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Ribonucleoside Triphosphate Reductase from *Lactobacillus leichmannii:* Kinetic Evaluation of a Series of Adenosylcobalamin Competitive Inhibitors, [ω-(Adenosin-5′-*O-yl*)alkyl]cobalamins, Which Mimic the Post Co-C Homolysis Intermediate

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A series of [ω-(adenosin-5′-O-*yl*)alkyl]cobalamins were examined for their inhibitory properties of ribonucleoside triphosphate reductase (RTPR) from *Lactobacillus leichmannii* in the presence of 5′-deoxyadenosylcobalamin (AdoCbl, Coenzyme B_{12}). These AdoCbl analogs, in which oligomethylene chains (C_3–C_7) were inserted between the corrin Co-atom and a 5′-O-atom of the adenosine moiety, were designed to probe the Co-C bond posthomolysis state in AdoCbl-dependent enzymes, a state in which the Co and 5′-C distance is believed to be significantly increased. Experimentally, all five analogs were competitive inhibitors, with K_i in the range of 8–56 μM. The [ω-(adenosin-5′-O-*yl*)alkyl]cobalamin analog with C_5 methylene carbons was the strongest inhibitor. This same pattern of inhibition, in which the C_5-analog is the strongest inhibitor, was previously observed in the AdoCbl-dependent *eliminase* enzyme systems, diol dehydratase and glycerol dehydratase. However, in methylmalonyl CoA *mutase*, the strongest inhibition is by the C_6-analog. This supports the hypothesis that the cobalamin posthomolysis intermediate in the eliminase enzymes differs from that in the mutase enzymes. These findings led, in turn, to an examination of the visible spectra of enzyme-bound cob(II)alamin in these two subclasses of AdoCbl-dependent enzymes. The results reveal an additional insight into the difference between the two classes: in the *eliminases*, the γ-band of bound cob(II)alamin is shifted from the 473 nm for free cob(II)alamin to *longer* wavelengths, 475–480 nm. However, in *mutases*, the γ-band of bound cob(II)alamin is shifted to shorter wavelengths, 465–470 nm. Overall, the results (a) provide strong evidence that two subclasses of AdoCbl-dependent enzymes exist, (b) give insights into the probable posthomolysis state in RTPR and other eliminases, and (c) identifies the C_5-analog as the tightest-binding analog for crystallization and other biophysical studies.

INTRODUCTION

An essential process in the catalytic cycle of 5′-deoxyadenosylcobalamin-dependent (AdoCbl; Coenzyme B_{12}) enzymatic reactions is the reversible homolytic cleavage

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of the AdoCbl Co-C bond to produce cob(II)alamin and a 5′-deoxyadenosyl radical or a cysteinyl thiyl radical (*1–3*). The mechanisms by which AdoCbl-dependent enzymes activate, cleave, and reform the Co-C bond during catalysis have been the focus of a wide variety of research efforts (*4–8*), especially since chemical precedent studies of AdoCbl Co-C bond cleavage revealed the $10^{12\pm1}$ enzymic acceleration of this step throughout the AdoCbl-dependent enzymes (*9–12*).

In order to better understand the structure of the intermediate species and the mechanisms involved, a series of five novel adenosylcobalamin analogs, [*ω*-(Adenosin-5′-O-*yl*)alkyl]cobalamins, were designed and previously synthesized with the goal of probing the posthomolysis state geometry in AdoCbl-catalyzed reactions (*13*). These [*ω*-(adenosin-5′-O-*yl*)alkyl]cobalamins have oligomethylene chains (C_3–C_7) inserted between the corrin Co-atom and the 5′-O-atom of adenosine (Fig. 1). Depending on the length of the oligomethylene chain, these novel analogs are expected to act as stronger or weaker inhibitors of AdoCbl-dependent reactions via their stronger or weaker binding to the posthomolysis conformation of the enzyme. The tightest binding of these AdoCbl analogs also allows an estimate of the separation distance in the enzyme between cob(II)alamin and 5′-deoxyadenosine following Co-C bond homolysis. This series of [*ω*-(adenosin-5′-O-*yl*)alkyl]cobalamin analogs has also been

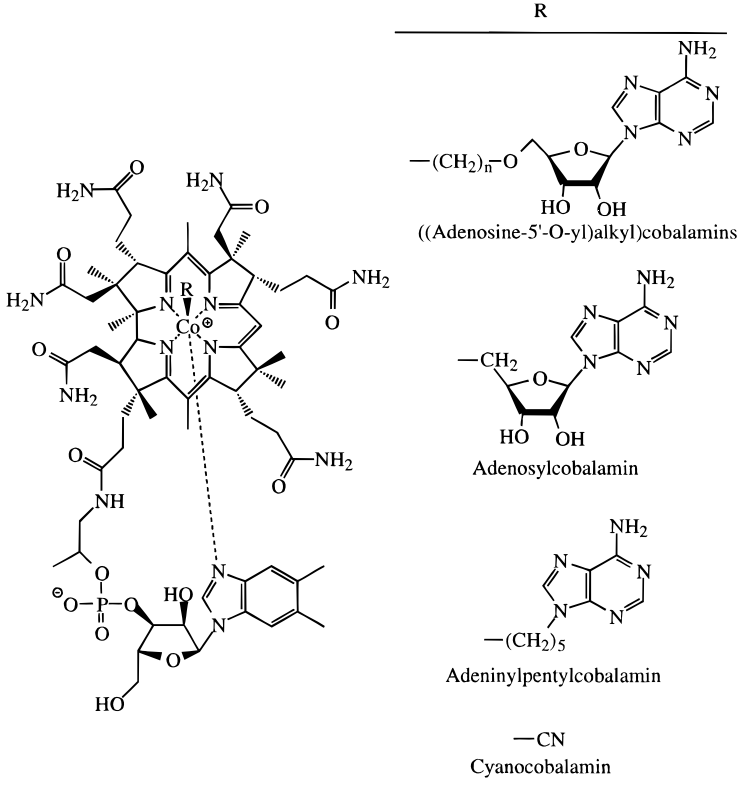

FIG. 1. Schematic structures of the AdoCbl analogs used in the present studies.

examined in the AdoCbl-dependent enzyme systems of methylmalonyl-CoA mutase from *Propionibacterium shermanii* (*14*), glycerol dehydratase from *Citrobacter freundii* (*15*), and diol dehydratase from *Salmonella typhimurium* (*15*).

Herein we extend the analysis of the inhibitory properties of this series of [ω-(adenosin-5′-O-*yl*)alkyl]cobalamins by examining them with RTPR and in competition against AdoCbl. The resulting competitive inhibition pattern is compared to that observed for the three other, previously studied AdoCbl-dependent enzymes. Our results, in combination with the literature (*14*), provide the clearest evidence to date in support of the hypothesis that the posthomolysis intermediates found in the AdoCbl-dependent eliminase enzymes are different than those in the mutase enzymes. The results also yield an estimate for the Co–C separation distance in the posthomolysis intermediate and identify the C_5-analog as the tightest-binding analog for biophysical studies of the AdoCbl(analogs)•RTPR holoenzyme complex.

EXPERIMENTAL PROCEDURES

Materials. The [ω-(adenosin-5′-O-*yl*)alkyl]cobalamins were synthesized, purified, and characterized according to Poppe *et al.* (*13*). The *L. leichmannii* ribonucleotide triphosphate reductase was isolated from overexpressing *Escherichia coli* cells (*16*) and purified using our recent shortened protocol (*17*), one including a dGTP-Sepharose affinity column (*18*). AdoCbl, hydroxycobalamin, adenosine, ATP, dithiothreitol, diphenylamine, and all other materials were obtained from Sigma and used without further purification. Adeninylpentylcobalamin (AdePeCbl) was synthesized by published procedures (*19–21*), with the following modification (full details are available elsewhere (*12*)): since adeninylpentylchloride was initially too impure to crystallize from aqueous methanol as reported (*21*), adeninylpentylchloride was purified using a Chromatotron (Harrison Research Co.) with a 4-mm silica gel disc (Merck, 7749) using dichloromethane/methanol (13:1, v/v) as the developing solvent. The resultant adeninylpentylcobalamin was 97% pure by HPLC (Alltech Versapack C_{18} column, 4.1 × 300 mm, 10-μm particle size, 60 Å pore size, eluted at 0.5 ml/min for 30 min with a linear gradient of 10–44% CH_3CN in 0.085 M H_3PO_4, adjusted to pH 3 with triethylamine). A single ^{31}P resonance was observed at −0.5 ppm vs a H_3PO_4 standard. The expected molecular weight was observed by mass spectroscopy (FAB-MS, glycerol matrix: found, M + H = 1533.6 Da; calculated, M + H = 1532.7 Da).

Methods. The analogs were tested as inhibitors of RTPR's ability to convert ATP to dATP in the presence of various AdoCbl concentrations. The concentration of each stock cobalamin solution (~2 mM) was determined using the literature extinction coefficients for [ω-(adenosin-5′-O-*yl*)alkyl]cobalamins (*13*), adeninylpentylcobalamin (*20*), or aquocobalamin (*23*). The activity of RTPR was assayed using our modification of the diphenylamine procedure (*17*), an assay which measures the amount of dATP produced from ATP.

In a typical kinetic experiment, all procedures were done under dim red light to avoid photolysis of AdoCbl's sensitive Co-C bond. The pH of the stock assay reaction solution (containing 83.3 mM potassium phosphate buffer (pH 7.3), 1.67 M sodium acetate, 16.7 mM ATP, 50 mM DTT) was adjusted to pH 7.3 with NaOH. The final reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.3), 1 M sodium acetate, 10 mM ATP, 30 mM DTT, prechosen amounts of AdoCbl (1, 2, 3, 8 μM)

and analogs (1, 5, 10, 15, 20 μM), and 0.34 μM ribonucleotide reductase (7 μg) in a total volume of 0.25 ml. All of the components, except for the RTPR, were mixed in a 13 $\times$ 100-mm glass test tube on ice. A series of deoxyribose standards were prepared by adding dAMP (150–500 μM) as the deoxyribose source. RTPR was added to all of the tubes except those containing the dAMP standard, and the reduction of ATP by RTPR was initiated by incubation at 37°C in a circulating water bath. Following a 10-min incubation, the reaction mixtures were placed on ice and 0.2 ml of 0.5 M 2-chloroacetamide in 0.25 M potassium phosphate (pH 7.5) was added (to derivatize free thiols which interfere with the assay's color development (*24*)). Each tube was then vortex mixed, capped with a marble, and heated at 100 °C in a heat block for 30 min. After cooling briefly on ice, 1 ml of diphenylamine reagent (*25,26*) was added. The diphenylamine reagent (made by mixing 0.5 g diphenylamine, 25 g glacial acetic acid, 750 μl conc. H_2SO_4, and 250 μl 50 mM cupric acetate), which contains 0.5 mmol cupric acetate to accelerate color development, was freshly prepared, since its storage causes a precipitate to form in the samples. The absorbance at 594 nm was measured after incubation at 37 °C for 2 h, during which the clear colorless solutions change to purple and then blue. The amount of deoxyribose generated was calculated from the dAMP standard point calibration curve (*27*). For the determination of the K_m and V_{max} of AdoCbl with RTPR, the above assay was run using only AdoCbl (i.e., no inhibitors) at various concentrations (0.1, 0.3, 0.5, 1, 1.5, 2, 3, 8 μM).

To generate cob(II)alamin, aquocobalamin was reduced by an excess of thiol to cob(II)alamin (*28–30*) under the assay conditions (30 mM DTT, pH 7.3); aquocobalamin was completely converted to cob(II)alamin by dithiothreitol within 1 min, and the resultant cob(II)alamin was stable for the duration of the assay period. Cob(II)alamin inhibition was evaluated in the absence and presence of an equivalent amount of adenosine in the assay solution, entries 6 and 7 in Table 1, respectively.

The K_m and V_{max} for AdoCbl were estimated by the preferred direct linear-plot method (*31*). At each inhibitor concentration, a direct linear plot was used to determine K_m^{app} and V^{app}, and the inhibition type (competitive, pure noncompetitive, mixed, or

TABLE 1

Apparent Inhibition Constants, K_i^{app}, for AdoCbl Analogs in RTPR Determined by the Direct Linear Method

Cobalamin Analog	K_i (μM)	Inhibition type
[3-(Adenosin-5′-*O-yl*)propyl]cobalamin (C_3)	55.8 ± 0.4	Competitive
[4-(Adenosin-5′-*O-yl*)butyl]cobalamin (C_4)	18.9 ± 0.4	Competitive
[5-(Adenosin-5′-*O-yl*)pentyl]cobalamin (C_5)	7.7 ± 0.2	Competitive
[6-(Adenosin-5′-*O-yl*)hexyl]cobalamin (C_6)	24.6 ± 0.5	Competitive
[7-(Adenosin-5′-*O-yl*)heptyl]cobalamin (C_7)	12.8 ± 0.4	Competitive
Cob(II)alamin	20.8 ± 0.2	Competitive
Cob(II)alamin + Adenosine	14.3 ± 0.2	Competitive
Cyanocobalamin (CNCbl)	42.6 ± 0.6	Competitive
Adeninylpentylcobalamin (AdePeCbl)	1.3 ± 0.9	Mixed

uncompetitive) was evaluated (*31,32*). The inhibition constants (K_i) were then determined in the usual fashion (*31–33*) from plotting K_m^{app}/V^{app} against the inhibitor concentration in the competitive inhibitor cases ([ω-(adenosin-5′-O-*yl*)alkyl]cobalamins, cob(II)alamin, and cyanocobalamin), and from plotting $1/V^{app}$ against the inhibitor concentration in the pure noncompetitive inhibition case (adenylpentylcobalamin).

Supporting information available. The following additional control and other experiments are available in the PhD Thesis of Robert K. Suto (*22*): synthesis and characterization of adeninylpentylcobalamin; kinetic analysis of AdoCbl interaction with RTPR; all the direct linear plots for each of the nine AdoCbl analogs and determination of apparent inhibition constants, K_i^{app}, in RTPR.

RESULTS

[ω-(Adenosin-5′-O-yl)alkyl]cobalamin inhibition kinetic studies. Under the conditions used, the kinetic values determined for AdoCbl interacting with RTPR are: $K_m = 1.5\ \mu M$, and $V_{max} = 1.8\ \mu M$, as determined by the direct linear-plot method (Fig. 2). The K_m value is consistent with the range of values observed previously by others ($K_m = 1.3\ \mu M$ (*16*), 1.1 μM (*34*), 1.59 μM (*34*), and 8.3 μM (*35*)).

The abilities of the individual [ω-(adenosin-5′-O-*yl*)alkyl]cobalamin analogs to inhibit RTPRs interaction with AdoCbl were measured and compared with several other cobalamins (Table 1). The K_i constants were calculated and reported using the method of direct linear plots (*31–33*); analysis of the data using the classical Dixon linearization (*36,37*) and Lineweaver–Burk linearization (*38*) methods resulted in values similar to the direct linear plot (available elsewhere (*22*)). The direct linear-plot data for AdoCbl shown in Fig. 2 is a representative example of the data (*22*). Figure 3 shows the plot used to determine K_i for the C_5 analog, again as a representative plot.

All of the [ω-(adenosin-5′-O-*yl*)alkyl]cobalamin analogs affected the K_m^{app} but not the V^{app}; hence, they are competitive inhibitors (*31–33*). In RTPR, an odd/even pattern in the oligomethylene chain number and in the observed K_i is observed in which the odd-numbered chain analogs, C_5 and C_7, are stronger inhibitors than the even-numbered chain analogs, C_4 and C_6. The C_5 analog is the strongest inhibitor of the series. The C_3-analog was a relatively poor inhibitor, suggesting that, in this analog, the 3-(adenosin-5′-O-*yl*)propyl group does not contribute significantly to binding.

As expected, none of the [ω-(adenosin-5′-O-*yl*)alkyl]cobalamin analogs were active coenzymes of RTPR when examined in the absence of AdoCbl. All of the analogs were stable in the presence of RTPR; in no case was Co-C homolysis observed (i.e., no detectable cob(II)alamin was produced).

Control experiments examining cob(II)alamin, cyanocobalamin, and adenylpentylcobalamin inhibition kinetic studies. Previously, Yamada *et al.* (*29*) found cob(II)alamin to be a linear competitive inhibitor, with an apparent K_i of $37 \pm 2\ \mu M$ in the absence of 5′-deoxyadenosine, and an apparent K_i of $3.0 \pm 2\ \mu M$ in the presence of 50 μM 5′-deoxyadenosine (and under their exact conditions). They also found that 50 μM adenosine significantly increased the binding of cob(II)alamin, but decreased the activity to roughly half. Since cob(II)alamin can also mimic the posthomolysis state, but now without the formation of the Co-C cleavage hydrogen abstraction product 5′-deoxyadenosine, and thus without generation of the cysteine radical (*39,40*), we too have examined cob(II)alamin's inhibition properties (and with our more highly

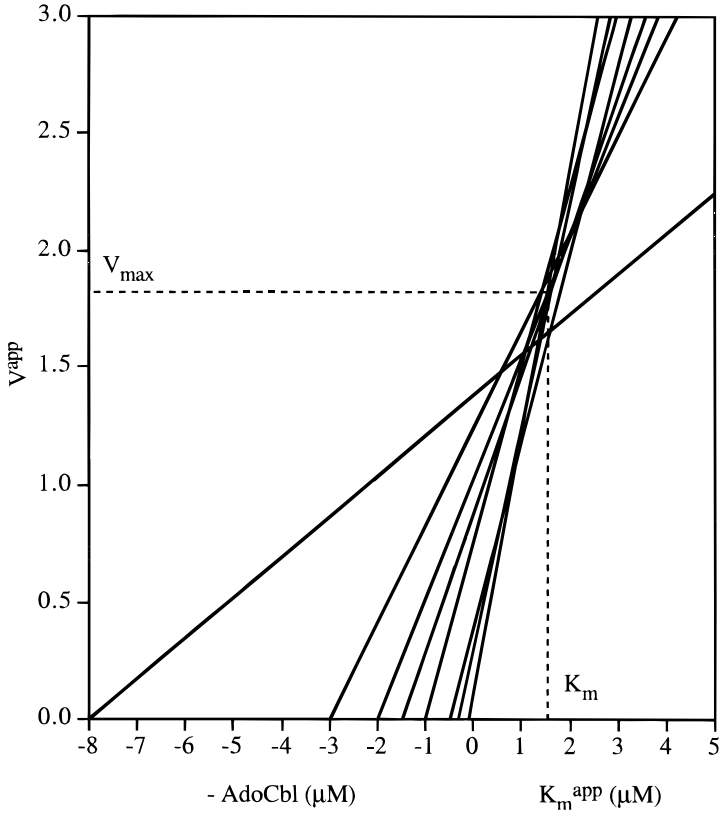

FIG. 2. Direct linear plot of the K_m and V_{max} for AdoCbl with RTPR showing the data (solid lines) and K_m and V_{max} (dotted lines).

purified, dGTP-based affinity column purified RTPR (*17*)). Cob(II)alamin proved to be a competitive inhibitor in our hands as well, with apparent K_i of 14.3 $\pm$ 0.2 μM (in the presence of an equivalent amount of adenosine) and 20.8 $\pm$ 0.2 μM (in the absence of adenosine). Adenosine was chosen for this present work, rather than 5′-deoxyadenosine, due to the fact that a 5′ oxygen is already present in the (adenosin-5′-O-*yl*)alkylcobalamin series (see Fig. 1). Thus, the presence of a stoichiometric amount of adenosine, at least at the concentrations of adenosine used (1–20 μM), lowered the K_i of the cob(II)alamin inhibition. The result that cob(II)alamin binds tightly is fully consistent with the knowledge that the corrin half of the posthomolysis intermediate is cob(II)alamin, and the idea that tight binding of cob(II)alamin and 5′-deoxyadenosine provides an important part of the driving force for homolytic cleavage of the Co-C bond (*12*,*30*,*41*).

The use of cyanocobalamin (CNCbl, Vitamin B_{12}, where a cyano group is the R group in Fig. 1) resulted in weaker competitive inhibition. CNCbl has also been found to be a moderate inhibitor in diol dehydratase and glycerol dehydratase (*15*).

Last, we also examined adeninylpentylcobalamin (AdePeCbl; Fig. 1), an analog