Cofactor Editing by the G-protein Metallochaperone Domain Regulates the Radical B₁₂ Enzyme IcmF**

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IcmF is a 5′-deoxyadenosylcobalamin (AdoCbl)-dependent enzyme that catalyzes the carbon skeleton rearrangement of isobutyryl-CoA to butyryl-CoA. It is a bifunctional protein resulting from the fusion of a G-protein chaperone with GTPase activity and the cofactor- and substrate-binding mutase domains with isomerase activity. IcmF is prone to inactivation during catalytic turnover, thus setting up its dependence on a cofactor repair system. Herein, we demonstrate that the GTPase activity of IcmF powers the ejection of the inactive cob(II)alamin cofactor and requires the presence of an acceptor protein, adenosyltransferase, for receiving it. Adenosyltransferase in turn converts cob(II)alamin to AdoCbl in the presence of ATP and a reductant. The repaired cofactor is then reloaded onto IcmF in a GTPase-gated step. The mechanistic details of cofactor loading and offloading from the AdoCbl-dependent IcmF are distinct from those of the better characterized and homologous methylmalonyl-CoA mutase/G-protein chaperone system.

Acyl-CoA mutases are a group of enzymes that utilize 5′-deoxyadenosylcobalamin (AdoCbl) or coenzyme B₁₂ as a cofactor for catalyzing carbon skeleton rearrangement reactions (1, 2). Isobutyryl-CoA mutase is a member of this enzyme family that is found in bacteria and catalyzes the reversible rearrangement of isobutyryl- and butyryl-CoA mutase (Fig. 1A) (3). A chemically similar interconversion of methylmalonyl-CoA and succinyl-CoA is catalyzed by methylmalonyl-CoA mutase (MCM) (4), which is the only AdoCbl-dependent enzyme found in mammals. AdoCbl-dependent isomerization reactions are initiated by the homolytic cleavage of the cobalt-carbon bond of AdoCbl yielding cob(II)alamin and the working 5′-deoxyadenosyl radical, which initiates the chemical reaction by hydrogen atom abstraction from the substrate. AdoCbl thus serves as a latent radical reservoir (5), and substrate binding accelerates the homolytic cleavage rate in AdoCbl-dependent enzymes by a factor of ~10^12 (6, 7). At the end of each catalytic cycle, the 5′-deoxyadenosyl radical and cob(II)alamin recombine, thus regenerating the resting AdoCbl form of the cofactor (Fig. 1A). During catalytic turnover, the occasional loss of the 5′-deoxyadenosine moiety renders MCM prone to inactivation (8) and necessitates its dependence on a repair system for reentry into the catalytic cycle. Two proteins, adenosyltransferase (ATR) (9) and a G-protein chaperone (10), are needed for cofactor repair and reloading. The Methylobacterium extorquens orthologs of MCM, ATR and the G-protein (known as MeaB), have been characterized extensively (8, 11–17), and our understanding of the mechanism of cofactor repair derives primarily from studies on this system. The importance of the chaperone-dependent cofactor loading and repair mechanisms is underscored by the existence of disease-causing mutations in both human ATR and the G-protein chaperone (18, 19).

IcmF is a naturally occurring fusion in which the G-protein chaperone is inserted between the B₁₂- and substrate-binding domains of isobutyryl-CoA mutase (20). In addition to the canonical reaction, IcmF also catalyzes the interconversion of pivalyl-CoA and isovaleryl-CoA, albeit inefficiently (21). Both the isomerization reactions are very prone to inactivation, which can be monitored by the oxidation of cob(II)alamin to aquocobalamin (OH₂Cbl) under aerobic conditions (20–22). The M. extorquens MCM inactivates at an ~40-fold slower rate than IcmF (21), and in the presence of GTP and MeaB (which binds to MCM with nanomolar affinity) (14), it releases cob(II)alamin into solution. MeaB can also use the non-hydrolyzable analog, GMPPNP, to trigger ejection of cob(II)alamin from MCM, indicating that the binding energy of GTP rather than the chemical energy of hydrolysis drives cob(II)alamin release (8). In contrast, GTP neither affects the rate of IcmF inactivation with its natural substrates (21) nor causes release of cob(II)alamin into solution under aerobic or anaerobic conditions. Hence, the molecular mechanism for cob(II)alamin unloading by IcmF appears to be distinct from the MCM/MeaB system.

Crystal structures of the Cupriavidus metallidurans IcmF provided the first architectural views of the G-protein chaperone–mutase molecular machinery, which controls the fidelity

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The abbreviations used are: AdoCbl, 5′-deoxyadenosylcobalamin; ATR, adenosyltransferase; OH₂Cbl, aquocobalamin; MCM, methylmalonyl-CoA mutase; MCM, methylmalonyl-CoA mutase; GMPPNP, guanosine-5′-[(β,γ)-imino]triphosphate; GMPPCP, guanosine-5′-[(β,γ)-methylene]triphosphate.
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In this study, we report that IcmF uses a more elaborate mechanism for cofactor repair than previously described for the MCM-MeaB system. Offloading inactive cofactor from IcmF requires the presence of ATR to receive it and is driven by GTP hydrolysis. ATR, in turn, catalyzes an adenosylation reaction converting cob(II)alamin in the presence of a reductant to the active AdoCbl cofactor, which can be reloaded onto IcmF. In addition, a GTP-dependent switch gates cofactor loading from ATR to IcmF.

Results

GTP/GDP Are Allosteric Ligands of ATR—Purified C. metallidurans ATR migrated as a single peak by gel filtration chromatography and eluted with an apparent molecular mass of ~72 kDa (data not shown), consistent with it being a homotrimer (subunit molecular mass ~22.6 kDa). As shown previously with other PduO-type ATRs (26–28), the C. metallidurans ATR binds AdoCbl in the base-off conformation with a characteristic peak at 458 nm (Fig. 2A, red trace). However, unlike the M. extorquens ATR, which binds AdoCbl at only two of three available active sites per trimer and with unequal affinity (13), the C. metallidurans ATR can bind 3 mol of AdoCbl of cofactor loading/unloading (Fig. 1B) (23, 24). Conserved elements used for signaling by G-proteins that were disordered in the structures of the “stand-alone” MeaB (17, 25) are organized in the G-protein domain of IcmF. The structural ordering of these signaling elements in IcmF results from their interactions with the substrate- and B\textsubscript{12} binding modules of the mutase domain (24). These interactions create the molecular tracts for bidirectional signal transduction between the mutase and G-domains that are used for sensing and responding to cofactor occupancy and activation state. AdoCbl is bound in the base-off conformation with His-39 serving as the lower axial ligand to the cobalt (Fig. 1C). The IcmF structures reveal “open” and “closed” conformations that might be associated with promoting cofactor loading/unloading and catalysis, respectively (24). The G-domain moves as a rigid body with the B\textsubscript{12} domain as IcmF switches between the open and closed conformations with the GTP-binding site being strategically located in a hinge within the G-domain. These structures provide a useful framework for understanding the molecular mechanism of nucleotide-gated cofactor ingress and egress. However, the mechanism of cofactor loading/offloading remained known.
simultaneously with equal affinity (5.5 ± 0.8 μM) (Table 1). Surprisingly, both GTP and GDP bind to ATR with similar affinity (272 ± 23 and 247 ± 34 μM) and enhance the affinity for AdoCbl ~5-fold (Table 1). In contrast, binding of GTP or GDP to apo-ATR, i.e. in the absence of AdoCbl, was not detectable. Neither apo-ATR nor ATR-AdoCbl exhibits detectable GTPase activity (data not shown).

Because the $K_D$ for ATP (25 ± 1 μM) is significantly lower than the cellular ATP concentration (~1–3 mM), the protein is expected to be predominantly in the ATR-ATP state, ready to bind cob(II)alamin. The adenosine groups of ATP and of AdoCbl occupy the same position in the ATR site (29, 30), and their binding is mutually exclusive. As expected, in the presence of ATP, a higher ratio of ATR/AdoCbl is needed to saturate the cobalamin-binding site (Fig. 2B).

**Binding of ATP and Cob(II)alamin to ATR Is Mutually Cooperative**—ATR binds cob(II)alamin weakly in the absence of ATP, and the resulting absorption spectrum has a $\lambda_{max}$ of 470 nm ($\epsilon = 9 \text{ mm}^{-1} \text{ cm}^{-1}$), which is characteristic of five-coordinate cob(II)alamin (Fig. 3A, black spectrum). In the presence of ATP, a dramatic spectral change is observed with a shift in the $\lambda_{max}$ to 464 nm accompanied by an increase in absorption ($\epsilon = 19 \text{ mm}^{-1} \text{ cm}^{-1}$) (Fig. 3A, red spectrum). The presence of ATP (but not GTP or GDP) increases the affinity of cob(II)alamin for ATR, and stoichiometric binding is observed (Fig. 3B). The spectral changes are similar to those reported previously for the *Lactobacillus reuteri* ATR (31) and are consistent with the formation of a 4-coordinate cob(II)alamin species lacking an axial water ligand (32, 33). The EPR spectrum of cob(II)alamin (Fig. 3C) displays $g$ and hyperfine ($A$) tensor anisotropies (see Fig. 3, legend) that are relatively large among the ATRs (34) and have been reported previously only for the EutT type ATR from *Salmonella enterica* (35). The EPR parameters are characteristic of the 4-coordinate species, in which separation of the $\beta$-axial ligand from cob(II)alamin leads to lowering of the $3d_{z^2}$ orbital energy and is associated with an increase in cob(II)alamin/cob(II)alamin redox potential (36). This facilitates the reductive alkylation reaction leading to AdoCbl formation.

In the presence of the electron donor, Ti(III) citrate, ATR converts cob(II)alamin to a mixture of species with a broad absorption peak between 400 and 600 nm (Fig. 3A), consistent with the presence of base-off ATR-bound AdoCbl ($\lambda_{max} = 456$ nm) and base-on free AdoCbl ($\lambda_{max} = 525$ nm). AdoCbl formation by ATR was verified by HPLC analysis (Fig. 4A). OH$_2$Cbl, the air oxidation product of cob(II)alamin, was observed in the presence of ATR + ATP only, and AdoCbl was observed upon addition of Ti(III) citrate. ATP and cob(II)alamin bind ATR with the same stoichiometry (Figs. 3B and 4B). Kinetic analysis of the ATR-catalyzed conversion of cob(II)alamin to AdoCbl yielded a $k_{cat}$ of 3.8 ± 0.1 min$^{-1}$ and $K_m$ for ATP of 2.3 ± 0.3 μM (Fig. 4C), which is 10-fold lower than the $K_D$ for ATP value of apo-ATR (Table 1).

**Cobalamins Modulate the GTPase Activity of IcmF—**IcmF exhibits GTPase activity ($k_{cat}$ = 6.0 ± 0.1 min$^{-1}$ at 25 °C) as reported previously (21). The GTPase activity is significantly decreased in the presence of excess of AdoCbl (0.7 ± 0.1 min$^{-1}$) or cob(II)alamin (1.4 ± 0.2 min$^{-1}$), indicating that the G-domain senses the presence and identity of the cofactor in the mutase active site (Fig. 5).

**GTP Hydrolysis Is Required for Full Loading of IcmF with AdoCbl from ATR—IcmF binds AdoCbl in the base-off/His-on conformation ($\lambda_{max} = 528$ nm), which is readily distinguishable from the base-off conformation in the ATR active site ($\lambda_{max} = 456$ nm) (Fig. 6A, upper panel).** IcmF binds 2 eq of AdoCbl from solution with a $K_D$ of 0.22 ± 0.08 μM, which is entropically driven (Table 2). The affinity for AdoCbl is unaffected by the presence of GDP. GDP binding ($K_D$ of 0.37 ± 0.05 μM) is accompanied by favorable enthalpic and entropic changes and is unaffected by ATR (Table 2). Surprisingly, the non-hydrolyzable analog, GMPPCP, weakens the affinity of IcmF for AdoCbl (~60-fold and allows loading of only one of two cofactor-binding sites. AdoCbl binding in the presence of GMPPCP is primarily enthalpically driven (Table 2).

Because AdoCbl binds to IcmF with a 25-fold higher affinity than to ATR, thermodynamics favor cofactor transfer to the mutase active site. Indeed, when ATR-AdoCbl is mixed with IcmF ± GTP or GDP, AdoCbl is transferred to IcmF (Fig. 6A, lower panel) as evidenced by the spectral shift from 456 to 528 nm. The slight inhibition of AdoCbl transfer in the presence of GDP or GDP is likely due to the higher affinity of ATR for AdoCbl in the presence of these nucleotides (Table 1). ATP (1 mM) enhanced AdoCbl transfer from ATR to IcmF in the pres-
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TABLE 1
Binding parameters of ATR with ligands determined by isothermal titration calorimetry
The data represent the mean ± S.D. of at least three independent experiments.

| Injectant         | $K_D$ ($\mu$M) | N (per trimer) | $\Delta H$ (kcal/mol) | $\Delta S$ (kcal/mol/K) | $\Delta G$ (kcal/mol) |
|-------------------|----------------|---------------|-----------------------|-------------------------|----------------------|
| AdoCbl            | 5.5 ± 0.8      | 2.9 ± 0.5     | 4.3 ± 0.2             | -2.8 ± 0.2              | -7.1 ± 0.1           |
| AdoCbl (+GTP)     | 1.2 ± 0.1      | 3.0 ± 0.6     | -13.2 ± 0.2           | 5.2 ± 0.2               | -8.0 ± 0.1           |
| AdoCbl (+GDP)     | 1.1 ± 0.4      | 3.0 ± 0.3     | -12.7 ± 1.0           | 4.7 ± 0.9               | -8.0 ± 0.2           |
| AdoCbl (+GMPPCP)  | 1.9 ± 0.4      | 3.1 ± 0.2     | -10.3 ± 0.6           | 2.6 ± 0.8               | -7.6 ± 0.1           |
| ATP               | 25 ± 1         | 2.8 ± 0.5     | -20.6 ± 0.7           | 14.4 ± 0.8              | -6.2 ± 0.1           |
| GTP (+AdoCbl)     | 272 ± 23       | 3.1 ± 0.2     | -9.0 ± 0.6            | 4.2 ± 0.6               | -4.8 ± 0.1           |
| GDP (+AdoCbl)     | 247 ± 34       | 3.2 ± 0.5     | -7.1 ± 0.9            | 2.4 ± 1.0               | -4.9 ± 0.1           |

* The concentrations of nucleotides were 1.5 mM.
* The concentrations of AdoCbl were 0.5 mM.

FIGURE 3. Binding of cob(II)alamin to ATR. A, changes in the UV-visible absorption spectrum of cob(II)alamin (20 μM) upon binding to ATR (60 μM) in Buffer B. Black trace, cob(II)alamin + ATR, $\lambda_{\text{max}} = 470$ nm; red trace, cob(II)alamin + ATR + 1 mM ATP, $\lambda_{\text{max}} = 464$ nm; blue trace, cob(II)alamin + ATR + 1 mM ATP + 600 μM Ti(III) citrate. The data are representative of at least three experiments. B, modulation of cob(II)alamin (10 μM) binding to ATR (1.8–90 μM) by nucleotides (1 mM). Unbound cob(II)alamin was separated by filtration and quantified by UV-visible spectroscopy. The data represent the mean ± S.D. of three independent experiments. C, CW-EPR spectrum of cob(II)alamin (300 μM) + ATR (450 μM) + 1 mM ATP (black trace) and simulated spectrum (red trace). Simulation parameters: $g_x = 3.424$, $g_y = 2.479$, and $g_z = 1.792$; $A_x = 1147$, $A_y = 685$, and $A_z = 529$ MHz; $g$-strain: $\alpha_x = 0.177$, $\alpha_y = 0.0472$, and $\alpha_z = 0.0381$.

ence of GDP/GTP (Fig. 6B). Addition of GMPPCP (or GMP-PNP, not shown) blocked AdoCbl transfer, which was alleviated ~50% by ATP (Fig. 6B). This result is consistent with the isothermal titration calorimetry data, which indicate that only 1 eq of AdoCbl binds per IcmF dimer in the presence of GMPPCP (Table 2). IcmF is likely to be nucleotide-loaded in the cell because the concentration of GTP (37) is substantial transfer of AdoCbl from IcmF to ATR in the absence of ATP. This result is consistent with the lower affinity of IcmF ($K_D = 12$ μM) than ATR ($K_D = 1.1$ μM) for AdoCbl in the presence of GMPPCP. ATP blocked the transfer of AdoCbl from IcmF to ATR as expected for competitive binding of their adenosine moieties in the active site of ATR. Approximately 30% of AdoCbl was transferred to ATR in the presence of 1 mM GMPPCP, whereas <20% of AdoCbl was ejected into solution (data not shown). GTP Hydrolysis Gates Ejection of Cob(II)alamin from IcmF to ATR—The UV-visible spectrum of cob(II)alamin bound to IcmF exhibits a $\lambda_{\text{max}}$ of 474 nm (Fig. 7A). In the presence of
ATR/ATP and GTP (1 mM), cob(II)alamin translocates from IcmF to ATR as evidenced by the spectral shift to 464 nm and an increase in intensity. Upon addition of Ti(III) citrate, ATR-bound cob(II)alamin was converted to AdoCbl, allowing subsequent quantification by HPLC of the fraction of transferred cob(II)alamin (Fig. 4A). In the absence of GTP or in the presence of GDP, ~20% of cob(II)alamin was transferred from IcmF to ATR-ATP (Fig. 7B). In contrast, quantitative transfer was observed in the presence of GTP. Transfer was blocked by GMPPCP. Importantly, GTP did not trigger release of cob(II)alamin from IcmF into solution (Fig. 7C). The $K_{\text{act}}$ for GTP for cob(II)alamin transfer was estimated to be 460 ± 90 μM (Fig. 7D). Collectively, these results indicate that ATR is required to receive cob(II)alamin as it is ejected from the IcmF site in a step that is gated by GTP hydrolysis.

Transfer of Inactive Cob(II)alamin from ATR to IcmF Is Disfavored—Cob(II)alamin, the substrate for ATR, represents an inactive form of the cofactor for IcmF, and its transfer from ATR to IcmF would be detrimental. Small spectral changes were observed when IcmF was added to ATR-cob(II)alamin (Fig. 8A). In contrast, IcmF binds cob(II)alamin efficiently from solution (Fig. 7C). Quantitative analysis revealed that ~30% of cob(II)alamin bound to ATR was transferred in the absence of nucleotides (Fig. 8B), which was blocked by GTP or GMPPCP. This behavior is similar to the MCM-MeaB system, in which GTP binding but not hydrolysis, shields MCM from being loaded with cob(II)alamin (8).

Discussion

Metallochaperones are critical guardians of cellular function, ensuring the fidelity of active cofactor assembly and delivery to target metalloproteins that represent ~30% of the proteome. With some $B_12$ enzymes, these metallochaperones double in a repair process regulating offloading of inactive cofactor and regenerating active enzyme (8). AdoCbl is a metabolically
expensive organometallic cofactor, which is assembled de novo by some bacteria in an ~30-step process (38). In mammals, it is a rare cofactor, which is obtained from the diet (39). Hence, the trafficking of AdoCbl, a high value product, is carefully regulated. Fusion of the mutase and its G-protein chaperone in a single polypeptide in IcmF simplifies investigation of the switches that signal and gate movement of the B12 cofactor between it and ATR. The latter synthesizes AdoCbl and delivers it to its target mutase (11). In this study, we demonstrate its additional role in cofactor offloading from an inactivated mutase and provide molecular insights into a more complex pattern of allosteric regulation of AdoCbl loading and repair than was previously known (8, 11, 13, 16, 17).

The structures of IcmF reveal that the switch I loop important for G-protein signaling resides at the interface between the B12- and G-domains, which move together as the protein equilibrates between closed and open conformations (24). In the latter, IcmF is primed to receive AdoCbl from ATR, and the

FIGURE 6. Transfer of AdoCbl between ATR and IcmF. A, transfer of AdoCbl from ATR to IcmF. Upper, UV-visible spectra of AdoCbl (20 μM) bound to ATR (33 μM) or IcmF (20 μM); lower, changes in the UV-visible absorption spectrum of ATR (33 μM)-AdoCbl (20 μM) mixed with IcmF (20 μM) in Buffer B. B, fraction of AdoCbl transferred from ATR to IcmF in the presence of 0 –10 mM GDP, GTP, or GMPPCP, in the presence or absence of 1 mM ATP. C, fraction of AdoCbl remaining bound to IcmF when IcmF (20 μM)-bound AdoCbl (20 μM) was mixed with ATR (33 μM) in the presence of nucleotides. The data are representative of at least three experiments or the mean ± S.D. of three independent experiments.

TABLE 2
Thermodynamic parameters for binding of ligands to IcmF
The data represent the mean ± S.D. of at least three independent experiments.

| Injectant | KD N (per dimer) | ΔH kcal/mol | −TΔS kcal/mol | ΔG kcal/mol |
|-----------|------------------|--------------|----------------|-------------|
| AdoCbl    | 0.22 ± 0.08      | 9.6 ± 0.5    | −18.6 ± 0.7    | −9.0 ± 0.3  |
| AdoCbl (+ GDP) | 0.19 ± 0.01      | 16.1 ± 1.3   | −25.1 ± 1.4    | −9.0 ± 0.1  |
| AdoCbl (+ GMPPCP) | 12.4 ± 0.4 | −9.7 ± 0.4 | 3.1 ± 0.4 | −6.6 ± 0.1 |
| GDP       | 0.37 ± 0.05      | −7.7 ± 0.8   | −1.0 ± 0.7     | −8.6 ± 0.1  |
| GDP (+AdoCbl) | 0.48 ± 0.05      | −5.5 ± 0.1   | −3.0 ± 0.1     | −8.5 ± 0.1  |
| GMPPCP    | 0.73 ± 0.01      | −10.6 ± 0.6  | 2.4 ± 0.6      | −8.2 ± 0.1  |
| GMPPCP (+AdoCbl) | ND | | | |

a The concentration of nucleotides was 1.5 mM.
b The concentration of AdoCbl was 0.2 mM.
c ND means is not detectable, i.e. the heat change was below noise level (0.04 μcal/s).
loop carrying His-39 in IcmF, which is important for cofactor docking (11), is exposed. Nucleotide switches regulate the movement of AdoCbl from ATR to IcmF. First, the C. metallidurans ATR is potentially allosterically regulated by GTP/GDP, which enhances its affinity for AdoCbl 5-fold. Although the $K_d$ values for GTP and GDP are high ($\sim 272$ and $247 \mu M$, respectively), allosteric regulation by GTP could be relevant at cellular concentrations of this nucleotide. Alternatively, the triphosphate tails of GTP and ATP might compete for the same binding site. Although ATP and AdoCbl cannot bind simultaneously to the same active site in ATR due to steric clashes, it is interesting that GTP and AdoCbl are able to bind (Table 1) suggesting that their nucleoside moieties have distinct binding modes. The structure of human ATR, which also belongs to the PduO-type group as the C. metallidurans ATR, reveals that ATP binds at the base of the active site, which is sealed upon

FIGURE 7. Ejection of cob(II)alamin from IcmF to ATR. A, changes in the UV-visible absorption spectrum of IcmF (20 $\mu M$) containing cob(II)alamin (20 $\mu M$) in Buffer B mixed with ATR (72 $\mu M$) and ATP (1 mM) in the presence of GTP (1 mM). Black traces, IcmF-cob(II)alamin; red traces, IcmF-cob(II)alamin + ATP + ATR + GTP; blue traces, IcmF-cob(II)alamin + ATR + ATP + Ti(III) citrate (600 $\mu M$). B, cob(II)alamin transfer from IcmF to ATR is enhanced by GTP. The fraction of cob(II)alamin transferred from IcmF to ATR was monitored in the presence of the indicated concentrations of nucleotide derivatives. C, minimal release of cob(II)alamin occurs from IcmF into solution. IcmF (20 $\mu M$) was mixed with cob(II)alamin (20 $\mu M$), and the fraction of cofactor released into solution in the presence of nucleotides (1 mM) was measured. D, dependence of cob(II)alamin (20 $\mu M$) transferred from IcmF (20 $\mu M$) to ATR (72 $\mu M$)-ATP (1 mM) on GTP concentration. The data presented here are representative of at least three experiments (D) or the mean ± S.D. of three independent experiments (B and C).

FIGURE 8. Back-transfer of cob(II)alamin from ATR-ATP to IcmF. A, changes in the UV-visible absorption spectrum of ATR (33 $\mu M$)-cob(II)alamin (20 $\mu M$)-ATP (1 mM) mixed with IcmF (20 $\mu M$). Black trace, ATR-cob(II)alamin-ATP; red trace, ATR-cob(II)alamin-ATP + IcmF; blue trace, IcmF-cob(II)alamin. B, fraction of cob(II)alamin that remained bound to ATR following mixing of ATR-cob(II)alamin-ATP with IcmF in the presence of nucleotides (1 mM each). The data are representative of at least three experiments or the mean ± S.D. of three independent experiments.
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binding of cob(II)alamin (29) consistent with an ordered binding mechanism (40).

ATR is a homotrimer with three active sites located at the subunit interfaces (27). The C. metallidurans ATR binds 3 eq of AdoCbl unlike the M. extorquens enzyme, which binds only two (13). IcmF is a homodimer, and although the two mutase sites are indistinguishable when AdoCbl binds from solution ± GDP, they can be distinguished in the presence of GMPPCP (Table 2). The latter weakens the affinity for the first equivalent of AdoCbl ~60-fold and blocks binding of the second equivalent of AdoCbl (Table 2). These data suggest that transfer of the first equivalent of AdoCbl from ATR to IcmF can occur in the presence of GTP or GDP, whereas transfer of the second equivalent requires GTP hydrolysis (Fig. 9). Cellular GTP concentration is higher than that of GDP, and the two nucleotides bind IcmF with equal and high affinity. However, GTP is not stably bound due to the intrinsic GTPase activity of IcmF. Hence, we predict that IcmF is predominantly loaded with GDP when it cycles through catalytic turnover (Fig. 9).

The molecular mechanism for GTP-driven signaling between ATR and IcmF is not known. It has been speculated that the γ-phosphate of GTP, which is positioned in a solvent-exposed region between the switch I and II domains of IcmF, might be involved in contacting ATR (24). Alternatively, GTP hydrolysis could stabilize the open conformation, which is consistent with the higher affinity of IcmF-GDP versus IcmF-GMPPCP for AdoCbl (Table 2).

It is imperative that cob(II)alamin and the working 5′-deoxyadenosine radical which are formed during the catalytic cycle of acyl-CoA mutases are recombined back to the resting AdoCbl form of the cofactor (Fig. 1A). All radical acyl-CoA mutases are prone to inactivation due to the occasional loss of the 5′-deoxyadenosine intermediate from the active site, which leads to subsequent oxidation of cob(II)alamin bound to the protein. The C. metallidurans IcmF under both aerobic and anaerobic conditions is susceptible to very rapid inactivation (21). We therefore sought to investigate a possible role of the G-domain of IcmF in the repair mechanism, which is analogous to the MeaB chaperone for MCM (8). The GTPase activity of IcmF is responsive to cofactor occupancy and is higher in the apo-enzyme state and lower when AdoCbl or cob(II)alamin are bound (Fig. 5). GTP blocks the transfer of cob(II)alamin to IcmF from ATR (but not from solution), thus averting its reconstitution with inactive cofactor (Fig. 8B). However, it is unlikely that the concentration of free cofactor in organisms that do not synthesize it is high enough to interfere with chaperone-mediated assembly. Overall, the process of AdoCbl loading from ATR to IcmF is similar to that reported for the M. extorquens proteins (8, 11, 13). Analysis of genomic databases reveals examples of many organisms that harbor several acyl-CoA mutases but only a single copy of the PduO-type ATR. This suggests that a single ATR is involved in the delivery of AdoCbl to multiple mutases (23).

The mechanism of cofactor repair is, however, surprisingly more complex than previously seen (Fig. 9). In the MCM/MeaB system, the enzyme is inactivated by loss of the 5′-deoxyadenosine moiety (8). In IcmF, ejection of cob(II)alamin requires ATR-ATP, which is expected to predominate in the cell because the $K_a$ for ATP (25 μM) ≪ intracellular [ATP] (~3 mM) (37). ATP promotes tight binding of cob(II)alamin to ATR (Fig. 3). Egress of cob(II)alamin from the IcmF active site requires GTP hydrolysis (Fig. 7B). In turn, the cob(II)alamin received back in the active site of ATR is readily converted to the active AdoCbl form in the presence of ATP and a reductant (Fig. 7). The physiological reducing partner of ATR is not known. It would be interesting to see whether the repair of inactive human MCM is similar to that of the MCM/MeaB or to the IcmF system. Our work also sets the stage for studies aimed at elucidating the mechanism of cross-talk between the G-protein (domain)/ATR repair system and the mutase active site where different acyl-CoA substrates bind.

**Experimental Procedures**

**Materials**—AdoCbl, GTP, GDP, GMPPCP, and ATP were purchased from Sigma.

**Cloning of ATR from C. metallidurans**—The full-length PduO-type ATR gene from C. metallidurans strain CH34 was amplified from genomic DNA (ATCC, Manassas, VA) using the following primers, and the PCR product was subcloned into the ligation-independent cloning vector, pMCSG7: forward TATCAGGACTCCCGCTCACGCTGCCAG-3′ and reverse 5′-TACTTCCAATCCAATGCCATGGGTAATCGCCTGTC-CAAATATTGC-3′ and reverse 5′-TTATCCACTTCCAATGCTC-TATCAGGACTCCCGCTCACGCTGCCAG-3′.

**Expression and Purification of C. metallidurans ATR—Escherichia coli** BL21(DE3) cells containing the ATR expression plasmid were grown at 37 °C in Luria-Bertani medium supplemented with 100 μg/ml ampicillin until the absorbance at 600 nm was 0.6–0.8. ATR expression was induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside, and the cells were grown for 20 h at 15 °C. Following centrifugation, the cell pellet (10 g from 2 liters of culture) was resuspended in 120 ml of lysis buffer (50 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, and 30 mM imidazole) containing 1 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride, and 1 tablet of the protease inhibitor mixture (Roche Diagnostics) and disrupted by sonication on ice. The cell lysate was centrifuged, and the supernatant was subjected to Ni-NTA affinity chromatography (Qiagen, Valencia, CA) and then gel filtration (Sephadex G-200).
was loaded onto a 2.5 × 8-cm nickel-Sepharose 6 Fast Flow column (GE Healthcare). The column was washed with 300 ml of lysis buffer, and eluted with a 260-ml linear gradient ranging from 30 to 350 mM imidazole in the lysis buffer. Fractions containing ATR were pooled, concentrated, and loaded onto a 1.6 × 60-cm Superdex 200 column (GE Healthcare) equilibrated with 50 mM HEPES, pH 7.5, 100 mM NaCl. Fractions containing ATR were pooled, concentrated, frozen in liquid nitrogen, and stored at −80 °C until further use. ATR is a homotrimer, and the protein concentration is expressed in terms of subunit concentration throughout this study.

Expression and Purification of C. metallidurans IcmF—Recombinant wild-type IcmF was expressed and purified as described previously (21). IcmF is a homodimer, and its protein concentration is expressed in terms of subunit concentration throughout this study.

GTPase Activity Assay—The GTPase activity of IcmF (4 μM) was determined in the presence of saturating concentrations of GTP (1 mM) at 25 °C in 0.6 ml of 50 mM HEPES buffer, pH 7.5, 100 mM NaCl and 10 mM MgCl2 (Buffer A). Aliquots (100 μl) were removed at varying times (1−15 min), quenched with 10 μl of 1 M trichloroacetic acid (10% v/v), and centrifuged to remove precipitated protein. To test the effect of cob(II)alamin derivatives (AdoCbl and cob(II)alamin), apo-IcmF was pre-incubated for 5 min at 25 °C with a 10-fold excess of each cofactor, and the reaction was started by adding GTP. The nucleotides were analyzed by HPLC using an ion exchange column, μBondapak NH2 300 × 3.9-mm column (Waters). HPLC conditions were essentially the same as described previously (20).

Isothermal Titration Calorimetry—Binding of AdoCbl or nucleotides (GDP or GMPPCP) was monitored by titrating IcmF (5−10 μM) with 29 × 10-μl injections of AdoCbl (100−150 μM) or nucleotides (100−150 μM) in Buffer A at 20 °C. The concentrations of nucleotides added to IcmF:AdoCbl or AdoCbl added to IcmF-GDP are listed in Tables 1 and 2. Binding of AdoCbl or GDP to ATR was determined by titrating ATR (10−20 μM) with 29 × 10-μl injections of AdoCbl (100−200 μM) or GDP (3.0−3.5 mM) in Buffer A at 20 °C. The Kd value was determined using a single-site binding model and represents the average of three independent experiments.

Binding of Cobalamins to IcmF or ATR—Cob(II)alamin was generated by photolysis of 100−200 μM AdoCbl in 200 mM HEPES buffer, pH 7.5, containing 100 mM NaCl and 10 mM MgCl2 (Buffer B) under anaerobic conditions. IcmF (20 μM) was mixed with cob(II)alamin (20 μM) in the presence or absence of 1 mM GTP, GDP, or GMPPCP. The concentration of AdoCbl and OH2Cbl were determined from their UV-visible spectrum. To quantify the amount of cob(II)alamin transferred to ATR, Ti(III) citrate (600 μM) was added to the reaction mixture after 5 min resulting in the synthesis of AdoCbl by ATR. The bound fraction was estimated using a Δε value at 525 nm of 6.1 mM−1 cm−1.

Electron Paramagnetic Resonance Spectroscopy—Continuous-wave EPR spectra were collected on a Bruker Elexsys E500 spectrometer by using a Super High Q Cavity (Bruker ER 4122SHQE) and Bruker ER4131VT system with nitrogen-flow cooling for temperature control. A nitrogen-flow cooling system was used. The following experimental conditions were used: modulation frequency, 100 kHz; modulation amplitude, 10 G; microwave power, 20 milliwatts; temperature; 120 K. Samples were transferred to 4-mm outer diameter quartz EPR tubes in an argon-filled glove bag under dim red light. Simulations of the experimental spectra were performed by using the EasySpin toolbox run in MATLAB (version R2015a; Mathworks, Natick, MA) (41).

Synthesis of AdoCbl by ATR—ATR (40−66 μM) was mixed with cob(II)alamin (20 μM) in the presence of 1 mM ATP in Buffer B (total volume = 120 μl) under anaerobic conditions. Titanium (Ti(III) citrate, 600 μM final concentration) was added to start the reaction. After incubation for 5 min at room temperature, the reaction was stopped with 30 μl of 10% (v/v) trifluoroacetic acid to obtain a final concentration of 2% (v/v). Precipitated protein was removed by centrifugation at 16,000 × g at 4 °C for 10 min, and the supernatant was used for HPLC analysis. AdoCbl and OH2Cbl (i.e. the oxidation product of cob(II)alamin) were separated on an Alltima HP C18 HL column (Grace, 250 mm × 4.6 mm, 5 μm) at a flow rate of 1 ml min−1. The mobile phase consisted of solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile). A gradient from 8 to 32% solvent B was used, and the absorbance was monitored at 254 and 350 nm.

To measure the rate of AdoCbl synthesis, ATR (0.3−0.6 μM) was mixed with cob(II)alamin (50 μM), ATP (0−100 μM), and 600 μM Ti(III) citrate in Buffer B (total volume = 120 μl) under anaerobic conditions. The reactions were stopped by adding 30 μl of 10% trifluoroacetic acid at 0−5 min. The amount of AdoCbl in the assay mixture was determined using the HPLC method described above. AdoCbl and OH2Cbl eluted at 26 and 18 min, respectively, and were monitored at 350 nm. The concentrations of AdoCbl and OH2Cbl were determined from their peak areas at 350 nm.

Cob(II)alamin Transfer between IcmF and ATR—IcmF (20 μM) was mixed with cob(II)alamin (20 μM) in Buffer B under anaerobic conditions, and ATP (1 mM) and ATR (66 μM) were then added to the mixture at room temperature. Transfer of cob(II)alamin from IcmF to ATR was initiated by the addition of GTP (1−5 mM) and monitored by changes in the UV-visible spectrum. To quantify the amount of cob(II)alamin transferred to ATR, Ti(III) citrate (600 μM) was added to the reaction mixture after 5 min resulting in the synthesis of AdoCbl by ATR. The concentration of AdoCbl formed was determined by HPLC.
as described above. The efficiency of transfer was calculated by comparing the concentration of AdoCbl formed and cob(II)alamin initially added to the reaction mixture. The transfer of cob(II)alamin from ATR to IcmF was monitored by mixing cob(II)alamin (20 μM), ATP (1 mM), ATR (33 μM), and GTP, GDP, GMPPNP, or GMPPCP (1 mM). IcmF (20 μM) was then added to initiate the transfer. After the samples were incubated for 5 min under anaerobic conditions, Ti(III) citrate (600 μM) was then added to the reaction mixture to allow synthesis of AdoCbl from ATR-cob(II)alamin-ATP to estimate the amount of cob(II)alamin that was not transferred from ATR. The concentration of AdoCbl formed was determined by HPLC as described above.

Transfer of AdoCbl between ATR and IcmF—ATR (33 μM) was mixed with 20 μM AdoCbl in the presence or absence of ATP, GTP, GDP or GMPPCP (1–10 mM each) in Buffer B. IcmF (20 μM) was then added to initiate AdoCbl transfer at room temperature and monitored by a change in the absorption spectrum from 456 nm (ATR-bound) to 528 nm (IcmF-bound). The extent of AdoCbl transfer was estimated from the increase in absorbance at 528 nm using a Δε = 7.5 mm−1 cm−1. To monitor the back transfer of AdoCbl from IcmF to ATR, IcmF (20 μM) was mixed with 20 μM AdoCbl and 1 mM of the nucleotides described above. ATR (33 μM) was added to initiate the transfer, which was monitored as described by UV-visible spectroscopy.

**Author Contributions**—Z. L. designed, performed, analyzed the experiments, and wrote the manuscript. V. C. and K. K. purified IcmF and ATR and conceived and performed some of the initial experiments. K. K. performed the GTase activity assay. D. C. helped conceive the experiments, analyzed the data, and co-wrote the manuscript. All authors approved the final version of the manuscript.

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