·NO [47, 48, 49] (*Figure 4*). Since ·NO is like O_2 a haem diatomic ligand, NOS is subject to feedback inhibition by ·NO [50, 51, 52]. Consequently, the competitive dynamics of ·NO/ O_2 haem binding, which vary between the NOS, and are determined by their varying rate of ferric haem reduction, Fe[III]-·NO dissociation, and Fe[II]- ·NO oxidation, mean that iNOS and nNOS, in particular, partition catalysis into productive and futile cycles, yielding variable ·NO and nitrate/nitrite species [38]. This is a delicate balance, which may be all too easily disturbed by immune/inflammatory challenge and/or low cofactor availability.

Other structural and dynamic chemistry problems in the NOS active site

L-arginine is bound at a distance to haem, (its central N-guanidine 3.8Å away from, and coplanar to haem [2]). X-ray crystal data show that the dense network of hydrogens binding L-arginine to the NOS protein orients L-arginine and its NOHA intermediate rigidly in relation to the haem, with its hydroxylimine oxygen and guanidinium carbon distant from the haem iron by 4.3Å and 4.4Å respectively. This distance is too long for an Fe-O bond H-bonded to the propionate group of the haem pyrrole ring D [53, 54]. Moreover, the substrate restricts O_2 binding [55, 54]. Thus direct ligation to the haem is precluded, and NOHA is bound by NOS as the *anti*-stereoisomer to the supposed catalytic haem (*Figures 2 & 9*) [2].

There is also a distance of >15Å between the flavoprotein domain and the haem to which electrons must transfer [56, 57]. Moreover, the distance between the supposed catalytic haem's FMN module docking site for electron transfer/exchange between FMN and the cofactor, H_4B, is even greater: H_4B being at least 17Å away from the proposed FMN docking site, so that a "through-haem" model for H_4B radical reduction has been proposed [58], the electron equivalent of jumping through hoops!

Therefore a *non-haem* iron has been proposed as the NOS catalyst [59], and it has been argued that H_4B is too far away from the haem to directly participate in the chemistry of the reaction [59, 60] (*Figures 2 & 9*). Something is needed to bridge all these gaps.

5'-deoxyadenosylcobalamin

The enzymatically active form of vitamin B_{12}, AdoCbl/$AdoCH_2$-Co[III], (*Figure 6*) is known as cofactor for only one mammalian enzyme, mitochondrial methylmalonyl CoA mutase (MU), the enzyme which catalyses the isomerisation of methylmalonyl CoA to succinyl CoA [61]. However, in microorganisms, AdoCbl participates in a number of important, enzymatic reactions, including those of ribonucleotide reductase [62], glutamate mutase, 2-methyleneglutarate mutase [63], diol dehydratase, glycerol dehydratase [64], ethanolamine ammonia-lyase [65] and aminomutases [66].

AdoCbl's unique carbon-cobalt bond is potentially labile, and, with the exception of ribonucleotide reductase in *L leichmannii* [67], AdoCbl's mode of catalytic interaction within its dependent enzyme characteristically involves enzyme-induced homolysis of the Co-C bond with formation of a 5'-deoxyadenosyl radical species, $AdoCH_2$· [68].

AdoCbl-dependent enzymatic reactions fall into three classes: Class I involve reversible carbon skeleton re-arrangements; Class II catalyse irreversible heteroatom eliminations; and Class III catalyse re-arrangements with migration of an amino group [69]. All have in common the 1,2-interchange of a hydrogen atom with a substituent group on adjacent carbon atoms of the substrate [68]. If AdoCbl takes part in NOS catalysis, it seems theoretically plausible that it may do so in a manner generally consistent with its characteristic chemistry in known AdoCbl-dependent enzymes.

Cobalamin and ·NO associations

AdoCbl is known to be produced at a rate in excess of the needs of MU [70], is not all protein bound [70, 71, 72, 73], and is found in up to four-fold higher concentrations than the MS coenzyme, MeCbl, in most mammalian cells [70, 72, 73, 74, 75, 76, 77,], (with the exception of fibroblasts where MeCbl predominates [78]). Endothelial cells contain four-fold higher concentrations of AdoCbl (compared to cytosolic MeCbl), in their mitochondria [75] where mitochondrial NOS is found [79]. It has been observed *in vitro* that a significant proportion of exogenously administered cobalamin, after partial conversion to AdoCbl, is found constantly associated with membranes in murine L1210 cells [70]. Endothelial cells also express high levels of (membrane-bound) eNOS [80, 81].

Table 1: Coincidental Actions of Nitric Oxide and Cobalamin

NO AND COBALAMIN (·NO references are in black: Cbl references are in red)

are involved in DNA repair, [99, 100, 101].
protect DNA from single strand break formation/ micronuclei induction, [99, 102, 103, 104, 105, 106, 107, 108, 109].
inhibit mammalian ribonucleotide reductase, [110, 85, 86].
inhibit DNA synthesis, [111, 112, 90, 113, 96, 114].

are both promoted by the transcription factor Sp1 (via TCII upregulation for Cbl), [115, 116].

at low levels activate, at higher levels ultimately inhibit:
IL-1β, [117, 118, 119], **via, COX-2 regulation,** [120, 119], **TNF-α,** [121, 122, 119],
NF-κB, [123, 124, 125, 126, 127, 119], **iNOS,** [128, 7, 119].

decrease total plasma homocysteine and increase erectile function in type 2 diabetic rats, in whom high cobalamin levels correlate with high NOS protein levels and NO activity, [129, 129].

regulate EGF/ EGFr, [130, 131, 132, 122, 133], **VEGF,** [134, 119], **bFGF,** [135, 136].

mediate cell protective effects via ERK1/2 and Akt, [137, 138, 139, 140, 141, 142].

induce heat shock protein 70, [143, 144, 145, 146],
and heat shock protein 32/haem oxygenase-1, [147, 148, 149, 150, 137, 151, 152, 153, 154, 127, 146].
down-regulate Mdr-1, [93, 95].

inhibit lipid peroxidation, [155, 156, 157, 145].
have antioxidant properties, [158, 159, 160, 127, 161].
promote GSH synthesis, [162, 163, 164, 165, 166].
are associated with increased glutathione reductase activity, [167, 168, 169].

regulate platelet aggregation, [170, 171, 172, 173].

reversibly inhibit cP450, [174, 175, 176 (AdoCbl only)].

have potent anti-viral action, [177, 178, 179], **including anti-hepatitis action**, [180, 181, 182, 183, 184, 185, 186].
enhance anti-bacterial action of immune system, [179, 187, 188, 189].
including phagocytosis, [190]; in which, AdoCbl is superior to HOCbl/CNCbl, [191].

Cbl supplementation of vegetarians with low Cbl status significantly increases eNOS NO release in the brachial artery [82].

Exogenous adenosine supplementation (which may increase AdoCbl synthesis) increases synthesis of ·NO [83] Circulating leukocytes, responsible for high iNOS ·NO in immune responses, have the most extraordinarily high cobalamin concentrations: 38-fold higher than in erythrocytes, [84].

In mammals, AdoCbl inhibits ribonucleotide reductase [85, 86], but the mechanism by which it does this is unknown. ·NO also inhibits ribonucleotide reductase [87], and during the cell growth cycle the levels of both cobalamin and ·NO

rise and fall together [88, 89, 90, 91, 92]. Therefore, ·NO induction down-regulates the Multi-Drug exporter-1 gene [93], the cellular cobalamin exporter [94]. Mdr-1 downregulation is also an effect of cobalamin [95]. It is notable too that both high ·NO and AdoCbl cause cell cycle arrest at the *G1-S* phase boundary [90, 91, 92, 96], the p53 cell cycle arrest checkpoint [97]. Whilst accumulation of ·NO leads to high expression of p53, which in turn inhibits iNOS/·NO [98] post *G1-S*-phase, with cobalamin levels falling in tandem [88, 89, 90, 91, 92]. Such associations (*Table 1*) may not be coincidental.

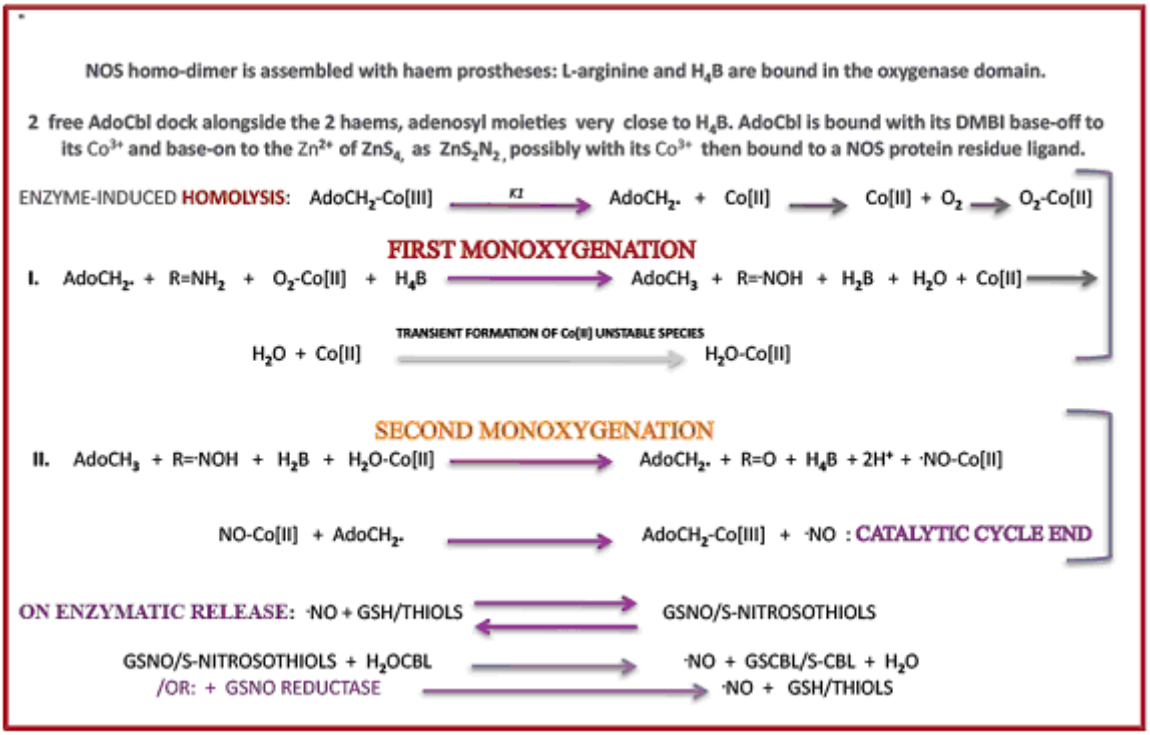
Hypothesis

The accelerated rate of AdoCbl synthesis, with the generally high intracellular AdoCbl/MeCbl ratio, are both specifically dictated by the need to supply AdoCbl, not just for the isomerisation of methylmalonyl CoA to succinyl CoA, but for regulated NOS catalysis and coupled ·NO synthesis. The AdoCbl-NOS-catalysed ·NO then both directly, by activation of DNA methyltransferase 1 [117] and SAM, with corollary ·NO regulatory effects on MS [192] and MeCbl, (the latter also known to act as a methyl donor [193] and, indirectly, via cell-signaling networks, regulates global methylation.

Since there is a SAM/MS cycle, /cystathionine-β-synthase/GSH cycle switch that is determined by levels of homocysteine [194], the MS/CBS cycles have hitherto been viewed as intimately and critically interlinked, along with the MS-dependent folate cycle.

It may be time, however, to pan out further and take a larger overview: the AdoCbl-catalysed NOS form a critical circuit together with MU/the TCA cycle, MS/the SAM cycle, the folate cycle, and the CBS/GSH cycle, all continuously feeding into each other, and ultimately all regulated, like all key eukaryotic, biologic processes, by AdoCbl-catalysed ·NO.

Figure 16: Adenobylcobalamin-mediated NOS catalysis. In mono-oxygenation I, R=L-arginine. In mono-oxygenation II, R=·Nω-L-hydroxyarginine, and then citrulline. Brackets indicate concerted reactions. The catalytic reactions below will, of course, be in duplicate, as they occur in the NOS homodimer.



AdoCbl-based NOS catalysis

When the cofactor, H_4B, is bound within each half of the NOS dimer, two molecules of free, base-off $AdoCH_2$-Co[III] dock in the immediate vicinity, closer to H_4B than the two thiolate-bonded haems in the oxygenase domain, which they effectively block. The porphyrin rings of the haems act as templates against which the the corrins of $AdoCH_2$.Co[III] correctly stack up in the active site. NOS adopt a closed conformation as each $AdoCH_2$.Co[III] DMB simultaneously extends perpendicularly away from the active site, displacing the two upper zinc tetrathiolate cysteine bonds, to form the zinc finger, ZnS_2N_2, which then functions as an ·NO responsive 'on-off' switch for catalysis, as in matrix metalloproteinases [195, 196], (*Figure 15*).

In the presence of the bound substrate, L-arginine, and H_4B, homolytic cleavage of $AdoCH_2$-Co[III]'s carbon-cobalt bond takes place, generating two paramagnetic centres: a 5'deoxy-5'adenosyl radical, $AdoCH_2$· and Co[II]. In a concerted fashion, Co[II] binds O_2. As homolysis of $AdoCH_2$-Co[III]'s Co-C bond in AdoCbl-dependent enzymes does not require an electron transfer, but is enzyme activated [197], it seems unlikely that this will differ in the NOS. The tight fit of $AdoCH_2$.Co[III]'s initially concave/butterfly-shaped, corrin, coupled with the dense necklace of hydrogen bonds around H_4B, which trap the adenosine moiety, as they do in diol dehydratase [198], together with the substrate binding energy, and perhaps some *trans* effect of the bulky, extended DMB base-off, should be sufficient to accelerate the homolytic scission of $AdoCH_2$-Co[III], by a factor of 10^{-10} to 10^{-12}, a "trillion-fold acceleration", typical of the effect on binding of $AdoCH_2$-Co[III] in most of its known dependent enzymes [16, 197, 198, 199, 200, 201]. Thus, at this point in AdoCbl-mediated NOS catalysis, NADPH-derived electron transfer from the NOS reductase domain is redundant.

Molecular Ping-Pong

As depicted in *Figures 5 & 16*, during the first monoxygenation, almost simultaneously with cleavage of the Co-C bond, the high energy, $AdoCH_2$· radical abstracts a hydrogen from the terminal guanidino amino group of L-arginine, which, together with one atom of dioxygen, bound by Co[II] (a thermodynamically privileged binding in 5-coordinate cobalamins by comparison with Co[III] [202]), and H_4B, yield: the intermediate, N^{ω}-hydroxylated-L-arginine, as a substrate radical, ·NOH, $AdoCH_3$, oxidized dihydrobiopterin, the quinoid H_2B, together with H_2O and Co[II]. The latter two products immediately combine as a (conveniently) highly unstable species [203], H_2O-Co[II]. This is the source of the single atom of oxygen required in the next step to convert ·NOH to citrulline and ·NO. The immediate proximity of a highly deprotonating agent, the Zn^{2+} of ZnS_2N_2, to the active site may, indeed, also be intended to ensure rapid deprotonation for this purpose.

In the second monoxygenation, $AdoCH_3$ plus ·NOH, plus H_2B, plus H_2O-Co[II] initially yield: citrulline (R=O) and, (since H_2O-Co[II], or Co[II] alone, though, pertinently, *not AdoCbl*, can combine with ·NO [204]), ·NO-Co[II], plus $AdoCH_2$· and two protons. Given ·NO's very high formation constant/binding affinity for Co[II] (k = 1.0 ± 0.5 x 10^8 M^{-1}) [205], it seems likely that ·NO will, prior to its enzymatic release, react rapidly with Co[II], transiently forming ·NO-Co[II] (k = 7.4 x 10^8 M^{-1} s^{-1}, K_{eq} ≈ 1 x 10^8 M^{-1}, 25ºC) [203, 205], a highly reversible reaction, found to be nearly diffusion controlled [203]. Within the NOS enzyme, this should prevent premature inhibition of NOS by build-up of ·NO. Further, if, for any reason, Co[II] is oxidised during NOS catalysis to H_2O-Co[III] or HO-Co[III], which would also inactivate the enzyme [15]), ·NO will immediately reduce it to Co[II], and thence will bind it with transient formation of ·NO-Co[II] [203]. The possibility of this latter reaction has been established chemically on an electrode surface [205]. Such a catalysis-related ·NO -Co[II] transience *within* NOS might, incidentally, also explain the failure to detect endogenous NOCbl intracellularly/ *in vivo* to date [75].

To conclude: almost simultaneously to the formation of ·NO-Co[II], radical collision with $AdoCH_2$· displaces NO from ·NO-Co[II], thus avoiding the NOS futile cycle, and enabling the geminate reformation of $AdoCH_2$-Co[III]. This brings the catalytic cycle to an end, with the subsequent enzymatic release of ·NO (*Figures 5 & 16)*.

Conclusion

The NOS reductase domain may be intended primarily to sustain the haem-based reserve reaction, and thus redundant (*Figures 4, 5, 15 & 16)*, in the presence of $AdoCH_2$-Co[III], and H_4B. (That is, unless Co[II] actually binds O_2 once more, rather than H_2O, for the second monoxygenation, in which case 2NADPH would be required).

In terms of Cbl-dependent enzymes, there is some analogy between the NOS reductase as back-up for

the haem reaction when cobalamin is in short supply, and the role of the similarly flavin-based methionine synthase reductase, which chaperones MS and periodically reactivates it when the catalytic Co[I] is reduced to Co[II] [206].

However, given the relatively greater importance of NOS even than MS to sustaining mammalian life, Nature takes no risks, which may be why eukaryotic NOS has a reductase domain inbuilt. (Unlike most prokaryotic NOS, with the exception of *Sorangium cellulosum* NOS, which is phylogenetically related to eukaryotic NOS [207]. Prokarytes also do not have a separate dedicated NOS reductase [208]. But then, most prokaryotes either synthesise, or have ample access to, AdoCbl.)

Nature's choice of H_4B as a catalytic cofactor is equally brilliant. For it is versatile enough to function either as a 1-electron donor in the haem-based, back-up reaction, or as a 2-electron donor in the primary $AdoCH_2$-Co[III]-based reaction.

In iNOS, with rising high levels of ·NO, the AdoCbl DMBs will ultimately be permanently displaced from their zinc anchor, as ·NO is known to displace *trans*-imidazole ligands, consistent with the known mechanism for NOS inactivation by ·NO [50, 51, 52]. Moreover, since ·NO directly increases activity of DNAmt-1 [117], it is likely that the iNOS gene will ultimately also be hyper-methylated or silenced [209].

This hypothesis thus offers a fundamental mechanism for the complete *biphasic* regulation – early promotion and later inhibition- of the NOS by increased levels of intracellular Cbl (effected by ·NO responsive/Sp1-transcriptionally promoted, Trans-cobalamin II upregulation [116]) during normal inflammatory responses and immune challenge. Indeed, my colleagues and I recently observed just such a biphasic regulation of the NOS, with promotion of initial levels of iNOS/eNOS protein by high dose cobalamin, during the immune response to endotoxaemia, with corollary regulation of principal inflammatory mediators, IL-1, TNFα, COX 2 and HMGB1, and consequent, beneficial, *in vivo* survival outcome, [119].

If this hypothesis is correct, the problem of designing NOS inhibitors for malfunctioning NOS/ONOO- derived pathology is redundant. The clinical use of high dose parenteral cobalamin should suffice to regulate the NOS.

Testing the hypothesis: existence of a partial proof?

If AdoCbl does take part in NOS catalysis, as a third mammalian cobalamin coenzyme, it should be possible to detect this by studying activity of the whole NOS enzyme both *in vitro* and *in vivo*, comparing NOS activity and ·NO synthesis in cobalamin deprived environments with the subsequent impact of AdoCbl addition.

It is noteworthy that a number of *in vivo* NOS studies have habitually/unwittingly expressed NOS in AdoCbl-rich hosts. Thus, rat nNOS expressed in AdoCbl-rich *E. coli* shows a 4.5 fold greater turnover activity, in the L-arginine to citrulline ·NO synthesis assay, of 450 nmol per minute per mg of NOS protein, as compared to 107 nmol per minute per mg of NOS protein, in purified rat nNOS *in vitro*, [12, 14]. Similarly, rat nNOS from cerebellar extracts, transfected into human kidney 293 cells –with significantly higher cobalamin than brain [210, 211]-, demonstrate a 10-fold increase in NOS activity, and protein, over the cerebellar extracts [13]. My colleagues and I have also seen a significant increase in NOS protein with various cobalamins in endotoxaemic mice [119]. Since administration of cobalamin is known to increase protein levels of MS [212], this may be another instance of the cobalamin cofactor increasing coenzyme assembly [7].

It is also notable that studies of ·NO synthesis by prokaryotic NOS have only shown high ·NO output when done *in vivo*, as in *Streptomyces turgidiscabies* [36] (which contains ample AdoCbl [213, 214]), and in *B. subtilis* expressed in *E.coli* [208]. Since whole *B. subtilis, in vivo* in the human gut, has an extraordinarily high uptake of cobalamin [215], and an even slower Fe[II]-NO dissociation rate *in vitro* than mammalian NOS [216], I would predict that an *in vitro* study of isolated *B. subtilis* NOS would, upon addition of AdoCbl, show both high ·NO synthesis and a high Fe[II]-NO (or rather, ·NO-Co[II]) dissociation rate, when compared to isolated *B. subtilis* controls without AdoCbl. The same should be true of mammalian NOS.

In AdoCbl NOS enzymatic catalysis studies, EPR and UV-visible spectroscopy can be used to detect predicted accumulation of the Co[II] species in the NOS following L-arginine/H_4B//AdoCbl binding and AdoCbl enzymatic homolysis.

In all studies ESR with spin-traps should be used to detect true ·NO, and care should be taken to discriminate between ·NO, and $ONOO^-$/related species, including superoxide products of the NOS, with and without AdoCbl. (The Griess assay is *not* discriminatory.)

In pathologies associated with cobalamin deficiency, functional or otherwise, the ·NO/ $ONOO^-$ ratio should be low, with high superoxide, decreased, or unproductive, NOS ·NO activity, and low levels of NOS protein. (However, due to "relaxed control" of mRNA when methyl groups/cobalamin are in short

supply [217, 218], mRNA may, paradoxically, be high; a likely source of confusion, along with the Griess assay, as to the real status of $^{\cdot}$NO levels in pathology.) Conversely, treatment with high dose, parenteral AdoCbl, (or MeCbl, which will interconvert partially to AdoCbl), should restore efficient NOS activity/ a regulatory high $^{\cdot}$NO/ $ONOO^{-}$ ratio, and resolve inflammation.

This "wild imagining" is offered as a possible new clue to the truth of still mysterious NOS catalysis, in the hope that, as Keats once wrote: "The Imagination may be compared to Adam's Dream: he awoke, and found it Truth" [219].

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Conflict of interest.

The author declares that she has no conflict of interest.

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[Note: Italian and Spanish journal references have been read by the author in the original language published. Russian papers are translated for the author by Mark Pallant-Tripp.]