Binding of Cob(II)alamin to the Adenosylcobalamin-dependent Ribonucleotide Reductase from Lactobacillus leichmannii

IDENTIFICATION OF DIMETHYLBENZIMIDAZOLE AS THE AXIAL LIGAND*

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The ribonucleotide triphosphate reductase (RTPR) from Lactobacillus leichmannii catalyzes the reduction of nucleoside 5'-triphosphates to 2'-deoxynucleoside 5'-triphosphates and uses coenzyme B12, adenosylcobalamin (AdoCbl), as a cofactor. Use of a mechanism-based inhibitor, 2'-deoxy-2'-methylencytidine 5'-triphosphate, and isotopically labeled RTPR and AdoCbl in conjunction with EPR spectroscopy has allowed identification of the lower axial ligand of cob(II)alamin when bound to RTPR. In common with the AdoCbl-dependent enzymes catalyzing irreversible heteroatom migrations and in contrast to the enzymes catalyzing reversible carbon skeleton rearrangements, the dimethylbenzimidazole moiety of the cofactor is not displaced by a protein histidine upon binding to RTPR.

There are three classes of adenosylcobalamin (AdoCbl)1 (Fig. 1, 1) requiring enzymes (1): those that catalyze reversible carbon backbone rearrangements (class I), those that catalyze irreversible heteroatom elimination reactions (class II), and those that catalyze rearrangements involving migration of an amino group (class III). All three classes initiate their radical-dependent reactions by catalyzing carbon–cobalt bond homolysis. The mechanism(s) by which these enzymes catalyze C–Co homolysis of 1, the identification of the axial ligand trans to the C–Co bond that is cleaved, and its role in this process have recently been a topic of interest in many laboratories (2). The x-ray structures of methylmalonyl-CoA (MmCoA) mutase (3, 4) and EPR studies of cob(II)alamin (2) bound to other class I enzymes (1, 5, 6) revealed that in each case the dimethylbenzimidazole (DBM) moiety of the cofactor is replaced by a protein-derived histidine. On the other hand, in diol dehydrase (DD), typical of the class II enzymes, DBM remains ligated to 2 (7, 8). Unique among the AdoCbl-requiring enzymes is ribonucleotide reductase, the only enzyme that does not catalyze a rearrangement reaction and in which C–Co homolysis occurs concomitant with the formation of a protein-based thyl radical (9–11). The mechanism of homolysis and the identity of the axial ligand in this enzyme have not previously been addressed and are the subjects of this paper.

Our laboratory has long been interested in the ribonucleotide triphosphate reductase (RTPR) from Lactobacillus leichmannii. In the presence of a nucleoside 5'-triphosphate (NTP) substrate, a reducing system, and a 2'-deoxynucleoside 5'-triphosphate (dNTP) allostERIC effector, RTPR catalyzes homolysis of the C–Co bond of 1 to generate 2, 5'-deoxynadinosine, and a thyl radical at Cys-408 of RTPR in a kinetically competent and concerted fashion (9, 10, 12–14). This thyl radical is then proposed to initiate the nucleotide reduction chemistry by abstraction of the 3'-hydrogen atom of the NTP (15). In this paper we report studies with a mechanism-based inhibitor of this protein, 2'-deoxy-2'-methylencytidine 5'-triphosphate (MdCTP, 3). These studies in conjunction with [U,15N]RTPR and AdoCbl specifically labeled in the nitrogens of its DMB moiety ([15N]AdoCbl) have provided the vehicle to identify DMB as the axial ligand during catalysis.

EXPERIMENTAL PROCEDURES

Materials and Methods—Alkaline phosphatase (calf intestine) was purchased from Boehringer Mannheim; all other reagents were purchased from Sigma and used without further purification. EPR tubes (4-mm outer diameter, 2.4-mm inner diameter) were purchased from Wilmad Glass Company. "Hepes buffer" corresponds to 25 mm Hepes, 4 mm EDTA, 1 mm MgCl2, pH 7.5. Thioredoxin and thioredoxin reductase were purified from overproducing strain SK3981 and K91/pMR14, respectively, as described previously (16, 17). RTPR (specific activity of 1.5 units mg−1) and prereduced RTPR were prepared as described previously (18). One unit of activity is defined as the amount of enzyme required to reduce 1 μmol of CTP min−1. RTPR uniformly labeled with [15N][U,15N]RTPR was prepared by the growth of Escherichia coli JM105 transformed with the plasmid pSQUIRE on M9 minimal medium supplemented with [15N]NH4Cl as described previously (19), and incorporation of 15N was analyzed by laser desorption mass spectrometry. AdoCbl containing 50% 15N in each nitrogen atom of the DMB moiety of AdoCbl ([15N]AdoCbl) was prepared, and the isotopic composition was determined by 1H NMR spectroscopy as described previously (7).

All operations involving covalamins were performed in the dark or in dim red light. Scintillation counting was performed on a Beckman LS 6500 multipurpose scintillation counter using Packard Scint-A-X scintillation fluid. EPR spectra at 9 GHz were acquired on a Bruker ESP-300 spectrometer at 100 K using the following parameters: microwave power, 10 milliwatts; modulation frequency, 100 kHz; modulation amplitude, 4 G; time constant, 164 ms. Spin quantization was achieved by double integration and comparison to a 1.0 mm CuSO4, 2 m NaClO4, 0.01 m HCl, 20% (v/v) glycerol standard (g = 2.18) (20).

Time-dependent Inactivation of RTPR by MdCtP—The inactivation solution contained in a volume of 100 μl 5 μl prereduced RTPR, 20 μM AdoCbl, 120 μM dATP, 20 μM thioredoxin, 0.12 μM thioredoxin reduc-
Axial Ligand of RTPR

RESULTS

Time-dependent Inactivation of RTPR by MdCTP—RTPR catalyzes the conversion of NTPs to dNTPs, requiring the presence of a dNTP allosteric effector and an appropriate reducing system, e.g., thioredoxin, thioredoxin reductase, and NADPH. MdCTP (3) was designed and synthesized (23) as a potential mechanism-based inhibitor of RTPR and as a probe for nucleotide radical intermediates. Incubation of 3 with RTPR, AdoCbl (1), an allosteric effector (dATP), and a reducing system gave the results shown in Fig. 2. A replot of this data reveals that only 1.5 eq of 3 are required for complete inactivation. No inactivation was observed in the absence of 3. Given the potency of inhibition, we decided to investigate the mechanism of this reaction in detail.

Generation of Cob(II)alamin and Paramagnetic Species during the Inactivation Reaction—Previous studies with mechanism-based inhibitors of RTPR have shown that inactivation is initiated by C–C bond homolysis. To examine if inactivation by 3 is initiated by a similar mechanism, we performed stopped flow UV-visible and rapid freeze quench EPR experiments. These studies revealed formation of 2 with a $k_{obs}$ of 10 s$^{-1}$ and of several additional paramagnetic species on the same time scale (data not shown). These results are in accord with our hypothesis of a radical mechanism of nucleotide reduction (15). The EPR spectra obtained are complex and contain multiple species. The structural identification of these species will require isotopic labeling of 3; studies are now in progress. However, at times from 250 ms to 30 s, a prominent feature of the EPR spectra is cob(II)alamin (2) uncoupled from other paramagnetic species (Fig. 3A). The rich spectrum associated with cob(II)alamin (Fig. 3A) is described by a $g_x$ of 2.27 and a $g_y$ of 2.01. Associated with $g_z$ are 8 hyperfine features (114 G) derived from the cobalt nucleus ($I = 7/2$). Furthermore, each cobalt hyperfine feature is associated with additional $^{15}$N hyperfine features ($I = 1$) apparent as triplets with 18 G coupling. The nitrogen hyperfine is associated with an axial ligand to cob(II)alamin (24), either an imidazole from the DMB moiety of the cofactor (Fig. 1) or a histidine-derived protein ligand. This cobalamin species is tightly bound to RTPR as the spectrum is substantially different from cob(II)alamin in solution taken

![Fig. 1. Structures of AdoCbl (1), cob(II)alamin (2), and MdCTP (3). The DMB and 5'-deoxyadenosyl moieties of 1 are highlighted in boxes A and B, respectively.](image)

![Fig. 2. Time-dependent inactivation of RTPR by MdCTP. RTPR (5 μM) was incubated with varying concentrations of MdCTP. ●, 0 μM; ○, 2.5 μM; ×, 5 μM; ■, 10 μM; □, 20 μM. At the indicated times, aliquots were removed and assayed for remaining RTPR activity.](image)
under identical conditions (Fig. 3D). Substitution of $^{14}$N(I = 1) with $^{15}$N (I = 1/2) provides a method to identify the axial ligand if the appropriate compounds can be generated.

**Inactivation Experiments Using** $[{U-^{15}N}]$RTPR or $[^{15}N]$AdoCbl—$[U-^{14}N]$RTPR was prepared from minimal media supplemented with $^{15}$NH$_4$Cl, and the $^{15}$N content of the purified enzyme was analyzed by laser desorption mass spectrometry. Wild type RTPR grown under the same conditions gave a m/z of 81,600 ± 300 (expected, 81,852), whereas $[{U-^{15}N}]$RTPR gave a m/z of 82,600 ± 300 (expected, 82,840). A sample of AdoCbl containing $^{15}$N in the DMB moiety ($[^{15}N]$AdoCbl) was prepared as described previously (7). The label is distributed equally between the two nitrogens of the imidazole ring; hence there is 50% isotope incorporation (7). In separate experiments, $[U-^{15}N]$RTPR with unlabeled AdoCbl and RTPR with $[^{15}N]$AdoCbl were incubated with MdCTP and frozen after ~30 s. The EPR spectra obtained in comparison with the control using RTPR and AdoCbl are shown in Fig. 3, A–C. The more highly resolved nitrogen hyperfine features in comparison with cob(I)-alamin in the absence of RTPR are apparent (compare Fig. 3, A–C to D), indicating, as noted above, that cob(I)-alamin is enzyme bound. A comparison of the nitrogen hyperfine structures observed under these conditions (see Ref. 24).

The spectrum from $[U-^{15}N]$RTPR is identical to that obtained with unlabeled RTPR (Fig. 4, A and B) and simulated using $^{14}$N hyperfine coupling parameters (Fig. 4C). In contrast, the experiment with $[^{15}N]$AdoCbl revealed altered nitrogen hyperfine interactions (Fig. 4D). Simulation of these interactions was successful assuming the axial ligand contains 50% $^{15}$N and 50% $^{14}$N, the same value as in the starting cofactor (Fig. 4E). These results demonstrate that the DMB moiety of AdoCbl is ligated to cobalt during its conversion to cob(I)-alamin at the active site of RTPR in the presence of MdCTP.

**DISCUSSION**

The means by which B$_{12}$-requiring enzymes effect the remarkable 10$^{10}$–10$^{12}$ rate acceleration of C-Co bond cleavage is not understood and is an ongoing focus in many research laboratories. A number of proposals addressing this rate acceleration have been forwarded based on model studies and theoretical considerations (26). One hypothesis advocates an upward flexing of the corrin ring, thereby applying a steric strain to the upper axial ligand resulting in a weakening of the C–Co bond (27–31). A recent resonance Raman study of MmCoA mutase, albeit in the absence of substrate, revealed a significant change in the conformation of the corrin ring of I upon binding to the enzyme but little perturbation of the Co–C force constant (32).

A second hypothesis emphasizes the importance of the axial nitrogen ligand trans to the C–Co bond that is cleaved. Model studies have indicated that a weaker nitrogen donor ligand decreases the dissociation energy of the C–Co bond (33–35). Interest in this second hypothesis has been stimulated by extended x-ray absorption fine structure spectroscopy and x-ray crystallography studies on MmCoA mutase, which indicate displacement of the DMB lower axial ligand of I by a protein histidine in cob(II)alamin-bound MmCoA mutase, with a long Co–N bond (3, 4, 36). EPR studies on other AdoCbl-requiring carbon skeleton mutases reveal that these class I enzymes also utilize a histidine as their axial ligand (1, 6) as do most of the cobalamin-dependent methyltransferases thus far examined (37–39). In contrast, the AdoCbl enzymes catalyzing irreversible heteroatom rearrangements, typified by DD, utilize the DMB moiety of the cofactor as the axial ligand (7, 8).

It was initially thought based on sequence alignments that if a given protein possessed the putative cobalamin binding motif DXXLXXG-(41–42 amino acids)-SXL-(22 amino acids)-GG (40) or a modification thereof then it would utilize H as the axial ligand, as revealed in the x-ray structures of MmCoA mutase.
Nitrogen hyperfine interactions in these signals have revealed whether the axial ligand is in fact the same during substrate turnover. The exchange coupling observed in many of the kinetically competent intermediates and the lack of detectable nitrogen hyperfine interactions in these signals have neither a necessary nor a sufficient condition for the protein histidine mode of ligation.

In our continuing efforts to understand the mechanism of C–Co bond homolysis catalyzed by RTPR, we have now determined the identity of the lower axial ligand in a cob(II)alamin-bound form of the enzyme as DMB. RTPR thus appears to use the same mode of cofactor binding as the other class II proteins such as DD. Furthermore the similarities between RTPR and DD extend to the first steps in the chemistries these enzymes catalyze that involve hydrogen atom abstraction followed by an irreversible loss of water. However, despite these similarities only RTPR chemistry seems to require a protein-based radical. Recent studies have begun to shed light as to how RTPR manages to perturb the rate and equilibrium constants for the C–Co bond homolysis so dramatically. First, the enzyme has been shown to couple the thermodynamically unfavorable C–Co bond homolysis step with a thermodynamically favorable hydrogen atom abstraction from Cys-408, the first enzymatic example of molecule-induced homolysis (10, 15). Second, a kinetic and thermodynamic analysis has revealed unexpectedly that entropy plays a key role in both ground state stabilization/destabilization and transition state stabilization (15, 42). Whether these factors are in play in other B12-requiring proteins remains to be determined.

The significance of nature’s choice of DMB as the axial cobalamin ligand in RTPR and related enzymes such as DD on the one hand and a protein histidine in the carbon skeleton mutants on the other also remains to be established. Finally, it should be noted that in all investigations into the nature of the axial ligand in AdoCbl-requiring proteins thus far, a substrate analog, a coenzyme analog, or photolysis of 1 have been required to generate the spectroscopic handle of cob(II)alamin. It remains to be established whether the axial ligand is in fact the same during substrate turnover. The exchange coupling observed in many of the kinetically competent intermediates and the lack of detectable nitrogen hyperfine interactions in these signals have required use of alternative approaches to identify the axial ligand (9).

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