

The ATP:Co(I)corrinoid Adenosyltransferase (CobA) Enzyme of *Salmonella enterica* Requires the 2'-OH Group of ATP for Function and Yields Inorganic Triphosphate as Its Reaction Byproduct*

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The specificity of the ATP:corrinoid adenosyltransferase (CobA) enzyme of *Salmonella enterica* serovar Typhimurium LT2 for its nucleotide substrate was tested using ATP analogs and alternative nucleotide donors. The enzyme showed broad specificity for the nucleotide base and required the 2'-OH group of the ribosyl moiety of ATP for activity. ³¹P NMR spectroscopy was used to identify inorganic triphosphate (PPP_i) as the byproduct of the reaction catalyzed by the CobA enzyme. Cleavage of triphosphate into pyrophosphate and orthophosphate did not occur, indicating that triphosphate cleavage was not required for release of the adenosylcorrinoid product. Triphosphate was a strong inhibitor of the reaction, with 85% of CobA activity lost when the ATP/PPP_i ratio present in the reaction mixture was 1:2.5.

Adenosylcobalamin (AdoCbl,¹ coenzyme B₁₂) (Fig. 1) is required in diverse metabolic reactions (1–3). Formation of the Co–C bond between the corrin ring and the upper ligand requires the cobalt ion of the ring to be in its Co(I) oxidation state before the 5'-deoxyadenosyl moiety of ATP can be transferred to it. The set of reactions (two 1-electron reductions and one adenosyl transfer) that convert corrinoids to their adenosylated

forms is known as the corrinoid adenosylation pathway, and evidence for the existence of this pathway was first described using crude cell-free extracts of *Propionibacterium freundenreichii* (4) and *Clostridium tetanomorphum* (5).

Our current understanding of the corrinoid adenosylation pathway in *S. enterica* is shown in Fig. 2. In this bacterium, corrinoid adenosylation is required for both synthesis and use of corrinoids. It is known that *de novo* synthesis of the corrin ring proceeds via adenosylated intermediates and that salvaging of exogenous, incomplete corrinoids also requires a functional corrinoid adenosylation pathway (6, 7). In *S. enterica*, the inability to adenosylate cobalamin prevents the expression of the ethanolamine utilization (*eut*) genes (8, 9), thus blocking growth on ethanolamine as carbon and energy source (10). In *Escherichia coli*, the inability to adenosylate Cbl results in the unregulated, constitutive expression of the *btuB* gene, which directs the synthesis of the outer membrane protein responsible for translocating exogenous corrinoids into the periplasmic space of the cell (11–13).

Biochemical analysis of the reaction catalyzed by the CobA enzyme (Fig. 2) suggested the nucleoside triphosphate bound to the enzyme before the corrinoid substrate (14). Consistent with this observation, the three-dimensional structure of the ternary complex between hydroxocobalamin (HOCbl) and MgATP shows that the corrinoid substrate is bound at the surface of the active site, atop of a cavity that encloses the MgATP molecule (15). The CobA enzyme contains a P-loop motif of the sequence GNGKGGKT defined by amino acids Gly³⁶–Thr⁴² (16, 17). This motif coordinates the α -, β -, and γ -phosphates of the nucleotide (15). The three-dimensional structure of CobA shows the phosphates of ATP coordinated in opposite orientation to that found in other nucleotide hydrolases, *i.e.* the position normally occupied by the γ -phosphate is occupied by the α -phosphate. This unusual mode for nucleotide binding is proposed to correctly position the C-5' of the ribosyl moiety for the nucleophilic attack by Co(I).

The work reported in this study was undertaken to establish the identity of the phosphate byproducts of the reaction and to begin the identification of moieties of the substrate that are important for catalysis. The data obtained indicate PPP_i is the byproduct of the CobA reaction and demonstrate the importance of the 2'-OH group of the ribosyl moiety of ATP to the adenosyltransfer reaction.

EXPERIMENTAL PROCEDURES

Reagents—Unless otherwise stated, all reagents were obtained from Sigma. The ATP analog 5-mercapto-5'-deoxy-ATP (A(S)TP) was a kind gift from G. D. Markham (Institute for Cancer Research, Philadelphia, PA).

Purification of the CobA Enzyme—CobA was purified as described previously without modifications (15).

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¹ The abbreviations used are: AdoCbl, adenosylcobalamin; Cbl, cobalamin; HOCbl, hydroxocobalamin; PPP_i, triphosphate; Fpr, ferredoxin (flavodoxin):NADP⁺ reductase (EC 1.18.1.2); FldA, flavodoxin; CobA, ATP:co(I)corrinoid adenosyltransferase (EC 2.5.1.17); APPPP, adenosine-5'-tetraphosphate; AMPPNP, adenylyl-5'-imidodiphosphate; AMPCPP, α,β -methyleneadenosine-5'-triphosphate; AMPPCP, β,γ -methyleneadenosine-5'-triphosphate; A(S)TP, 5-mercapto-5'-deoxyadenosine-5'-triphosphate.

Corrinoid Adenosylation Assays—ATP analogs and alternative nucleotide donors were tested as substrates for the CobA enzyme using the potassium borohydride (KBH_4)-dependent corrinoid adenosylation assays as described (14, 18). Briefly, reaction mixtures contained (final volume, 1 ml): KBH_4 , 74 μmol ; HOCl , 50 nmol; CoCl_2 , 420 nmol; MgCl_2 , 1 μmol ; Tris-Cl, pH 8.0, at 37 °C, 200 μmol ; CobA, 0.4 nmol, and 400 nmol of each nucleotide (unless otherwise stated). Reaction mixtures were incubated for 15 min at 37 °C. The amount of product generated was quantified as described (14). The effect of triphosphate on CobA activity was investigated by including increasing amounts of triphosphate (0–3 mM) in the reaction mixture.

^{31}P NMR Spectroscopy and Preparation of CobA Reaction Products for ^{31}P NMR—For ^{31}P NMR analysis, ferredoxin (flavodoxin):NADP⁺ reductase (Fpr) enzyme and flavodoxin (FldA) protein substituted for KBH_4 as the reducing system for the generation of the co(I)rrinoid substrate of CobA (19). The reaction mixture contained (final volume, 1 ml): HOCl (1 μmol), ATP (2 μmol), MgCl_2 (4 μmol), NADPH (2 μmol), Fpr (21 nmol), FldA (24 nmol), CobA (12 nmol) in Tris-Cl (200 μmol) buffer, pH 8.0, at 37 °C. Reaction mixtures were incubated at 37 °C for 2 h. Samples from reaction mixtures were transferred into 5-mm (internal diameter) NMR tubes (Wilma Glass, Buena, NJ). EDTA was added to a 10 mM final concentration. The final volume of the sample was brought up to 0.6 ml with 100% D_2O (Cambridge Isotope Labs, Andover, MA).

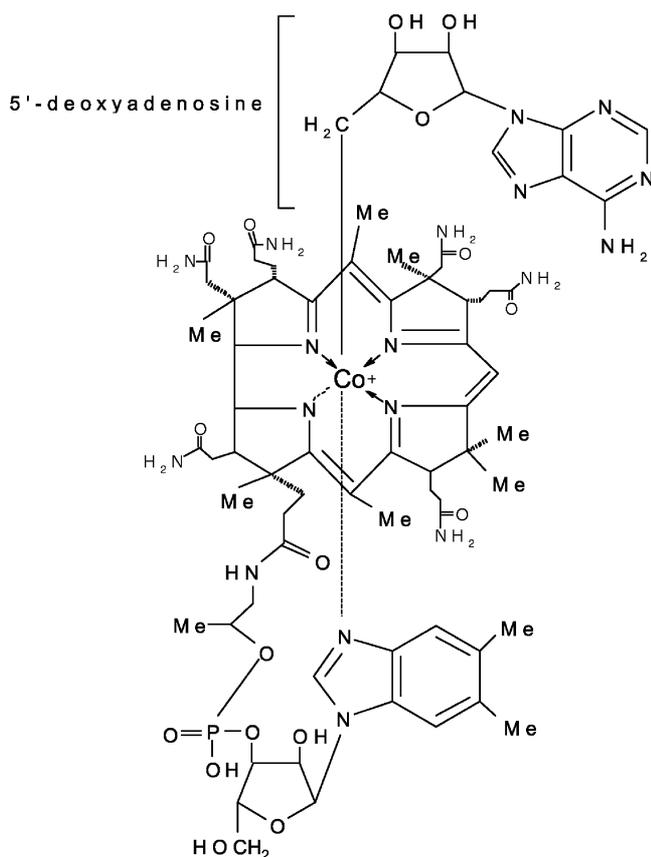


FIG. 1. Chemical structure of AdoCbl.

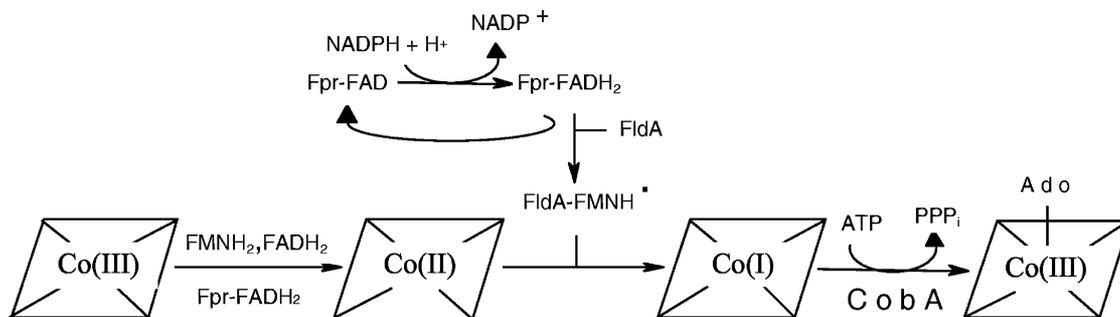


FIG. 2. Corrinoid adenosylation pathway in *S. enterica*. In this model $\text{NADPH} + \text{H}^+$ is the source of reducing power to convert Co(III) to Co(I) using ferredoxin (flavodoxin):NADP⁺ reductase (Fpr) and flavodoxin A (FldA) proteins to perform the reductions (19). Fpr-FAD, oxidized form of Fpr; Fpr-FADH₂, hydroquinone form of Fpr; FldA-FMN, oxidized form of FldA; FldA-FMNH[•], semiquinone form of FldA; PPP_i, triphosphate.

NMR Data Acquisition— ^{31}P NMR spectra of corrinoid adenosylation assay products were obtained at 161.98 MHz using a Bruker Instruments DMX-400 Avance console spectrometer equipped with a 9.4 T wide-bore magnet (Nuclear Magnetic Resonance Facility, University of Wisconsin-Madison). Spectra were Fourier-transformed with 3-Hz line broadening. Chemical shifts were referenced to H_3PO_4 set to 0.0 ppm.

RESULTS AND DISCUSSION

Differences in the Base of the Nucleotide Substrate Have No Effect on CobA Activity—To identify functional groups of the ribose and base moiety of the nucleotide that are important for activity, the specificity of CobA for the donor nucleotide was assessed (Table I). It was previously shown that CobA had

TABLE I
Use of alternative nucleotide donors by CobA

Nucleotide donor	% Rate ^a
	<i>nmol AdoCbl min⁻¹</i>
ATP (adenosine-5'-triphosphate)	100
AMP (adenosine-5'-monophosphate)	NM ^b
ADP (adenosine-5'-diphosphate)	NM
ITP (inosine-5'-triphosphate)	83
dATP (2'-deoxyadenosine-5'-triphosphate)	NM
dTTP (2'-deoxythymidine-5'-triphosphate)	NM
dCTP (2'-deoxycytidine-5'-triphosphate)	NM
dGTP (2'-deoxyguanosine-5'-triphosphate)	NM
CTP (cytidine-5'-triphosphate)	150
UTP (uridine-5'-triphosphate)	125
GTP (guanosine-5'-triphosphate)	45

^a Average rate for product formation under these conditions using ATP as substrate was 0.4 nmol of AdoCbl min⁻¹ ± 0.14 nmol of AdoCbl min⁻¹.

^b Not measurable under the assay conditions used.

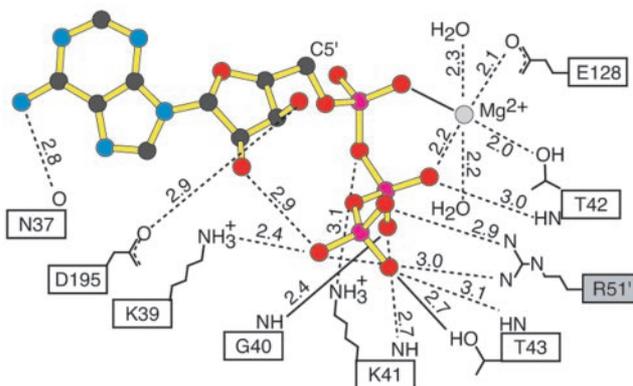


FIG. 3. Interactions of MgATP within the CobA active site. Schematic of the hydrogen bonding interactions around the MgATP molecule in the CobA active site. Residue R51' corresponds to the side chain of arginine from the 2-fold-related subunit. Color coding for different atoms: O (red), C (black), N (blue), P (magenta); distances shown in Å.

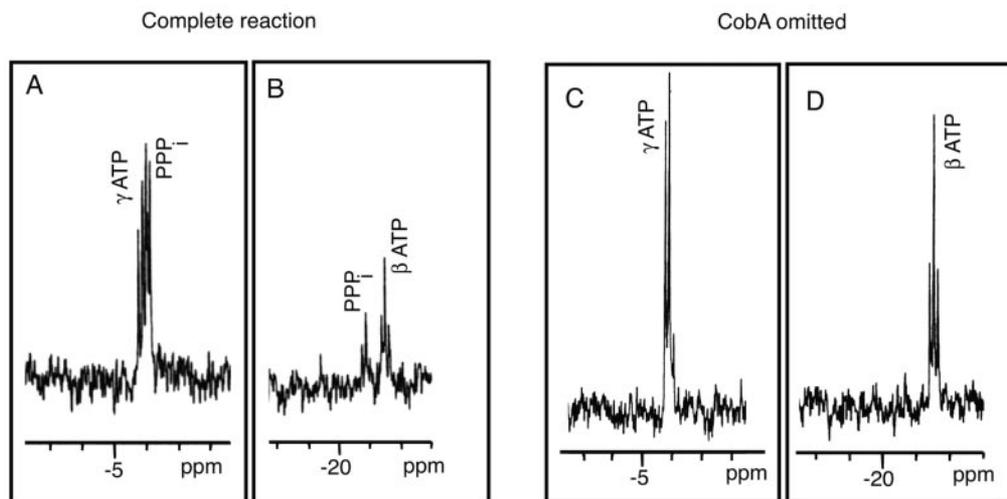


FIG. 4. Detection of ATP and its cleavage products in corrinoid adenosylation assay reaction mixtures. Panel A, chemical shifts observed for peaks corresponding to the γ -phosphate of ATP and the outer phosphates of PPP_i in a ^{31}P NMR spectrum of the complete reaction mixture. Panel B, chemical shifts observed for peaks corresponding to the β -phosphate of ATP and the center phosphate of PPP_i in a ^{31}P NMR spectrum of the complete reaction mixture. Panel C, chemical shift observed for the γ -phosphate of ATP in a ^{31}P NMR spectrum of a reaction mixture in which the CobA enzyme was omitted. Panel D, chemical shift observed for the β -phosphate of ATP in a ^{31}P NMR spectrum of a reaction mixture in which CobA was omitted.

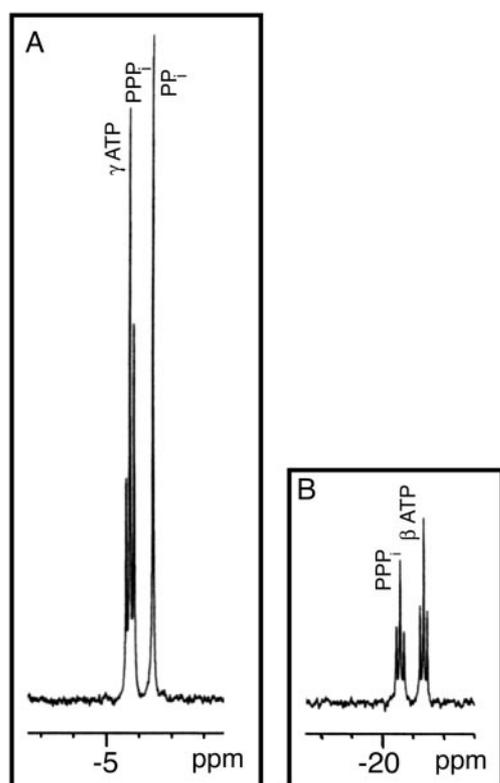


FIG. 5. Chemical shifts for standards added to the complete reaction mixture. Panel A, chemical shifts observed for the γ -phosphate of ATP, the outer phosphates of PPP_i and PP_i added to the complete reaction mixture. Panel B, chemical shifts observed for peaks corresponding to the β -phosphate of ATP and the center phosphate of PPP_i when these standards were added to the complete reaction mixture.

broad specificity for the donor nucleotide (14). When CTP, UTP, and GTP were tested as substrates, product formation (relative to the rate of ATP) was observed at rates that were 98, 88, and 37%, respectively. This broad specificity of CobA for its nucleoside triphosphate substrate was recently explained in light of the few interactions between the base and the polypeptide

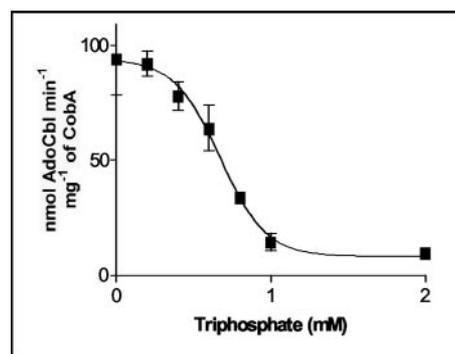


FIG. 6. Inhibitory effect of triphosphate. Reaction conditions were as described under "Experimental Procedures." Amount of product formed was calculated as described elsewhere (14, 19). The assay lacking PPP_i in the reaction mixture was done in quadruplicate, all other determinations were performed in duplicate.

TABLE II
ATP analogs used as substrates by the CobA adenosyltransferase enzyme

Analog	% Rate ^a
	<i>nmol AdoCbl min⁻¹</i>
ATP (adenosine-5'-triphosphate)	100
APPPP (adenosine-5'-tetraphosphate)	93
AMPPNP (adenylyl-5'-imidodiphosphate)	150
AMPCPP (α,β -methyleneadenosine-5'-triphosphate)	NM ^b
AMPPCP (β,γ -methyleneadenosine-5'-triphosphate)	NM ^b
A(S)TP (5-mercapto-5'-deoxyadenosine-5'-triphosphate)	NM ^b

^a Average rate for product formation under these conditions using ATP as substrate was $0.4 \text{ nmol of AdoCbl min}^{-1} \pm 0.14 \text{ nmol of AdoCbl min}^{-1}$.

^b Not measurable under the assay conditions used.

observed in the three-dimensional structure of the ternary complex between HOcbl , MgATP , and CobA (15). Measurements of the initial velocity of the reaction shown in Table I were consistent with the conclusion that the specificity of the enzyme for the base component of its nucleoside triphosphate substrate is not high.

The 2'-OH of the Ribosyl Moiety of ATP Is Pivotal for Enzyme Activity—At enzyme concentrations appropriate for the measurement of initial reaction velocity, 2' deoxyribonucleotides failed to serve as substrates for CobA. At high enzyme concentrations ($\sim 1.3 \mu\text{M}$), the rate of product formation was detectable (relative to the rate of the reaction when ATP was used as substrate) in the following order of preference: dATP (39%), dCTP (8%), dGTP (0.7%), and dTTP (not detectable). These results can be explained in light of the three-dimensional structure of the CobA-MgATP complex (Fig. 3). As shown in Fig. 3, the nucleotide is folded onto itself with the 2'-OH group of the ribosyl moiety interacting with the γ -phosphate of the nucleotide. This hydrogen bond is proposed to be critical for exposure of the C-5' carbon of the ribose to the Co(I) nucleophile. It appears that the 2'-OH group is involved in generating a torsional angle that is important for achieving the proper conformation of ATP for the nucleophilic attack by the co(I)rrinoid. The interactions of the CobA polypeptide with MgATP (Fig. 3) also help explain the lack of enzyme activity observed when ADP or AMP was used as substrate. The mixture of orthophosphate and ADP did not substitute for ATP, suggesting an important structural role for the β - γ phosphate bond in generating the correct conformation of the nucleotide substrate. AMP is likely further affected by the lack of Mg^{2+} coordination.

PPP_i Is the Byproduct of the CobA Reaction—Fig. 4 illustrates chemical shifts observed in ^{31}P NMR spectra of products of the CobA reaction. The triplets observed at -20.9 ppm ($J_{\text{P-P}} = 21.1 \text{ Hz}$) and -21.5 ppm ($J_{\text{P-P}} = 19.7 \text{ Hz}$) in the spectrum obtained for the complete system (Fig. 4, panel B) correlate well with the chemical shifts observed for the central phosphate of authentic PPP_i and the β -phosphate of ATP, respectively. In the same spectrum, doublets corresponding to the γ -phosphate of ATP and the outer phosphates of PPP_i were observed at -5.9 and -6.0 ppm , respectively (Fig. 4, panel A). The observed chemical shifts were confirmed by addition of authentic ATP and PPP_i to the sample (data not shown). Signals with chemical shifts corresponding to other phosphate-containing compounds present in the reaction mixture such as NADPH and Cbl were also observed (data not shown). The signals for PPP_i were absent in spectra obtained from a reaction mixture in which the CobA enzyme was omitted (Fig. 4, panels C and D). These signals were also absent in spectra obtained from a reaction mixture in which Cbl was omitted (data not shown). On the basis of these results, it was concluded that the byproduct of the CobA reaction was PPP_i. To investigate whether the PPP_i byproduct was further cleaved into PP_i and P_i during the reaction, authentic PP_i was added to the sample. A singlet with a chemical shift of -6.4 ppm , corresponding to this compound was observed (Fig. 5). This signal was not observed in the original spectrum obtained for the complete reaction mixture (Fig. 5, panel A). The reaction catalyzed by the CobA enzyme is one of only two known reactions in which an adenosyltransferase occurs (20). The ATP:L-methionine S-adenosyltransferase (S-adenosylmethionine (AdoMet) synthetase, EC 2.5.1.6) enzyme catalyzes the adenosyltransfer from ATP to the sulfur atom of methionine in a two-step reaction. The first step in this reaction involves cleavage of the triphosphate chain of ATP between the C-5' and the α -phosphate. In the second step, triphosphate is hydrolyzed into PP_i and P_i before the endproduct of the reaction, AdoMet is released (21). The above results obtained with the CobA enzyme suggested that unlike the AdoMet synthetase enzyme, CobA has no detectable triphosphatase activity. Further support for triphosphate as the byproduct of the reaction was obtained from inhibition studies. As shown in Fig. 6, triphosphate was a strong inhibitor of CobA enzyme activity, with 85% of the activity lost when the ATP/

PPP_i ratio in the reaction mixture was 1:2.5.

Results from Experiments with Non-hydrolyzable ATP Analogs Provide Evidence for Nucleophilic Displacement of PPP_i—Several ATP analogs substituted in the triphosphate chain of the nucleotide were tested as substrates for the CobA enzyme (Table II). Compounds APPPP and AMPNP were found to be efficient substrates for CobA at rates comparable to that of ATP, providing additional evidence that points at PPP_i as sole byproduct of the corrinoid adenosylation reaction. If CobA had triphosphatase activity associated with it, these compounds would be expected to be inactive as substrates for the enzyme or affect the rate of product formation, as has been demonstrated for AdoMet synthetase (21–23).

When α,β - and β,γ -methylene-substituted derivatives of ATP were tested as substrates for CobA, enzyme activity was not measurable. These compounds were poor inhibitors of the enzyme under the conditions tested, since no inhibition of CobA activity was detectable when they were present in the reaction mixture at relatively high concentrations (0.5 and 1 mM, data not shown). These results were in contrast to results obtained with other nucleotide hydrolases where these compounds have been found to be substrates and or inhibitors (22, 24–26). The analog A(S)TP, however, was a strong inhibitor of CobA activity with complete inhibition of enzyme activity observed at $3 \mu\text{M}$ A(S)TP. Inhibition by A(S)TP was expected because this analog contains a sulfur bridge rather than an oxygen bridge between the C-5' and the α -phosphate, making it non-hydrolyzable at the C-5' position.

Conclusions—The results described herein further our understanding of the reaction catalyzed by the *S. enterica* ATP:co(I)rrinoid adenosyltransferase (CobA) enzyme function, which is required for *de novo* biosynthesis of AdoCbl and for the assimilation of incomplete corrinoids. The crucial role of the 2'-OH group of the ribosyl moiety of ATP in CobA activity can be readily visualized in the three-dimensional structure of the binding site occupied by ATP. The γ -phosphate of ATP is critical for positioning the target (the C-5') for nucleophilic attack by Co(I). The data are consistent with nucleophilic displacement of the triphosphate chain of ATP. This mechanism appears to be similar to the reported one for the *C. tetanomophum* co(I)rrinoid adenosyltransferase but different than the mechanism reported for the *P. freundreichii* enzyme. Unlike AdoMet synthetase, CobA lacks any detectable triphosphatase activity. This difference between AdoMet synthetase may reflect differences in the ease of product release between these enzymes.

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