Large-scale Domain Dynamics and Adenosylcobalamin Reorientation Orchestrate Radical Catalysis in Ornithine 4,5-Aminomutase

Received for publication, September 24, 2009, and in revised form, November 16, 2009 Published, JBC Papers in Press, January 27, 2010, DOI 10.1074/jbc.M109.068908

Kirsten R. Wolthers, Colin Levy, Nigel S. Scrutton, and David Leys

From the Faculty of Life Sciences, University of Manchester, Manchester Interdisciplinary Biocentre, 131 Princess Street, Manchester M1 7DN, United Kingdom

D-Ornithine 4,5-aminomutase (OAM) from Clostridium sticklandii converts D-ornithine to 2,4-diaminopentanoic acid by way of radical propagation from an adenosylcobalamin (AdoCbl) to a pyridoxal 5'-phosphate (PLP) cofactor. We have solved OAM crystal structures in different catalytic states that together demonstrate unusual stability of the AdoCbl Co-C bond and that radical catalysis is coupled to large-scale domain motion. The 2.0-Å substrate-free enzyme crystal structure reveals the Rossmann domain, harboring the intact AdoCbl cofactor, is tilted toward the edge of the PLP binding triose-phosphate isomerase barrel domain. The PLP forms an internal aldimine link to the Rossman domain through Lys629, effectively locking the enzyme in this “open” pre-catalytic conformation. The distance between PLP and 5'-deoxyadenosyl group is 23 Å, and large-scale domain movement is thus required prior to radical catalysis. The OAM crystals contain two Rossmann domains within the asymmetric unit that are unconstrained by the crystal lattice. Surprisingly, the binding of various ligands to OAM crystals (in an oxygen-free environment) leads to transimination in the absence of significant reorientation of the Rossmann domains. In contrast, when performed under aerobic conditions, this leads to extreme disorder in the latter domains correlated with the loss of the 5'-deoxyadenosyl group. Our data indicate turnover and hence formation of the “closed” conformation is occurring within OAM crystals, but that the equilibrium is poised toward the open conformation. We propose that substrate binding induces large-scale domain motion concomitant with a reconfiguration of the 5'-deoxyadenosyl group, triggering radical catalysis in OAM.

Enzymes use conformational motion, from small molecular vibrations to the reorganization of active site residues, and occasionally through to large-scale domain movement, to achieve catalytic prowess (1–3). The coupling of dynamics to catalysis requires precise timing and control, and this is especially true of enzymes that house highly oxidative radical intermediates such as the adenosylcobalamin (AdoCbl)4 (coenzyme B12)-dependent isomerases. Ornithine 4,5-aminomutase (OAM; EC 5.4.3.5) belongs to this group of enzymes. OAM, from Clostridium sticklandii, functions in the oxidative fermentation of L-ornithine by conversion of D-ornithine to 2,4-diaminopentanoate (4). In addition to AdoCbl, the enzyme contains pyridoxal L-phosphate (PLP), which forms an internal aldimine link to Lys629 in the resting state of the enzyme (5). The incoming substrate induces transimination, whereby the migrating amine of the substrate forms an external aldimine link to PLP. Homolysis of the AdoCbl Co-C bond is triggered by formation of the external aldimine generating cob(II)alamin and the highly reactive carbon-centered 5'-deoxyadenosyl radical (Ado'). H' abstraction by Ado' from the PLP-substrate complex produces a substrate radical that isomerizes, possibly through a cyclic (azacyclopropylcarbinyl radical) intermediate (6) (Fig. 1). Re-abstraction of H' from 5'-deoxyadenosine (AdoH) by the product-like radical intermediate regenerates Ado', which in turn recombines with cob(II)alamin. The internal aldimine is then re-established commensurate with product release. In this new biological capacity, PLP is thought to promote catalysis by 1) introducing unsaturation into the migrating amine and 2) stabilizing of high-energy radical intermediates through electron withdrawal by the pyridine ring (7).

OAM is a α2β2 heterodimer comprising two strongly associating subunits, OraS (12.8 kDa) and OraE (82.9 kDa) (5), with the latter subunit showing sequence similarity to 5,6-lysine aminomutase, a second PLP- and AdoCbl-dependent aminomutase. Electron paramagnetic resonance (EPR) spectra of OAM and 5,6-LAM do not show the formation of radical intermediates during steady-state turnover with natural substrates. This is in marked contrast to related enzymes such as glutamate mutase (GM) (8), methylnalonyl-CoA mutase (MCM) (9), and ethanolamine ammonia lyase (10), which show the accumulation of cob(II)alamin during turnover. In OAM, the

Enzymes use conformational motion, from small molecular vibrations to the reorganization of active site residues, and occasionally through to large-scale domain movement, to achieve catalytic prowess (1–3). The coupling of dynamics to catalysis requires precise timing and control, and this is especially true of enzymes that house highly oxidative radical intermediates such as the adenosylcobalamin (AdoCbl)4 (coenzyme B12)-dependent isomerases. Ornithine 4,5-aminomutase (OAM; EC 5.4.3.5) belongs to this group of enzymes. OAM, from Clostridium sticklandii, functions in the oxidative fermentation of L-ornithine by conversion of D-ornithine to 2,4-diaminopentanoate (4). In addition to AdoCbl, the enzyme contains pyridoxal L-phosphate (PLP), which forms an internal aldimine link to Lys629 in the resting state of the enzyme (5). The incoming substrate induces transimination, whereby the migrating amine of the substrate forms an external aldimine link to PLP. Homolysis of the AdoCbl Co-C bond is triggered by formation of the external aldimine generating cob(II)alamin and the highly reactive carbon-centered 5'-deoxyadenosyl radical (Ado'). H' abstraction by Ado' from the PLP-substrate complex produces a substrate radical that isomerizes, possibly through a cyclic (azacyclopropylcarbinyl radical) intermediate (6) (Fig. 1). Re-abstraction of H' from 5'-deoxyadenosine (AdoH) by the product-like radical intermediate regenerates Ado', which in turn recombines with cob(II)alamin. The internal aldimine is then re-established commensurate with product release. In this new biological capacity, PLP is thought to promote catalysis by 1) introducing unsaturation into the migrating amine and 2) stabilizing of high-energy radical intermediates through electron withdrawal by the pyridine ring (7).

OAM is a α2β2 heterodimer comprising two strongly associating subunits, OraS (12.8 kDa) and OraE (82.9 kDa) (5), with the latter subunit showing sequence similarity to 5,6-lysine aminomutase, a second PLP- and AdoCbl-dependent aminomutase. Electron paramagnetic resonance (EPR) spectra of OAM and 5,6-LAM do not show the formation of radical intermediates during steady-state turnover with natural substrates. This is in marked contrast to related enzymes such as glutamate mutase (GM) (8), methylnalonyl-CoA mutase (MCM) (9), and ethanolamine ammonia lyase (10), which show the accumulation of cob(II)alamin during turnover. In OAM, the...
metallo-paramagnetic species is only observed with the addition of inhibitor, 2,4-diaminobutyrate (DAB) to the enzyme. DAB binds to PLP and radical abstraction by Ado' leads to formation of an overstabilized PLP-bound radical intermediate. This in turn leads to the stable formation of the cob(II)alamin in the active site (11). The EPR spectrum of the OAM-inhibitor complex reveals
strong electronic coupling between cob(II)alamin and the organic inhibitor-based radical indicating a distance of less than 6 Å between the two paramagnetic species, similar to that reported for GM (12) and MCM (13).

The crystal structures of GM (14), MCM (15), and diol dehydratase (DD) (16) show AdoCbl, housed in a Rossmann-like domain, positioned directly above the pore of a triose-phosphate isomerase (TIM)-barrel, a conformation that positions the 5′-deoxyadenosyl (Ado) group of AdoCbl close to the site of substrate binding. However, the crystal structure of 5,6-LAM, shows the Rossmann domain tilted toward the edge of the TIM barrel (17), effectively expelling AdoCbl from the active site and introducing a distance of ~25 Å between the Ado group and PLP (17). It is envisioned that substrate-binding triggers release of the Rossmann domain from its “locked” position by breaking the internal aldime bond, thereby allowing the domain to reposition over the active site for productive hydrogen transfer and radical propagation between the PLP-bound substrate and AdoCbl.

Here, we report the crystal structures of OAM in the resting state (substrate-free) and complexed with substrate (D-ornithine) and inhibitor (DAB). In the pre-catalytic form, OAM adopts an “open” conformation (similar to that of 5,6-LAM) with the Rossmann domain, harboring an intact AdoCbl cofactor, tilted toward the edge of the TIM barrel. We show that addition of substrate or inhibitor to protein crystals triggers movement of Rossmann domains that are unconstrained by lattice contacts, with the equilibrium between the open and “closed” states favoring the former conformation. Our studies point to a coupling of domain dynamics with reaction chemistry in OAM.

**EXPERIMENTAL PROCEDURES**

**Enzyme Preparation, Crystallization, and Soaking—Ornithine 4,5-aminomutase from C. sticklandii** was prepared from an overexpressing strain of *Escherichia coli* as previously described (11). A protein solution (8 mg/ml of 4,5-OAM, 5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 8.0, 2 mM PLP, and 2 mM AdoCbl) was mixed in a 1:1 ratio with precipitant solution (0.1 M Tris-HCl, pH 8.0, 0.2 M MgCl₂, 25% (w/v) polyethylene glycol 2000 monomethylether) under red light at room temperature. Crystals were grown by vapor diffusion at 20 °C in the dark. Several crystals were soaked for several minutes under ambient light in mother liquor with 50 mM D-ornithine or DL-2,4-diaminobutyrate in aerobic and anaerobic conditions before being flash frozen in liquid nitrogen (using mother liquor supplemented with 8% polyethylene glycol 200 as cryoprotectant). For the latter conditions, crystal trays were introduced into a Belle Technology glove box (O₂ < 5 ppm) 5 h before soaking the crystals with an anaerobic solution of substrate/ inhibitor. In this case, crystals were flash-cooled under anaerobic conditions prior to data collection.

**Data Collection, Model Building, and Refinement—** Data sets were collected from single cryofrozen crystals at ESRF (Grenoble) or Diamond (UK) synchrotron beamlines. Data were processed and scaled using iMOSFLM (18) and SCALA (19). The structure of the substrate-free form of OAM was solved using molecular replacement with the program PHASER (20) using the crystal structure of 5,6-LAM as a search model (17) (PDB code 1XRS). Following NCS averaging using DMmulti (21) and automatic rebuilding using ARP/wARP (22) further positional and B-factor refinement was performed with REFMAC5 (23). Alternate rounds of manual rebuilding was performed in COOT (24). The solvent model was created with ARP/warp (22). The structure of the resting state enzyme was used a starting model for refinement of the substrate/inhibitor-soaked structures.

All crystals belong to the P2₁ space group, and contain two α₁β₂ heterodimers that are replete with AdoCbl and PLP (supplemental Table S1). The refined OAM structure contains residues 4–114 of Orα (α) and residues 7–739 of Orβ (β). No electron density could be observed for residues 272–273, 416–417, and 506–509 of Orα. The OAM structure does not contain residues Gly²²⁰ to Asp²²² of Orα present in the previously published sequence (5). We do not find any evidence for these residues from *C. sticklandii* genomic DNA sequencing or by alignment between the OrαE protein sequences from *C. sticklandii* and *Clostridium difficile* (accession number ZP_05349629; 79% sequence identity), which also shows that the corresponding OrαE coding sequence from the latter organism also does not contain the three amino acid GID insert (a similar conclusion can be reached by alignment with various *Thermoanaerobacter* sp). The average B-factors (for all main chain atoms) in the substrate-free structure for the individual domains (averaged across the 4 OAM molecules present in the asymmetric units (AU)) are 19.8 Å² for the TIM barrel (residues 75–373 of OrαE), 28.8 Å² for the dimerization domain (residues 509–587 of OrαE), 27.8 Å² for the accessory clamp (residues 373–506 of OrαE and all residues of Orβ), and 36.0 Å² for the Rossmann domain (residues 591–740 of OrαE). The average B-factors for the cofactors are 21.8 Å² for PLP, 39.0 Å² for AdoH, and 27.4 Å² for cobalamin. Additional B-factors of the individual Rossmann domains and cofactors within the AU are listed in Table 1. Coordinates and associated structure factors have been deposited with the Protein Data Bank (PDB) with accession codes: 3KP1 (resting state OAM), 3KOW (back soaked OAM), 3KOZ (anaerobic complex with ornithine), 3KP0 (aerobic complex with 2,4-diaminobutyrate), and 3KOY (anaerobic complex with ornithine).

**Modeling of the Closed State—** A qualitative model for the OAM closed state was created by rigid body positioning of the TIM barrel domain and the AdoCbl binding Rossmann domain on corresponding domains of the evolutionary related glutamate mutase (PDB code 119C), using the secondary structure matching algorithm within Coot (24). The Rossmann domains could be superimposed with a root mean square deviation of 1.7 Å for 121 Ca atoms (28% sequence identity), whereas TIM barrel domains could be superimposed with a root mean square deviation of 2.2 Å for 294 Ca atoms (15% sequence identity). This results in a dramatic reorientation of the OAM Rossmann domain with respect to the corresponding TIM barrel. Nevertheless, the model is compatible with the OAM crystal lattice for both unconstrained Rossmann domains. This model of the OAM closed state has no bad contacts, except for severe VDW clashes that occur between the OAM PLP-bound substrate and the AdoCbl adenosine moiety. Reorientation of the latter from the conformation observed in the OAM open state to an anti...
and western conformation (as observed in the template structure glutamate mutase), places the C5 of AdoH within van der Waals distance of the PLP-bound substrate and removes obvious “bad” contacts. The closest contact is made with the C4 of the substrate from which H-abstraction is projected to take place. This observation serves to validate the model obtained, as it agrees with the EPR spectrum of the OAM-DAB complex (11), which points to a $\rho$ distance between the radical pair.

**RESULTS AND DISCUSSION**

The “Pre-catalytic” Conformation of OAM—The 2.0-Å resolution crystal structure of substrate-free OAM reveals two $\alpha_\beta\beta_2$ heterodimers within the AU. The large $\beta$ subunit (encoded by OraE) comprises a TIM barrel, the dimerization domain, and a Rossmann-like domain (Fig. 2A). The smaller $\alpha$ subunit (encoded by OraS) comprises an extended $\alpha$-helix followed by a four-helical knot, and forms part of the accessory clamp. PLP and AdoCbl, added exogenously to the protein prior to crystallization, are bound, respectively, to each of the four TIM barrels and Rossmann domains present within the AU. The $\alpha_\beta\beta_2$ heterodimer undergoes a domain swap, whereby the Rossmann domain of one $\beta$ subunit interacts with the TIM barrel of the adjacent $\beta$ subunit. A 15-residue long flexible linker connects the Rossmann domain and TIM barrel within a single polypeptide.

It is immediately evident from the overall structure that catalysis requires a significant re-orientation of the Rossmann domain. As shown in Fig. 2a, the Rossmann domain is pivoted away from the core of the TIM barrel in a configuration that projects the 5'-deoxyadenosyl moiety of AdoCbl toward the solvent and $\sim$23 Å away from the PLP. This “edge on” orientation of the Rossmann domain largely resembles the previously determined structure of 5,6-LAM (17). A glycine-rich loop, interrupting the second $\alpha$-helix of the Rossmann domain, contains the conserved Lys629. This residue anchors the Rossmann domain to the TIM barrel of the opposite monomer by forming an imine link to the PLP that is tightly bound to the TIM barrel surface. The side chains of Arg192 and Asn226 of the TIM barrel also bridge the two domains by forming salt bridges with both the PLP and residues Asp627 and Glu624 of the Rossmann domain. The total surface contact between the Rossmann domain of one monomer and the TIM barrel of the second monomer is $\sim$550 Å (1.6% of the total surface area) with a surface complementarity of 0.61 (25). In this open pre-catalytic conformation, several residues are involved in hydrogen bonding and electrostatic contacts between both domains: His119, Tyr191, Arg192, Asn226, Arg232, and Arg426 located on surface loops of the TIM barrel and Glu624, Asp627, and Glu634 located in the second $\alpha$-helix of the Rossmann domain. The PLP-binding determinants are largely conserved between 5,6-LAM and OAM, with the exception of
an additional residue (His-225) in the latter protein, which forms a hydrogen bond to the PLP phenolic group (supplemental Fig. S1).

Within the AU, the positions of only two of the four Rossmann domains are restrained by crystal packing contacts (subunits B and C), whereas the remaining two are located within relatively large solvent channels (supplemental Fig. S2). The presence of intact cofactors, and the unconstrained environment for two of the four AdoCbl-binding domains, suggests that OAM should be able to undergo domain motion upon substrate binding and thus adopt a closed conformation that would support catalysis within the crystals.

The OAM-AdoCbl Complex Is Unusually Stable—AdoCbl binds to OAM in the “base off” “His-on” mode whereby the imidazole side chain from His618 replaces the dimethylbenimidazole base as the lower axial ligand coordinated to the cobalt. The dimethylbenimidazole base extends into the hydrophobic cavity of the Rossmann domain, tightly anchoring the cofactor to the protein. This binding mode of AdoCbl was first observed in methionine synthase (26), and has been documented in GM (27), MCM (28), and 5,6-LAM.

The structures of AdoCbl-dependent enzymes, to date, indicate that photolytic cleavage of relatively weak AdoCbl Co-C occurs in the crystal, as there is a >3 Å separation between the C5 atom of Ado and the cobalt atom (17, 27, 28) in the solved structures. Interesting, the usually labile Co-C bond is intact in the OAM structure. The clearly defined omit density map (Fig. 2, bottom) shows that the distance determined between cobalt and the C5 atom of the Ado group is 2.0 Å, and this is the case for all four cofactors of the asymmetric units. This is a surprising result considering that crystal mounting, ligand soaks, and subsequent freezing in liquid N2 all took place under ambient light. In addition, no efforts were made to minimize x-ray exposure of the crystals during synchrotron data collection. To date, this is the first instance of a Co-C AdoCbl bond in a protein structure.

It is difficult to speculate at this time as to the origins of the increased Co-C bond stability in OAM, but it may be linked to the local environment of the cofactor. The deoxyadenosyl group is largely solvent exposed and in the syn conformation about the glycosidic bond. The 2'-OH and 3'-OH of the ribose and the N3 atom of the adenine ring all form hydrogen bonds with water molecules that in turn form polar contacts with the protein. The single direct protein contact is made between the N1 atom of the adenine ring and backbone carbonyl of Leu489. The adenine ring is also in van der Waals contact with C7 of the acetamide side chain extending from the B ring of the corrin macrocycle.

An overlay of the OAM-bound AdoCbl with the crystal structure of the free cofactor (29)(Fig. 2, bottom) reveals that the position and conformation of the deoxyadenosyl moiety is distinct between the two structures. In OAM, the Ado group is in the syn conformation and lies above the B ring of the corrin macrocycle (i.e., “eastern” position; N24-Co-A15-A14 torsion of −90°). Crystal structures of the free AdoCbl, on the other hand, show the Ado moiety in the anti conformation with the adenine ring over the C ring (i.e., “southern” position; N24-Co-A15-A14 torsion of −0°) (29, 30). Although this preferred solid-state conformer, a two-dimensional NMR study reveals that the free cofactor fluctuates between either form (31). Molecular dynamics simulations indicate that both the southern and eastern conformations are associated with local energy minima (32); thus, it not is immediately evident if the position of the Ado group with respect to the corrin ring influences the stability of the Co-C bond. Nevertheless, this serendipitous finding together with the unrestrained nature of certain Rossmann domains in the lattice should allow for catalysis to occur within the crystals.

Crystal Structure of Ligand-OAM Complexes under Anaerobic Conditions—Fig. 3 illustrates the proposed catalytic cycle of OAM involving large-scale domain motion. In this model, the substrate-free enzyme is locked in an open conformation, enforced by the presence of the internal aldimine link between PLP and Lys629 of the Rossmann domain. The incoming substrate breaks this covalent link and effectively “frees” the Rossmann domain to re-orientate over the TIM barrel into the proposed closed conformation. This conformation accommodates
all the ensuing chemical steps: homolysis of AdoCbl, H-atom abstraction by Ado, and radical-mediated isomerization (Fig. 1, steps 2–7). Reorientation of the Rossmann domain away from the core of the TIM barrel leads to opening of the active site, allowing release of product concomitant with reformation of the internal aldimine and the open conformation.

Under anaerobic conditions, the binding of D-ornithine and DAB to OAM in solution leads to product turnover for the substrate and formation of an overstabilized radical intermediate for the inhibitor. The addition of either D-orn or DAB to OAM crystals in an anaerobic environment leads to crystal structures wherein the ligand is bound via a Schiff base to all four PLP cofactors in the AU, with concomitant release of the Lys side chain. In the D-ornithine bound structure (Fig. 4), the δ-amine of the substrate is bound via an imine link to the PLP cofactor and Lys has moved away from the PLP C4 atom. The substrate is located in the TIM barrel cavity, in a direction opposite to that of the Lys side chain in the substrate-free structure. The D-ornithine α-carboxylic moiety forms hydrogen bonds and salt bridges with the side chains of Glu, Arg, and His, whereas the α-amine interacts electrostatically with Glu. The substrate analog (DAB) makes similar contacts with the protein, except His replaces His as the hydrogen-bond donor to the α-carboxylate. The PLP cofactor appears more mobile in the substrate/inhibitor bound forms compared with the internal aldimine state, with less clearly defined electron density, particularly for the C5 atom, which bridges the phosphate moiety with the pyridine ring (Fig. 4).

The extensive contacts made between side chains and the substrate within the core of the TIM barrel could potentially disrupt surface contacts (principally between Asp and Glu) made between the Rossmann domain and TIM barrel in the open conformation. Furthermore, the binding of ligands releases the restraints imposed by the PLP-Lys linkage on the position of the Rossmann domain. Despite this, both the unconstrained Rossmann domains clearly remain in the open conformation, and in all four Rossmann domains the Ado group remains coordinated to the cobalt atom.

Ligand binding does cause the mobility of the Rossmann domains to increase. This is apparent from an enlargement in the ΔB-factor values (defined as the difference in B-factor between Rossmann domains and the associated TIM barrel value) for the unconstrained domains compared with the restrained domains (Table 1). This observation suggests either that the closed catalytically relevant conformation cannot be adopted within the crystal lattice, or that the equilibrium between open and closed forms remains poised toward the open state in the crystalline state.

Crystal Structures of Aerobic OAM-Ligand Complexes—To investigate whether OAM can indeed reach the closed conformation within the confines of the crystal lattice, we made use of the fact that OAM catalysis is sensitive to molecular oxygen. In solution, the addition of DAB or D-ornithine in an aerobic environment leads to rapid and irreversible formation of hydroxy-cobalamin, whereby the cob(II)alamin generated by homolysis reacts with O to form the hydroxy derivative of the cofactor. Ado, formed in the reaction cycle, is unable to recombine with the cofactor and likely dissociates from the enzyme. Given the remarkable stability of the AdoCbl Co-C bond in OAM crystals, the loss of Ado following aerobic substrate/ligand

---

**TABLE 1**

ΔB factor values of individual Rossmann domains and cofactors within the OAM AU

| Structure       | Monomer | Rossmann domain ΔB | ΔB – ΔB_{substrate-free} |
|-----------------|---------|--------------------|--------------------------|
| Substrate-free   | A       | 22.9               | 0                        |
|                 | B       | 16.9               | 0                        |
|                 | C       | 6.8                | 0                        |
|                 | D       | 16.4               | 0                        |
| Anaerobic D-orn | A       | 42.5               | 19.6                     |
|                 | B       | 18.2               | 1.3                      |
|                 | C       | 11.9               | 5.1                      |
|                 | D       | 46.7               | 20.3                     |
| Anaerobic DAB   | A       | 31.9               | 9.0                      |
|                 | B       | 10.4               | –6.5                     |
|                 | C       | 11.4               | 4.6                      |
|                 | D       | 18.9               | 2.5                      |
| Aerobic D-orn   | A       | 50.0               | 27.1                     |
|                 | B       | 25.8               | 8.9                      |
|                 | C       | 9.6                | 2.8                      |
|                 | D       | 39.6               | 23.2                     |
| Aerobic DAB     | A       | ND                 | ND                       |
|                 | B       | 14.8               | –2.1                     |
|                 | C       | 9.3                | 2.5                      |
|                 | D       | ND                 | ND                       |
| Back soak       | A       | 43.1               | 16.7                     |
|                 | B       | 6.5                | –0.3                     |
|                 | C       | 14.7               | –2.2                     |
|                 | D       | 48.0               | 25.1                     |

* ND, not determined.
soaks serves as a marker for radical pair formation. In solution, the rate of DAB-catalyzed hydroxycobalamin formation is significantly faster than that with d-ornithine, which is likely due to overstabilization of the biradical state (i.e. the prolonged presence of cob(II)alamin active site provides more opportunity for it to react with molecular oxygen). As OAM shows an increased rate of non-productive catalysis (formation of hydroxycobalamin), with DAB, OAM crystals were soaked with the inhibitor under aerobic conditions in an effort to demonstrate the transition from open to closed conformation is indeed possible within the crystals. Crystal structures of aerobically DAB-soaked OAM reveal a complete lack of electron density for both unrestrained Rossmann domains, whereas the two domains involved in lattice contacts remain locked in the resting-state conformation (Fig. 5A). The absence of both Rossmann domains demonstrates that extreme disorder occurs following aerobic DAB soaks, which suggests the conversion of AdoCbl to OHChbl during radical formation in the presence of oxygen (and subsequent loss of AdoH from the enzyme) is responsible for the observed disorder. That loss of the AdoH leads to extreme disorder can be rationalized by the fact that the absence of this moiety reduces the size of the Rossmann domain-TIM barrel domain interface observed in the open conformation by ~20% (from ~550 to 450 Å²). Nevertheless, the presence of OHChbl and loss of AdoH (which would strongly suggest catalysis occurs within the OAM crystals) cannot be unequivocally demonstrated due to the extreme disorder observed.

Evidence for OAM Catalytic Turnover in the Crystalline State—To determine whether the mobile Rossmann domains are indeed able to adopt a closed conformation, and participate in radical transfer that would necessitate the formation of OHChbl (though a cob(II)alamin intermediate) and loss of AdoH, the OAM crystals were incubated with DAB (molar excess in mother liquor for 2 min) and “back soaked” to remove excess ligand to allow the internal PLP-Lys⁶²⁹ bond to reform. The crystal structure corresponding to ligand-free crystals that had been exposed to ligand under aerobic conditions indeed reveals all Rossmann domains occupying the open configuration. No electron density for any residual ligand could be observed, and the PLP has clearly reformed the imine linkage with Lys⁶²⁹. Significantly, the total lack of electron density for the AdoH group in one of the mobile Rossmann domains (and weak electron density for the Ado group in the other) indeed demonstrates AdoCbl converts to OHChbl under these conditions (Fig. 5B). Thus, we conclude OAM is able to adopt the closed conformation and initiate Co-C bond breakage within the OAM crystals. It is important to note that the loss of AdoH in both mobile Rossmann domains is not due to photolytic events or the thermal energy from the x-ray beam, which are known to labilize the Co-C bond (17) as both of the lattice-constrained Rossmann domains still contain intact AdoCbl. Similar to what is observed for the ligand-bound anaerobic OAM structures, the average ΔB-factors for the two unconstrained Rossmann domains remain high, indicating an increase in mobility when compared with the resting state enzyme structure (Table 1). We propose this increase in mobility is due to (partial) loss of the deoxyadenosyl group thus perturbing the Rossmann domain-TIM barrel interface in the open conformation. Clearly, the effects of breakage of the PLP-Lys⁶²⁹ bond and removal of the AdoH group on domain mobility are additive, leading to complete disorder of the Rossmann domains as observed for the DAB-soaked aerobic structure.

Modeling the OAM Closed Conformation—Due to the unfavorable position of the open to closed equilibrium, we were unable to capture OAM in the closed conformation in the crystalline form. To provide further insights into the proposed closed conformation and to establish whether this position could indeed be occupied within the OAM lattice constraints, we created a model based on the crystal structure of GM (PDB code 119C) (27). The latter enzyme contains a similar AdoCbl-binding Rossmann domain directly positioned over the substrate-binding TIM barrel (Fig. 6). Using the structural homology between the respective Rossmann and TIM barrel domains, we superposed the OAM domains onto the GM structure, leading to a model for the closed OAM state. Evidence for this closed conformation is supported by the EPR spectrum of the OAM-DAB complex (11), which points to a <6-Å distance between the radical pair. The OAM closed model indeed places the AdoCbl cofactor near the PLP cofactor. Interestingly, the syn and eastern conformation of the deoxyadenosine moiety,
observed in the OAM open state, is incompatible with substrate binding, as it would lead to direct overlap with the substrate in the active site. Reorientation to an anti and western conformation, as observed in GM (27) places the C5 of AdoH within van der Waals distance to the PLP-bound substrate, close to the optimal geometry for direct hydrogen transfer from the C4 atom of d-ornithine, consistent with the kinetic mechanism of Fig. 1. This model thus suggests that substrate binding is responsible for triggering the Rossmann domain open to closed transition (through release of Lys629), and for driving the reorientation of the Ado moiety concomitant with formation of the closed state.

Conclusions—The propensity for uncontrolled propagation of radical reactions combined with inherent reactivity of radical species underpins the relatively scarce use of radical reactions in biology. Nevertheless, several enzymes have been found to catalyze radical chemistry and thus successfully contain and control these reactions. The advantage of using radicals in a reaction cycle lies in their ability to make an otherwise chemically or thermodynamically challenging reaction possible. This is the case with vitamin B12, requiring enzymes, which use carbon-based free radicals to cleave C-C, C-N, and C-O bonds on otherwise unreactive (inert) substrates by H-abstraction from an unactivated carbon atom. For all of these enzymes, AdoCbl serves as a radical repository.

Interestingly, and unlike most AdoCbl-dependent enzymes or other radical-based enzymes studied to date, our present OAM crystal structures reveal that large-scale domain motion is required prior to radical pair formation. Our structures reveal that ligand binding establishes an open to closed conformational equilibrium in the enzyme that remains poised toward the open state. This strategy has obvious benefits, as it reduces the population of the reactive radical species to a minimum, as can be observed in solution studies. It is also possible that this (in part) underpins the unusual stability of the AdoCbl cofactor in OAM. Once the closed state is formed, however, AdoCbl homolysis and radical transfer needs to be tightly orchestrated with domain motion. Any domain motion that would occur during the radical pair state is likely to lead to dead-end reactions. On the other hand, a tight “locking-in” of the closed state that would safeguard from such events appears incompatible with the observed preference for the open state. Clearly, a detailed understanding of this apparent contradiction can only be acquired following insight into the structure of the closed state. However, modeling of the OAM closed state using the structurally related Class I isomerase glutamate mutase leads to a surprisingly plausible model for the OAM closed state. In this case, the AdoCbl cofactor is now located within van der Waals distance of the PLP cofactor. Interestingly, the syn conformation of the adenosine moiety as observed in the open state appears incompatible with substrate binding (direct overlap), whereas reorientation to an anti conformation (as observed for glutamate mutase) places the 5’ carbon within van der Waals distance of PLP-bound substrates. It is possible that a locking mechanism exists that safeguards the enzyme from returning to the open conformation when in the radical state. A reorientation of the Ado moiety from syn to anti and from eastern to northern appears to be required to allow radical catalysis, and might be coupled to such a locking mechanism. In this case, the substrate-bound active site could have significant affinity for anti 5’-deoxyadenosine (as opposed to syn), which in turn serves as “glue” between the Rossmann and TIM barrel domains. The existence of an adenine binding pocket has been suggested for diol dehydratase (33). In a similar vein, product dissociation could allow the 5’-deoxyadenosyl moiety to return to the eastern conformation, which could trigger the transition from the closed to open state. It is possible that a locking mechanism involving re-orientation of the Ado group exists that safeguards the enzyme from returning to the open conformation when in the biradial state.

Acknowledgments—Access to Diamond and ESRF beamlines is gratefully acknowledged.

REFERENCES
1. Henzler-Wildman, K. A., Lei, M., Thai, V., Kerns, S. J., Karplus, M., and Kern, D. (2007) Nature 450, 913–916
2. Agarwal, P. K., Billeter, S. R., Rajagopalan, P. T., Benkovic, S. J., and Hammes-Schiffer, S. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 2794–2799
3. Boehr, D. D., Dyson, H. J., and Wright, P. E. (2006) Chem. Rev. 106, 3055–3079
4. Barker, H. A. (1981) Annu. Rev. Biochem. 50, 23–40
5. Chen, H. P., Wu, S. H., Lin, Y. L., Chen, C. M., and Tsay, S. S. (2001) J. Biol. Chem.
Domain Dynamics in OAM

Chem. 276, 44744 – 44750
6. Chang, C. H., Ballinger, M. D., Reed, G. H., and Frey, P. A. (1996) Biochemistry 35, 11081 – 11084
7. Wetmore, S. D., Smith, D. M., and Radom, L. (2001) J. Am. Chem. Soc. 123, 8678 – 8689
8. Bothe, H., Darley, D. J., Albracht, S. P., Gerfen, G. J., Golding, B. T., and Buckel, W. (1998) Biochemistry 37, 4105 – 4113
9. Zhao, Y., Abend, A., Kunz, M., Such, P., and Rétey, J. (1994) Eur. J. Biochem. 225, 891 – 896
10. Schepler, K. L., Dunham, W. R., Sands, R. H., Fee, J. A., and Abeles, R. H. (1975) Biochim. Biophys. Acta 397, 510 – 518
11. Wolthers, K. R., Rigby, S. E., and Scrutton, N. S. (2008) J. Biol. Chem. 283, 34615 – 34625
12. Yoon, M., Patwardhan, A., Qiao, C., Mansoorabadi, S. O., Menefee, A. L., Reed, G. H., and Marsh, E. N. (2006) Biochemistry 45, 11650 – 11657
13. Mansoorabadi, S. O., Padmakumar, R., Fazliddinova, N., Vlasie, M., Banerjee, R., and Reed, G. H. (2005) Biochemistry 44, 3153 – 3158
14. Reitzer, R., Gruber, K., Jogl, G., Wagner, U. G., Bothe, H., Buckel, W., and Kratky, C. (1999) Structure 7, 891 – 902
15. Mancia, F., Keep, N. H., Nakagawa, A., Leadlay, P. F., McSweeney, S., Rasmussen, B., Bösecke, P., Diat, O., and Evans, P. R. (1996) Structure 4, 339 – 350
16. Masuda, J., Shibata, N., Morimoto, Y., Toraya, T., and Yasuoka, N. (2000) Structure 8, 775 – 788
17. Berkovitch, F., Bebshad, E., Tang, K. H., Enns, E. A., Frey, P. A., and Drennan, C. L. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 15870 – 15875
18. Collaborative Computation Project Number 4 (1994) Acta Crystallogr. D 50, 760 – 763
19. Evans, P. (2006) Acta Crystallogr. D 62, 72 – 82
20. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) J. Appl. Crystallogr. 40, 658 – 674
21. Cowtan, K. (1994) Joint CCP4 and ESF-EACBM Newsletter on Protein Crystallography 31, 34 – 38
22. Perrakis, A., Morris, R., and Lamzin, V. S. (1999) Nat. Struct. Biol. 6, 458 – 463
23. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. D 53, 240 – 255
24. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. D 60, 2126 – 2132
25. Lawrence, M. C., and Colman, P. M. (1993) J. Mol. Biol. 234, 946 – 950
26. Drennan, C. L., Huang, S., Drummond, J. T., Matthews, R. G., and Lidwig, M. L. (1994) Science 266, 1669 – 1674
27. Gruber, K., Reitzer, R., and Kratky, C. (2001) Angew. Chem. Int. Ed. Engl. 40, 3377 – 3380
28. Mancia, F., and Evans, P. R. (1998) Structure 6, 711 – 720
29. Ouyang, L., Rulis, P., Ching, W. Y., Nardin, G., and Randaccio, L. (2004) Inorg. Chem. 43, 1235 – 1241
30. Lenhert, P. G., and Hodgkin, D. C. (1961) Nature 192, 937 – 938
31. Summers, M. F., Marzilli, L. G., and Bax, A. (1986) J. Am. Chem. Soc. 108, 4285 – 4294
32. Brown, K. L., Cheng, S. F., and Marques, H. M. (1998) Polyhedron 17, 2213 – 2224
33. Toraya, T. (2003) Chem. Rev. 103, 2095 – 2127