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Human ATP:Cob(I)alamin Adenosyltransferase and Its Interaction with Methionine Synthase Reductase*

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The final step in the conversion of vitamin B_{12} into coenzyme B_{12} (adenosylcobalamin, AdoCbl) is catalyzed by ATP:cob(I)alamin adenosyltransferase (ATR). Prior studies identified the human ATR and showed that defects in its encoding gene underlie *cblB* methylmalonic aciduria. Here two common polymorphic variants of the ATR that are found in normal individuals are expressed in *Escherichia coli*, purified, and partially characterized. The specific activities of ATR variants 239K and 239M were 220 and 190 nmol min^{-1} mg^{-1}, and their K_m values were 6.3 and 6.9 μM for ATP and 1.2 and 1.6 μM for cob(I)alamin, respectively. These values are similar to those obtained for previously studied bacterial ATRs indicating that both human variants have sufficient activity to mediate AdoCbl synthesis *in vivo*. Investigations also showed that purified recombinant human methionine synthase reductase (MSR) in combination with purified ATR can convert cob(II)alamin to AdoCbl *in vitro*. In this system, MSR reduced cob(II)alamin to cob(I)alamin that was adenosylated to AdoCbl by ATR. The optimal stoichiometry for this reaction was ~4 MSR/ATR and results indicated that MSR and ATR physically interacted in such a way that the highly reactive reaction intermediate [cob(I)alamin] was sequestered. The finding that MSR reduced cob(II)alamin to cob(I)alamin for AdoCbl synthesis (in conjunction with the prior finding that MSR reduced cob(II)alamin for the activation of methionine synthase) indicates a dual physiological role for MSR.

The vitamin B_{12} coenzymes, adenosylcobalamin (AdoCbl)[1] and methylcobalamin (CH_3Cbl), are required cofactors for at least 15 different enzymes that have a broad but uneven distribution among living forms (1, 2). In humans, two B_{12}-dependent enzymes are known (3, 4). AdoCbl-dependent methylmalonyl-CoA mutase (MCM) catalyzes the reversible rearrangement of methylmalonyl-CoA to succinyl-CoA (5). MCM is found in the mitochondrial matrix and it is required for the complete catabolism of compounds degraded via propionyl-CoA including branched-chain amino acids, odd-chain fatty acids, and cholesterol (Fig. 1) (5). The second B_{12}-dependent enzyme known in humans is CH_3Cbl-dependent methionine synthase (MS). This enzyme is found in the cytoplasm where it catalyzes the conversion of methyltetrahydrofolate and homocysteine to tetrahydrofolate and methionine (6). In mammals, methionine is an essential amino acid, and MS plays a role in recycling the *S*-adenosylhomocysteine formed from *S*-adenosylmethionine-dependent methylation reactions (7).

Humans are incapable of synthesizing AdoCbl and CH_3Cbl *de novo* and depend on dietary sources of complex cobalamin precursors, such as vitamin B_{12} (cyanocobalamin) and hydroxycobalamin (HOCbl). The pathway by which these precursors are metabolized to the coenzyme forms is thought to be similar in both prokaryotes and eukaryotes (Fig. 1). For AdoCbl synthesis, cyanocobalamin is proposed to be converted to glutathionylcobalamin, reduced successively to cob(II)alamin and cob(I)alamin, respectively, and finally, adenosylated to AdoCbl (Fig. 1) (8–13). For the synthesis of CH_3Cbl, cob(II)alamin associated with MS is reductively methylated to form CH_3Cbl (6, 14, 15). For this reaction (which is also used for the reductive activation of MS following adventitious oxidation of the cobalamin cofactor), *S*-adenosylmethionine is the methyl-group donor and methionine synthase reductase (MSR) reduces cob(II)alamin to cob(I)alamin (16, 17).

In humans, inherited defects in the MCM or MS structural genes or in the genes needed for the synthesis of B_{12} coenzymes result in methylmalonic aciduria, homocystinuria, or combined disease. These rare disorders, which are often fatal in the first year of life, result from recessive autosomal mutations that fall into nine complementation groups (*mut*, *cblABCDEFGH*) (18, 19). *cblF*, *cblC*, and *cblD* defects lead to combined disease. Prior studies indicated that *cblF* mutations affect cobalamin transport from the lysosome to the cytoplasm, whereas *cblC* and *cblD* mutations impair the conversion of complex precursors to cob(II)alamin (20, 21). The *cblE* and *cblG* complementation groups correspond to the genes for MSR (MTRR) and MS (MTR), respectively, and defects in either gene results in homocystinuria (22, 23). The *mut* group corresponds to the gene that encodes MCM (MUT) (24). The *cblA* group (MMAA gene) encodes a protein proposed to protect MCM from inactivation (25) and recent studies have shown that the *cblB* complementation group (MMAB gene) encodes the mitochondrial ATP:cob(I)alamin adenosyltransferase (ATR) (26, 27). Mutations in the *cblH* complementation group also result in methylmalonic aciduria, but the nature of the underlying defect and the corresponding gene are unknown (28).

The human enzyme that catalyzes the reduction of cob(II)alamin to cob(I)alamin for AdoCbl synthesis has not been iden-

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[1] The abbreviations used are: AdoCbl, adenosylcobalamin; CH_3Cbl, methylcobalamin; MCM, methylmalonyl-CoA mutase; MS, methionine synthase; MSR, methionine synthase reductase; HOCbl, hydroxycobalamin; ATR, ATP:cob(I)alamin adenosyltransferase; DTT, dithiothreitol; CMCbl, carboxymethylcobalamin; HPLC, high performance liquid chromatography.

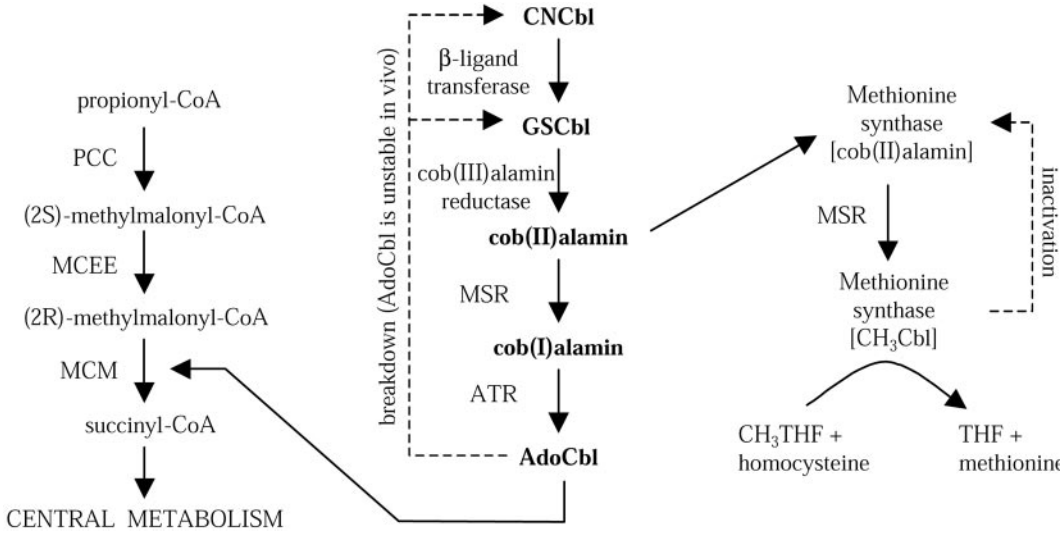

FIG. 1. **Propionyl-CoA metabolism, methionine synthesis and intracellular cobalamin metabolism.** In humans, the pathways shown are needed for the complete catabolism of compounds degraded via propionyl-CoA and for recycling homocysteine. Abbreviations: *PPC*, propionyl-CoA carboxylase; *MCEE*, methylmalonyl-CoA epimerase; *MCM*, AdoCbl-dependent methylmalonyl-CoA mutase; *CNCbl*, vitamin B_{12}; *GSCbl*, glutathionylcobalamin; *THF*, tetrahydrofolate.

tified. Recent studies showed that MSR mediates cob(II)alamin reduction for MS activation (17, 29), raising the possibility that this enzyme might play a similar role in AdoCbl synthesis. Here we describe the purification and initial biochemical characterization of the human ATR from recombinant *E. coli* and demonstrate that *in vitro* recombinant human MSR can reduce cob(II)alamin to cob(I)alamin for AdoCbl synthesis by the ATR enzyme.

MATERIALS AND METHODS

Chemicals and Reagents—Restriction enzymes and T4 DNA ligase were from New England Biolabs, Beverly, MA. Titanium III citrate was prepared as previously described (30). AdoCbl, HOCbl, β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), iodoacetic acid, flavin adenine dinucleotide (FAD), and bovine serum albumin were purchased from Sigma. Isopropyl β-D-thiogalactopyranoside, ultrapure ammonium sulfate, ATP, and dithiothreitol (DTT) were purchased from ICN Biomedicals, Inc., Aurora, OH. Pefabloc SC PLUS was from Roche Diagnostics. All other chemicals and reagents were from Fisher Scientific.

Bacterial Strains and Growth Media—The bacterial strains used were *E. coli* DH5α and BL21 DE3 RIL (Stratagene). Luria-Bertani (LB) was the rich medium used (Difco) (31). LB was supplemented with 25 μg/ml kanamycin and 20 μg/ml chloramphenicol or as indicated.

General Molecular and Protein Methods—Agarose gel electrophoresis and restriction enzyme digests were performed using standard protocols (32, 33). PCR products and plasmid DNA were gel purified using a QIAquick® gel extraction kit (Qiagen, Inc., Valencia, CA). DNA ligation was accomplished using T4 DNA ligase according to the manufacturer's instructions (New England Biolabs, Beverly, MA). Bacterial transformation and SDS-PAGE were performed as described (33).

ATP:Cob(I)alamin Adenosyltransferase Assays—ATR assays were performed as previously reported with some modifications (33). Reaction mixtures contained 200 mM Tris-HCl (pH 8.0), 2.8 mM $MgCl_2$, 10 mM KCl, 0.05 mM HOCbl, 0.4 mM ATP, and 1 mM titanium(III)citrate in a total volume of 2 ml. Reaction components (except for the ATR) were dispensed into cuvettes inside an anaerobic chamber (Coy Laboratories, Ann Arbor, MI). The cuvettes were sealed, removed from the chamber, and incubated at 37 °C for 2 min. Reactions were initiated by addition of purified recombinant ATR, and AdoCbl formation was measured by following the decrease in absorbance at 388 nm ($\Delta\epsilon_{388}$ = 24.9 cm^{-1} mM^{-1}).

Construction of ATR Expression Strains—Plasmid pNL166 (27) was restricted with BglII and HindIII to release the 645-bp fragment that encodes the ATR enzyme. This fragment was gel purified and ligated into a modified pET-41a expression vector, pTA925 (33). Ligation mixtures were used to transform *E. coli* DH5α by electroporation and transformants were selected by plating on LB kanamycin medium. Pure cultures were prepared from selected transformants and plasmid DNA isolated from these strains was analyzed by restriction digestion and DNA sequencing. Clones with the expected DNA sequence were transformed into *E. coli* strain BL21 DE3 RIL (Stratagene) for high-level protein production.

PCR was used to construct the ATR 239M polymorphic variant. Plasmid pNL166 (27) provided the template DNA. The primers used for amplification were 5′-GCCGCCAGATCTTATGCCTCAGGGCGTGGA-AGACGGG-3′ (forward) and 5′-GCCGCCAAGCTTTCAGAGTCCCTCA-GACTCGGCCGATGGGTCATTTTTCATGTATATTTTCTCTTGATTC-CC-3′ (reverse). These primers introduced BglII and HindIII restriction sites into the PCR product and were designed to change the lysine at position 239 to methionine. Ligation, transformation, and analysis of plasmid DNA were performed as described above. Clones with the expected DNA sequence were transformed into *E. coli* BL21 DE3 RIL for protein production.

Purification of the Human ATR Variants—Soluble cell extracts of the *E. coli* expression strains were used for purification of both ATR variants. The growth of cells and the preparation of cell extracts was carried out as described (33). For ammonium sulfate precipitation, cell extracts of ATR 239K (160 mg of protein) and 239M (180 mg of protein) were diluted to 1 mg/ml in 50 mM sodium phosphate (pH 7.0), 300 mM NaCl. Then, ultrapure ammonium sulfate was added as a fine powder with stirring. Precipitated proteins were centrifuged for 15 min at 12,000 × g_{max} using a Beckman JLA-10.500 rotor. The protein pellets were resuspended in 4 ml of 5 mM potassium phosphate (pH 6.8), and passed through a 0.45-μm filter. ATR 239K (60 mg) and 239M (35 mg) were applied to a 60-ml ceramic hydroxyapatite column (Bio-Rad) equilibrated with 5 mM potassium phosphate (pH 6.8). The column was washed with 60 ml of equilibration buffer and eluted with a 600-ml linear gradient of 5 to 200 mM potassium phosphate (pH 6.8) at a flow rate of 5 ml/min. Fractions containing ATR of the highest purity were pooled and exchanged into 10 mM potassium phosphate (pH 7.0), 50 mM KCl using a Vivaspin centrifugal concentrator with a molecular weight cutoff of 10,000 daltons (Viva Science, Binbrook, UK). Samples were filtered through a 0.45-μm pore size membrane and further purified using a Mono Q HR 10/10 anion exchange column (Amersham Biosciences). ATR 239K (10 mg) and 239M (9 mg) were applied to the column that had been equilibrated with 10 mM potassium phosphate (pH 7.0), 50 mM KCl. Then, the column was washed with 8 ml of equilibration buffer and eluted with a 160-ml linear gradient from 50 to 1000 mM KCl in 10 mM potassium phosphate (pH 7.0) at a flow rate of 4 ml/min. Protein elution was monitored by following the absorbance of the column effluent at 280 nm and 8-ml fractions were collected. Purified ATR was concentrated and stored in 10 mM phosphate buffer (pH 7.0), 130 mM KCl, and 50% glycerol at −20 °C.

MSR-ATR Assay—The basis of this assay is that MSR converts cob(II)alamin to cob(I)alamin, which is in turn converted to AdoCbl by the ATR enzyme. The conversion of cob(I)alamin to AdoCbl proceeds with an increase in absorbance at 525 nm that allows quantitation ($\Delta\epsilon_{525}$ = 4.8 cm^{-1} mM^{-1}). Assays contained 200 mM Tris (pH 8.0), 1.6

TABLE I
Purification table for human ATR variants 239K and 239M

Purification step	Total protein		Specific activity		Total activity		Yield		Purification	
	239K	239M	239K	239M	239K	239M	239K	239M	239K	239M
	mg		*nmol min^{-1} mg^{-1}*		*nmol min^{-1}*		*%*		*-fold*	
Cell-free extract[a]	160	180	49	35	7840	6300	100	100	1	1
Ammonium sulfate precipitate	60	35	110	70	6600	2450	84	39	2.2	2
Hydroxyapatite chromatography	10	9	118	97	1180	873	15	14	2.4	2.8
MonoQ chromatography	5	3	220	190	1100	570	14	9	4.5	5.4

[a] Cell-free extracts were prepared from recombinant *E. coli*.

mM potassium P_i, 2.8 mM $MgCl_2$, 100 mM KCl, 0.1 mM cob(II)alamin, 0.4 mM ATP, 1 mM DTT, 1 mM NADPH, and purified MSR and ATR as indicted in the text. The total assay volume was 2 ml and anaerobic procedures were used as described above for the ATR assay. Cob(I-I)alamin was prepared by exposing an anoxic solution of 1 mM AdoCbl to a 150 watt incandescent light at a distance of 15 cm for 30 min (33). ATR 239K was purified as described above and recombinant human MSR was purified as described previously (17). Reaction mixtures were incubated at 37 °C for 2 min prior to initiating reactions by the addition of an anoxic solution of NADPH.

Measurement of Cob(I)alamin with Iodoacetate—Iodoacetate reacts rapidly and quantitatively with cob(I)alamin to form carboxymethylcobalamin (CMCbl). This reaction proceeds with an increase in absorbance at 525 nm ($\Delta\epsilon_{525}$ = 5.3 cm^{-1} mM^{-1}) and hence provides a facile method for quantitating cob(I)alamin (34). Anoxic stock solutions of iodoacetate (40 mM) were prepared and used the same day. The stock solution was shielded from light using aluminum foil and was added to assay mixtures just prior to the initiation of reactions.

Separation and Quantification of Cobalamins by HPLC—HOCbl, AdoCbl, and CMCbl were separated and quantified by HPLC using a NovaPak C_{18} column (3.9 × 150 mm) equipped with a C_{18} Sentry guard column (Waters, Milford, MA). Samples (200 μl) were loaded onto the column and eluted with a 30-ml linear gradient from 10 to 90% methanol in 50 mM sodium acetate (pH 4.6) at a flow rate of 1 ml/min. The absorbance of column effluent was monitored at 365 nm, and analytes were quantified by comparison of peak areas to a standard curve. The CMCbl standard was prepared by incubating 2 ml of 200 mM Tris-HCl (pH 8.0), 1.6 mM potassium P_i, 2.8 mM $MgCl_2$, 100 mM KCl, 0.1 mM cob(II)alamin, 1 mM DTT, 0.1 mM FAD, and 0.4 mM iodoacetate at 37 °C for 1 h, which resulted in quantitative conversion of cob(II)alamin to CMCbl (data not shown). Standard curves were prepared using 5, 10, 20, 30, and 40 nmol of CMCbl, AdoCbl, or HOCbl. Peak areas were integrated using Waters Breeze software. The standard curves for CMCbl, AdoCbl, and HOCbl had regression coefficients (r^2) of 0.9985, 0.9993, and 0.9956, respectively. Solutions that contained CMCbl or AdoCbl were shielded from light to prevent photolysis.

Effect of Ionic Strength on the MSR-ATR System—The ionic strength of MSR-ATR assays was varied by the addition of KCl. Ionic strength was calculated using the formula $I = 1/2\Sigma\ (c_i z)^2$, where I is the ionic strength, c_i is the molar concentration of each type of ion, and z is the charge of each ion (35). For lower values of ionic strength, it was necessary to reduce the Tris-HCl concentration.

DNA Sequencing—DNA sequencing was carried out by University of Florida, Interdisciplinary Center for Biotechnology Research, DNA Sequencing Core facility, and University of Florida, Department of Microbiology and Cell Science, DNA Sequencing facility, as described previously (36).

RESULTS

Purification of the Human ATR Variants—The human ATR catalyzes the transfer of a 5′-deoxyadenosyl group from ATP to the central cobalt atom of cob(I)alamin to form AdoCbl (27). Two common polymorphic variants of the human ATR having either lysine or methionine as amino acid 239 (ATR 239M and 239K) were recently shown to be present in 54 and 46% of 72 control cell lines, respectively (26). We previously produced ATR 239K as a glutathione *S*-transferase fusion protein in *E. coli* (27). Here, subcloning and site-directed mutagenesis were used to construct *E. coli* expression strains that produce high levels of ATR 239K and 239M without fusion tags and lacking their predicted mitochondrial targeting sequences. Both ATR variants were produced at high levels and purified to apparent homogeneity from soluble cell extracts of recombinant *E. coli*. The purification consisted of three steps including ammonium sulfate precipitation, ceramic hydroxyapatite, and Mono Q chromatography (Table I). Both ATR variants precipitated between 40 and 50% ammonium sulfate saturation and eluted from the hydroxyapatite and Mono Q columns at 110–135 mM potassium P_i and 114–130 mM KCl. ATR 239K and 239M were purified 4.5- and 5.4-fold with specific activities of 220 and 190 nmol min^{-1} mg^{-1}, respectively. The yields were 14 and 9%. During purification, the inclusion of KCl in chromatography and storage buffers increased enzyme stability.

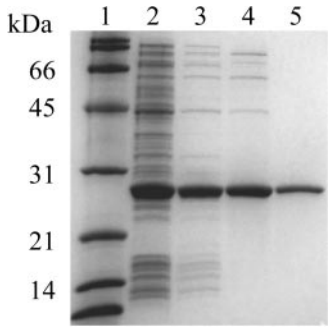

FIG. 2. **Purification of the human ATR.** SDS-PAGE was used to assess the purification of ATR 239K. *Lane 1*, molecular mass markers; *lane 2*, 10 μg of soluble cell-free extract from the *E. coli* expression strain; *lane 3*, 5 μg of ammonium sulfate precipitate; *lane 4*, 4 μg of hydroxyapatite eluate; *lane 5*, 2 μg of Mono Q fraction containing purified ATR. The purification of ATR 239M proceeded similarly (not shown).

SDS-PAGE was used to follow the purification of both ATR variants. Because purification of both enzymes proceeded similarly, only the gel used to assess the purity of ATR 239K is depicted (Fig. 2). Following the final Mono Q chromatography step, both variants appeared to be homogenous following staining with Coomassie Brilliant Blue.

Linearity of the ATR Reaction—The effect of ATR concentration on activity was determined. Results showed that the rate of adenosylation was proportional to ATR concentration from 5 to 115 μg for both variants and linear regression yielded r^2 values of 0.9995 and 0.9993 for ATR 239K and 239M, respectively.

ATR Reaction Requirements—To determine the ATR reaction requirements, key assay components were individually omitted. For both variants, there was no detectable activity in the absence of ATR, ATP, HOCbl, or titanium(III) citrate (the reducing agent used to produce cob(I)alamin). In the complete reaction mixture, there was 215 and 194 nmol min^{-1} mg^{-1} activity for ATR 239K and 239M, respectively. In the absence of $MgCl_2$, activity decreased by 20% for both ATR variants. There was no significant difference in activity in the absence of KCl.

Alternative Nucleotide Donors—The specificity of the ATR variants for ATP, CTP, GTP, UTP, ADP, and AMP was examined. ATP was found to be the best substrate giving a specific activity of 207 and 197 nmol min^{-1} mg^{-1} for ATR 239K and 239M, respectively. These values were set as 100% activity. When CTP, GTP, or UTP were tested as substrates for ATR 239K there was 9, 16, or 8% activity, respectively (Table II).

TABLE II
Use of alternative nucleotide donors by the human ATR

Nucleotide donor[a]	% Activity	
	ATR 239K	ATR 239M
ATP	100	100
ADP	ND[b]	ND
AMP	ND	ND
CTP	9	6
GTP	16	14
UTP	8	6

[a] ATP is the physiological substrate for the ATR and activity with this nucleotide was set to 100%
[b] ND, none detected.

Similar results were found for ATR 239M (6, 14, or 6% activity with CTP, GTP, or UTP, respectively). For both ATR variants, there was no detectable activity when AMP or ADP were substituted for ATP.

K_m and V_{max} Values for ATR 239K and 239M—ATR 239K and 239M displayed Michaelis-Menten kinetics with respect to both ATP and cob(I)alamin. Lineweaver-Burk double-reciprocal plots were used to calculate K_m and V_{max} values (35). For ATR 239K and 239M, the K_m values for ATP were 6.3 and 6.9 μM, and the K_m values for cob(I)alamin were 1.2 and 1.6 μM, respectively. The V_{max} values for ATR 239K and 239M were 250 and 200 nmol min^{-1} mg^{-1}, respectively. When the K_m values for cob(I)alamin were determined, saturating levels of ATP (400 μM) were added to assay mixtures while varying the concentration of cob(I)alamin. Similarly, saturating levels of cob(I)alamin (50 μM) were added to assays when the K_m values for ATP were determined. Each value used for kinetic calculations was the average of three measurements of the initial reaction rate.

MSR Reduces Cob(II)alamin to Cob(I)alamin for AdoCbl Synthesis—The human enzyme that catalyzes the third step of cyanocobalamin assimilation, the reduction of cob(II)alamin to cob(I)alamin for AdoCbl synthesis by the ATR enzyme, is unknown. Recently, recombinant human MSR was shown to catalyze cob(II)alamin reduction for MS activation (17). To test whether this enzyme can also reduce cob(II)alamin to cob(I)alamin for AdoCbl synthesis, an *in vitro* assay was used (MSR-ATR assay). In assays that contained standard components, cob(II)alamin, ATP, DTT, NADPH, 100 μg of purified MSR, and 10 μg of purified ATR, AdoCbl was formed at a rate of 0.96 nmol min^{-1}. Controls showed that no measurable AdoCbl was formed in the absence of ATR, MSR, ATP, or cob(II)alamin. When NADPH was excluded from the reaction mixture, 65% of the total activity remained indicating that DTT can partially replace NADPH as the electron donor for this reaction. When DTT was omitted, 100% activity remained showing that NADPH can function efficiently as the electron donor without the need for DTT. In addition, several further tests were used to establish that AdoCbl was the product of the reaction. The UV-visible spectrum of completed reactions was characteristic of AdoCbl, and the reaction product was photolyzed by a 30-min exposure to incandescent light with the formation of cob(II)alamin (Fig. 3). Moreover, the product of the MSR-ATR reaction co-migrated with authentic AdoCbl by reverse-phase HPLC following co-injection (not shown). Thus, results showed that the combination of the MSR and ATR enzymes converted cob(II)alamin to AdoCbl. Because prior studies have shown that the human ATR requires cob(I)alamin for AdoCbl synthesis and is inactive with cob(II)alamin, these results indicate that MSR reduced cob(II)alamin to cob(I)alamin for AdoCbl synthesis by ATR.

Cob(I)alamin Is Sequestered by the MSR-ATR System—Above, we presented evidence that MSR can reduce cob(II)alamin to cob(I)alamin for adenosylation by ATR. In principle, there are two ways in which this could occur. MSR could reduce cob(II)alamin to cob(I)alamin directly, which is subsequently released into solution and diffuses to ATR. Alternatively, MSR could reduce cob(II)alamin bound to ATR and the interaction between the two proteins could sequester cob(I)alamin.

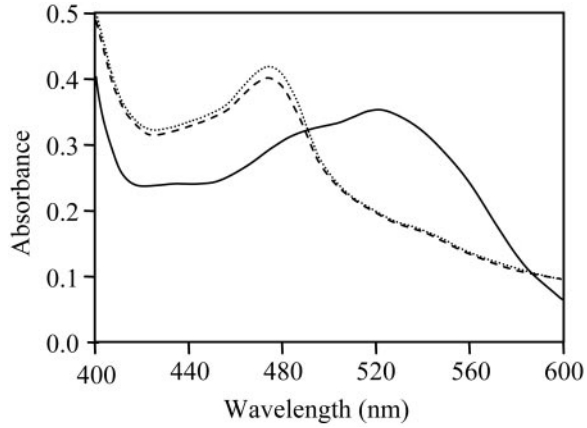

FIG. 3. **Absorbance spectra of an MSR-ATR assay.** Prior to the addition of NADPH the spectrum is that of cob(II)alamin (*dotted line*); 1 h after the addition of NADPH and incubation at 37 °C, the spectrum is that of AdoCbl (*solid line*); after photolysis, the spectrum is characteristic of cob(II)alamin (*dashed line*).

TABLE III
The MSR-ATR system sequesters cob(I)alamin

Variable assay components[a]	Amount detected		
	AdoCbl	CMCbl	HOCbl
		nmol	
NADPH, MSR, ATR, and iodoacetate	85	46	45
DTT, FAD, ATR, and iodoacetate	18	165	6.0
NADPH, MSR, and ATR	94	ND[b]	81

[a] Reaction mixtures contained 200 mM Tris (pH 8.0), 1.6 mM KP_i, 2.8 mM $MgCl_2$, 100 mM KCl, 0.1 mM (200 nmol) cob(II)alamin, 0.4 mM ATP, and the components indicated in the table in the following amounts: 10 μg of purified ATR, 150 μg of purified MSR; 1 mM NADPH, 1 mM DTT, 50 μM FAD. Cob(I)alamin was generated enzymatically using NADPH and purified MSR (first and third lines) or chemically by the combination of DTT and FAD, which necessarily generates free cob(I)alamin (second line). HOCbl, CMCbl, and AdoCbl were resolved and quantitated by HPLC. In the reverse-phase system used their retention times were 11.9, 13.8, and 15.3 min, respectively.
[b] ND, not determined.

To test whether cob(I)alamin was sequestered or released into solution during AdoCbl synthesis by the MSR-ATR system, a chemical trap for cob(I)alamin was used. Iodoacetate (which reacts rapidly and quantitatively with cob(I)alamin to form CMCbl) was added to an MSR-ATR assay at a 1000-fold excess compared with the ATR (400 μM compared with 0.4 μM). The assay was allowed to proceed to completion. Then, CMCbl and AdoCbl were resolved and quantitated by reverse-phase HPLC. Results showed that 35% of the cob(I)alamin formed was converted to CMCbl by reaction with iodoacetate and that 65% was converted to AdoCbl via the ATR enzyme (Table III, first line). If "free" cob(I)alamin had been produced under the conditions used, it is expected that the majority should have been converted to CMCbl by reaction with the large excess of iodoacetate prior to diffusion to the ATR for conversion to AdoCbl. Hence, the above results indicated that a significant portion of the cob(I)alamin was sequestered (protected from reaction with iodoacetate) during conversion of cob(II)alamin to AdoCbl by MSR and ATR.

As a control, assays were performed that were similar to those described above except that MSR was replaced with a

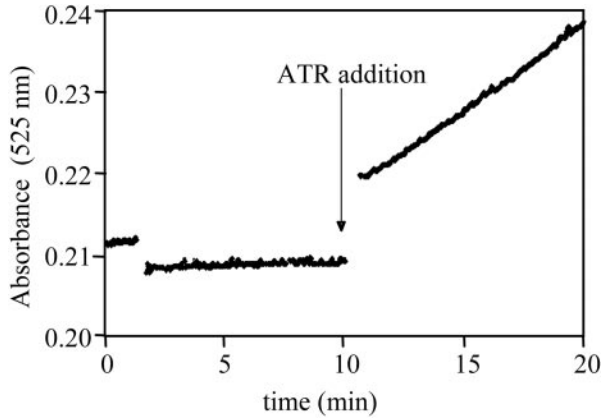

FIG. 4. **MSR produces little cob(I)alamin in the absence of the ATR enzyme.** During the first 10 min of the reaction little cob(II)alamin was reduced to cob(I)alamin. During this phase, iodoacetate was used to detect cob(I)alamin. Iodoacetate reacts chemically with cob(I)alamin to form CMCbl and this reaction proceeds with an increase in absorbance at 525 nm. At the 10-min time point, 10 μg of ATR was added to the assay mixture and the rate of cob(I)alamin production increased ~46-fold. This large increase in the rate of cob(I)alamin formation indicated a physical interaction between the MSR and ATR enzymes. The assay conditions used were similar to those for the MSR-ATR assay and controls showed that iodoacetate worked effectively under these conditions.

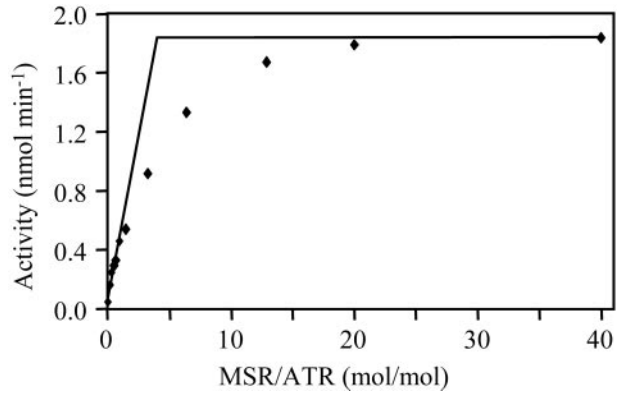

FIG. 5. **Stoichiometry of the MSR-ATR system.** The MSR-ATR assay was used for these studies. 10 μg (0.4 nmol) of purified ATR was used with the molar ratio of MSR indicated. Maximal activity required ~4 MSR/ATR.

combination of 1 mM DTT plus 50 μM FAD to chemically generate cob(I)alamin *in situ*. Chemical reduction of cob(II)alamin necessarily produces free cob(I)alamin, which must then diffuse to the ATR before it can be converted to AdoCbl. Under these conditions, only 10% of the cob(I)alamin formed was converted to AdoCbl, whereas 90% was converted to CMCbl (Table III, *second line*). This was in contrast to results obtained when MSR was used to generate cob(I)alamin for ATR where 65% of the cob(I)alamin formed was converted to AdoCbl (Table III, *first line*). Hence, results indicate that the MSR-ATR system sequesters cob(I)alamin during the conversion of cob(II)alamin to AdoCbl.

Interestingly, further studies showed that iodoacetate had relatively little effect on the total amount of AdoCbl formed by the MSR-ATR system. In the absence or presence of iodoacetate, 94 or 85 nmol of AdoCbl was produced, respectively (Table III). This indicated that the majority of the cob(I)alamin produced by the MSR-ATR system was sequestered.

MSR Produces Little Cob(I)alamin in the Absence of the ATR—Iodoacetate was also used to test whether MSR can produce free cob(I)alamin in the absence of ATR. Assays were prepared that were similar in composition to the MSR-ATR assay except that ATR was replaced with iodoacetate. During the first 10 min of the reaction, cob(I)alamin was formed at a very low rate (0.02 nmol min^{-1}) (Fig. 4). Then, at the 10-min time point, 10 μg of purified ATR was added to the assay and the rate of cob(I)alamin formation increased ~46-fold. These findings showed that the presence of ATR greatly enhanced the production of cob(I)alamin by MSR indicating a physical interaction between MSR and ATR. Control experiments showed that bovine serum albumin had no effect on the rate of cob(I)alamin formation when substituted for ATR in the second phase of the reaction shown in Fig. 4. Moreover, controls showed that iodoacetate was working effectively under our assay conditions. In similar reaction mixtures containing DTT and FAD, the cob(I)alamin formed was efficiently trapped by iodoacetate (data not shown).

Stoichiometry of the MSR-ATR System—The dependence of the ATR reaction on the molar ratio of MSR/ATR was determined at ratios ranging from 0 to 40 (Fig. 5). Maximal activity occurred at a ratio of ~4 mol of MSR (which is a monomer) per mol of ATR monomer. This ratio is similar to the optimal stoichiometry for activation of MS by MSR and is a reasonable value for a physiological process (17).

The data in Fig. 5 were found to fit well to Michaelis-Menten kinetics with a K_{act} for MSR of 0.69 ± 0.06 μM. This value is 8.6-fold higher than the previously reported K_{act} of MSR for MS (80.7 ± 13.7 nM) and is a physiologically relevant value (17).

Ionic Strength Dependence of Cobalamin Reduction and Adenosylation by the MSR-ATR System—Variation of the ionic strength from 15 to 720 mM had relatively little effect on the synthesis of AdoCbl by the MSR-ATR system (data not shown). Within this range, greater than 65% activity was retained. This is in contrast to the relatively narrow ionic strength dependence of MS activation by MSR (17).

DISCUSSION

Prior studies identified the human ATR and showed that defects in its encoding gene (MMAB) underlie *cblB* methylmalonic aciduria (26, 27). Investigations also identified two common polymorphic variants of ATR that are found in normal individuals (26). Here, both ATR variants were expressed in *E. coli*, purified, and found to have similar kinetic properties. The specific activities of variants 239K and 239M were 220 and 190 nmol min^{-1} mg^{-1}, and the K_m values were 6.3 and 6.9 μM for ATP and 1.2 and 1.6 μM for cob(I)alamin, respectively. These values are roughly similar to those previously reported for bacterial ATRs where specific activities range from 53 to 619 nmol min^{-1} mg^{-1}, and K_m values from 2.8 to 110 μM for ATP and from 3 to 5.2 μM for cob(I)alamin (33, 37, 38). The K_m values for the human ATR are appropriate to its physiological role because cellular levels of cobalamin are typically 1 μM or less in higher organisms and ATP concentrations are in the low millimolar range (1). Thus, the results reported here show that both common ATR variants are essentially wild-type and have kinetic properties sufficient to meet cellular needs for AdoCbl synthesis.

Three classes of ATRs that are unrelated in amino acid sequence have been identified, the PduO-type, CobA-type, and EutT-type (33). Members of the CobA- and PduO-types have been purified, partially characterized, and their crystal structures determined (33, 37–39). The CobA enzyme has a relatively broad specificity for nucleotide donors. It is active with ATP, CTP, GTP, UTP, and ITP and contains a modified P-loop for nucleotide binding (39). In contrast, the *Thermoplasma acidophilum* ATR, which is a PduO-type enzyme and lacks a recognizable P-loop, uses only ATP and deoxy-ATP as nucleotide donors (38). Thus, different modes of ATP binding are thought to account for observed differences in nucleotide spec-

ificity by CobA- and PduO-type enzymes. The human ATR is a PduO-type enzyme, but results presented here showed that its nucleotide specificity is intermediate to that of the CobA and *T. acidophilum* enzymes. In addition, the K_m of the human and *T. acidophilum* enzymes for ATP is 7 *versus* 110 μM, suggesting some differences between the two enzymes in their mode of ATP binding that might be expected because these enzymes are 32% identical in amino acid sequence.

Results reported here demonstrate that an *in vitro* system containing the human MSR and ATR converted cob(II)alamin to AdoCbl. In this system, MSR reduced cob(II)alamin to cob(I)alamin, which in turn was converted to AdoCbl by ATR. When 0.2 μM purified ATR and 0.6 μM purified MSR were used, the rate of AdoCbl was 0.96 nmol min^{-1}. It is probable that this level of activity is sufficient to meet physiological needs. Cells require only very small quantities of AdoCbl. Typical intracellular levels are about 1 μM, whereas other well known coenzymes are present at 100–1000-fold higher concentrations or more (40). Furthermore, the MSR-ATR system is about 10-fold more active than the analogous bacterial system, which consists of the Fpr, FldA, and CobA proteins (41). Thus, the MSR-ATR system mediates the conversion of cob(II)alamin to AdoCbl at a physiologically relevant rate. To our knowledge, this is the first reported evidence that MSR can catalyze the reduction of cob(II)alamin to cob(I)alamin for AdoCbl synthesis.

In addition to its role in AdoCbl synthesis, prior studies showed that MSR also reduces MS-bound cob(II)alamin to cob(I)alamin for the reductive activation of MS (16, 17). It is known that MS activation occurs in the cytoplasm but that AdoCbl synthesis occurs in the mitochondrion. Therefore, to carry out both functions MSR must reside in both compartments. Interestingly, recent studies have indicated that alternative transcript splicing leads to the production of MSR enzymes with and without mitochondrial targeting sequences providing further evidence of a dual role for this enzyme (29).

Findings reported here that indicate dual functionality for MSR bring to light an interesting parallel between cobalamin reduction in bacteria and in humans. Biochemical evidence indicates that *E. coli* flavodoxin (FldA) catalyzes the reduction of cob(II)alamin to cob(I)alamin for both the reductive activation of MS (MetH) and the synthesis of AdoCbl by the bacterial ATR (CobA) (14, 15, 42). Similarly, human MSR reduces cob(II)alamin for both MS activation (16, 17) and AdoCbl synthesis by the human ATR (this study). Thus, although the human MSR and ATR lack significant sequence similarity to their bacterial counterparts (FldA and CobA), evolution appears to have driven analogous dual physiological roles for FldA and MSR.

In this report, we also conducted studies to determine whether cob(I)alamin was sequestered or released free in solution during the conversion of cob(II)alamin to AdoCbl by the MSR-ATR system. Experiments in which iodoacetate was used as a chemical trap indicated that cob(I)alamin was sequestered. This is likely to be physiologically important. Cob(I)alamin is one of the strongest nucleophiles that exists in aqueous solution and an extremely strong reductant ($E'_0 = -0.61$ V) (43, 44). It oxidizes instantaneously in air and rapidly reduces protons to H_2 gas at pH 7 (45). Hence, within cells, sequestration of cob(I)alamin would be important for preventing nonspecific or deleterious side reactions.

The finding that cob(I)alamin was sequestered during the conversion of cob(II)alamin to AdoCbl also indicates a physical interaction between MSR and ATR. This raises the question of whether MSR interacts with both MS and ATR by a conserved mechanism. Prior studies of the MSR-MS reaction indicated that the optimal stoichiometry is ~4 MSR/MS and that activity is maximal over a narrow range of ionic strength indicating that electrostatic interactions are important to the MSR-MS interaction (17). Here we found that the optimal ratio for the MSR-ATR system is also ~4 MSR/ATR, but in contrast to the MSR-MS system, there was no strict dependence on ionic strength. Hence, although MSR has a conserved function in both systems (cob(II)alamin reductase), the details of the protein-protein interactions between MSR and either MS or ATR are apparently somewhat different.

The investigations reported here support prior studies indicating redundant systems for mitochondrial cobalamin reduction in mammals. Several lines of evidence indicated a role for MSR in the reduction of cob(II)alamin to cob(I)alamin for AdoCbl synthesis: the MSR and ATR enzymes converted cob(II)alamin to AdoCbl at a significant rate; the stoichiometry needed for maximal activity was reasonable (4MSR/ATR); and cob(I)alamin was sequestered as is expected for the physiologically relevant system. On the other hand, prior investigations showed that patients with inherited defects in MSR (*cblE* disorder) generally have homocystinuria with the exception of one reported case resulting in combined homocystinuria and mild methylmalonic aciduria (46). This indicates that *in vivo* cob(II)alamin reduction for AdoCbl synthesis can occur independently of MSR. Of the known complementation groups that result in methylmalonic aciduria, only the *cblH* and *cblA* groups have phenotypes consistent with a role in mitochondrial cobalamin reduction. Recent studies have indicated that the *cblA* group functions to protect MCM from inactivation (25) and there has been only one reported case of *cblH* methylmalonic aciduria (28, 47). Thus, at this time, there are no strong indications as to the identity of the alternative reductase(s). Redundant systems for cobalamin metabolism are known in bacterial systems (33, 48).[2] There, inducible systems provide extra capacity during times of high demand. Similarly, in humans multiple cobalamin reductases might be required for an efficient response to physiological stresses or diets that result in higher rates of propionyl-CoA formation. For example, MSR could be targeted to the mitochondria under circumstances where the diet is rich in amino acids metabolized via propionyl-CoA.

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