Orchestrating Improbable Chemistries: Structural Snapshots of B_{12}-Dependent Methionine Synthase's Catalytic Choreography

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Abstract

Cobalamin (vitamin B_{12}) and its derivatives play an essential role in biological methylation, with cobalamin-dependent methionine synthase (MS) serving as a canonical example. MS catalyzes multiple methyl transfers within a single, dynamic multi-domain architecture that has proven challenging to study, hampering efforts to elucidate its catalytic mechanism(s). Utilizing a thermostable MS homolog and non-native cobalamin cofactors, we have captured crystal structures of transient conformational states of MS, including those directly involved in folate demethylation and homocysteine methylation. These snapshots reveal the mechanistic significance of five-coordinate, His-off methylcobalamin in homocysteine methylation and highlight the crucial role of the folate-binding domain and interdomain linkers in orchestrating the intricate structural rearrangements required for catalysis. This expanded conformational ensemble, including the unexpected capture of novel 'Cap-on' conformations, underscores the remarkable plasticity of MS, exceeding previous estimations. Our findings provide crucial insights into the catalytic mechanism of MS, laying the foundation for harnessing cobalamin's biocatalytic potential and elucidating how nature exploits protein dynamics to facilitate complex transformations.

Main

Methionine synthase is a multi-modular enzyme capable of binding and activating three substrates (homocysteine, HCY; methyltetrahydrofolate, MTF/folate, FOL; and *S*-adenosylmethionine, SAM) to achieve three distinct methylations (Reaction I, II, and III, Fig. 1a). Reactivity is governed by access to the cobalamin cofactor and its oxidation state, with each reaction associated with the formation of three different catalytically competent ternary complexes and an active site between the Cob domain and its respective substrate domain (Fig. 1a)[1]. The cobalamin cofactor's oxidation state alternates between Co(III) and Co(I) states during the catalytic cycle (Reaction I and II, Fig. 1a) but the highly reactive Co(I) is oxidized to the catalytically inactive Co(II) once every 2000 turnovers[2,3]; MS can restore catalytic competency by entering the reactivation cycle, during which reductive methylation of Co(II) regenerates the catalytically competent methylcobalamin, Co(III) state, allowing for re-entry to the catalytic cycle (Reaction III, Fig. 1a)[3,4]. However, while rich biochemical studies exist on factors that govern and can trigger MS domain rearrangements, atomic level insights into what the conformations associated with the catalytic cycle look like has eluded structural characterization[5,6,7,8]. Using rigorous biochemical studies, Bandarian *et al.* (Fig. 2b) introduced a model where a minimal set of four conformations was proposed along with the notion that methylcobalamin serves as a key branching point in both the catalytic and reactivation cycles[7].

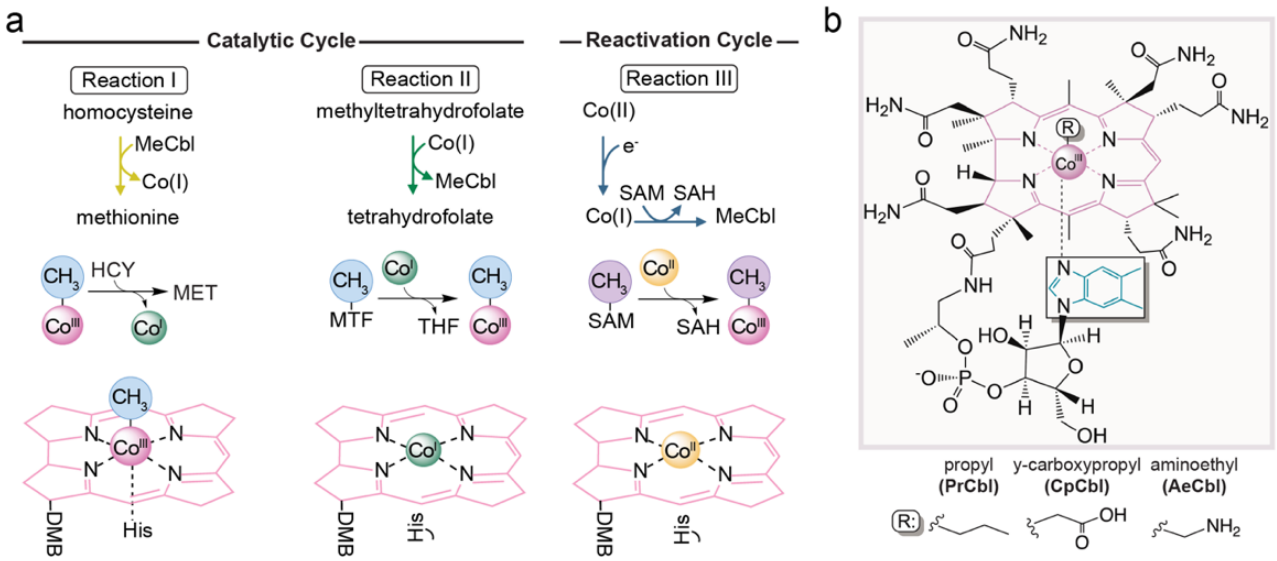

Figure 1. Methionine synthase harnesses its cobalamin cofactor to dictate chemical outcome. a The three distinct methyl transferase reactions catalyzed by methionine synthase (MS). The primary catalytic cycle allows for the cobalamin cofactor to alternate between the Co(III) and Co(I) states for homocysteine methylation (Reaction I) and folate demethylation (Reaction II), respectively. Due to the microaerophilic conditions present in vivo, MS can undergo inactivation when Co(I) is oxidized to the inactive Co(II) state. During the reactivation cycle, the active state of the cofactor is restored via reductive methylation with SAM/AdoMet, regenerating the Co(III) state and allowing for re-entry to the catalytic cycle (Reaction III). The coordination number, redox state, and His ligation status are particular to each methylation. **b** The structure of the cobalamin cofactor, showcasing the corrin ring (pink), its upper axial (α face) ligand and its lower axial (β face) ligating nucleotide tail (dimethylbenzimidazole, DMB). Methionine synthase binds its native cobalamin cofactor, methylcob(III)alamin (R group: CH_3, methyl) in the base-off, His-on form. Synthetic cobalamin cofactors with alternate upper axial ligands incapable of catalysis can and have been used to modulate function via cofactor mimicry and subsequent inhibition and can capture distinct conformations (R: propyl, γ-carboxypropyl, aminoethyl).

Previously, we used a thermophilic MS homolog (*t*MS) from *Thermus thermophilus* that displayed robust stability, allowing for exhaustive functional mutagenesis studies, purification as an apoenzyme, and facile reconstitution with non-native cobalamin cofactors to form novel holoenzymes (Fig. 1b)[9]. This model system allowed for the determination of the first full-length structure of MS (8SSC) captured via crystallography and the observation of holoenzyme formation *in crystallo* (8SSD, 8SSE)[9]. The ability to purify excised domain constructs, coupled with the ability to purify all mutants generated, allowed us to begin interrogating MS mechanistically using a combination of rationally chosen non-native cobalamins and *t*MS constructs[9].

Here, we leverage our *t*MS model to capture five new crystal structures of MS in action, three Cap-on conformations prior to catalysis and two catalytically competent conformations, that provide insights into the structural motifs used to guide and gate the large conformational rearrangements required for MS catalysis. We show that the MS conformational ensemble is more expansive than previously appreciated, adopting additional gating conformations[10,11] (Cap-on) that highlight the role of the Fol:Cap linker (Linker II, Fig. 2) in mediating structural transitions, where it acts as a hinge. The structures show that the "uncapping" transition required for cobalamin access (Cap:Cob linker, Linker III, Figs. 2b, c, and d) is predetermined, adopting the same placement relative to the Cob domain in all observed Cap-off structures. The two catalytic conformations (Fol-on, Reaction II; Hcy-on, Reaction I, Fig. 2b) provide the first structural blueprints of a B_{12}-dependent methyltransferase active site, one required for folate demethylation and the other for homocysteine methylation. Our biochemical data highlight the role the Fol domain plays in steering the conformational ensemble towards catalytic states, while also demonstrating that the cobalamin cofactor adopts a His-off, five-coordinate state during catalysis. Notably, we demonstrate the importance of the ligating His residue in signaling cofactor status (redox state, coordination

number) and guiding the conformational ensemble, positing that His-on ligation gates the ensemble into Cap-on states (gateway conformations), enabling MS to 'reset' and interconvert between these states, and His-off ligation allows for cobalamin flexibility and substrate-bound domain access after uncapping (Cap-off, catalytic/reactive). We propose a revised mechanistic and structural model for MS, one that emphasizes the role of a linker (Fol:Cap, Linker II), His-off ligation, and guided domain uncapping in gating and thereafter guiding rearrangements that allow for cofactor access required for catalysis.

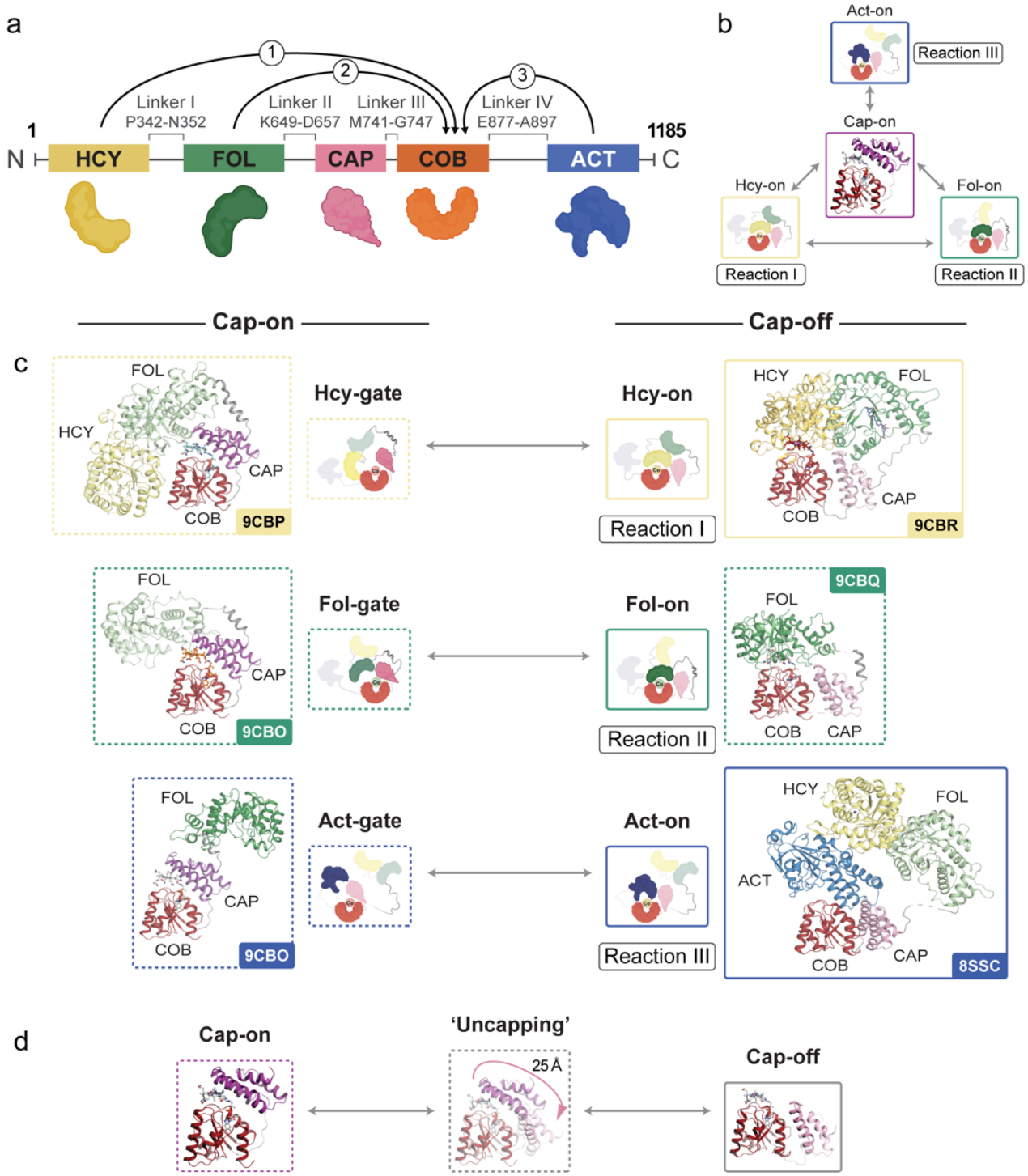

Figure 2. Methionine synthase conformational ensemble dictates chemical outcome. a Four compact substrate/cofactor binding (Hcy, Fol, Cob, and Act) and cofactor capping (Cap) domains are color-coded, with linkers displayed. **b** The minimum set of conformations MS must adopt during catalysis includes a Cap-on state that protects the cobalamin cofactor. Uncapping of the Cob domain allows for substrate domain access to the cobalamin cofactor,

with three unique ternary complexes required for each methylation (Hcy-on, homocysteine methylation, Reaction I; Fol-on, folate demethylation, Reaction II; Act-on, cobalamin reductive reactivation, Reaction III). **c** Experimentally determined Cap-On structures enable MS 'resetting', while Cap-Off structures facilitate ternary complex formation between the Cob and substrate binding domains as part of the catalytic cycle (Reactions I, II). Similarly, Cap-on to Cap-off transitions enable chemistry as a part of the reactivation cycle (Reaction III). **d** The Cap-on states protect the cobalamin cofactor, and the uncapping transition results in a ~25 Å displacement of the Cap domain to a predetermined position nestled to the side of the Cob domain, allowing for substrate-binding domain access and chemistry by enabling access to the cobalamin cofactor.

Cap-on gateway conformations expand Methionine Synthase's conformational ensemble

The ability to purify MS constructs that were previously unattainable provided an avenue for the ability to rationally sample and strategically limit the structural space MS could adopt via tactical truncations. By using the Fol:Cap:Cob tridomain construct, we envisioned capturing the ternary catalytic folate demethylation complex (Fol-on). Similarly, we employed the Hcy:Fol:Cap:Cob tetradomain construct in an attempt to capture the ternary catalytic homocysteine methylation complex (Hcy-on).

Accordingly, tMS^{FolCapCob} was crystallized bound to its native cofactor, methylcobalamin (MeCbl), and its structure was solved to 3.34 Å (Figs. 2 and 3a, Supplementary Fig. 1, and Supplementary Table 1, 9CBO); using propylcobalamin (prCbl), we were able to crystallize tMS^{HcyFolCapCob}, solving the structure to 2.45 Å (Figs. 2 and 4a, and Supplementary Table 1, 9CBP). The latter reveals a Cap-on conformation (Hcy-gate, Supplementary Fig. 1, 9CBP), while the former captured two novel Cap-on conformations in one crystal, distinct from the only other reported Cap-on conformation of a muti-domain MS (Hcy:Fol:Cap:Cob, 8G3H)[11], where the folate binding site is pre-positioned to interact with the Cob domain (Fol-gate, Supplementary Fig. 2, 9CBO). While a third novel Cap-on structure was captured in the same crystal as the termed Fol-gate structure, further work is underway to confirm our preliminary assignment as a gateway conformation (proposed Act-gate/resting conformation, Supplementary Fig. 1, 9CBO).

Capturing three Cap-on conformations, two of which are distinct/unique (Fol-gate, Act-gate/resting, Supplementary Fig. 1) and one that recapitulates 8G3H (Hcy-gate, Supplementary Fig. 1), indicates that the premise that 8G3H represents a "resting" state is an oversimplification and deficient/incomplete assignment of the overall and inherently complex mechanism that allows MS to interconvert between catalytically capable/relevant/competent conformations. Indeed, the three observed Cap-on states likely represent conformations that act as essential steps leading up to the three different methylations. While incapable of chemistry themselves, these reported Cap-on states act as gateways, allowing access to the three ternary conformations required for chemistry/catalysis to occur. While the authors termed their structure as *the* "resting" state, we argue that it represents one of at least three gateway/'resting' conformations. The capture of three distinct Cap-on states was wholly unexpected and indicates that the structural space MS samples prior to catalysis is more complex than previously thought, encompassing more than the predicted minimal set of four conformations (Fig. 2b)[1,6,7,8,11,12].

The defining feature of the Cap-on conformations is the structured Fol:Cap linker (Linker II), which was found as an unstructured loop in the full-length tMS structure (8SSC)[9]. In the three gateway states presented here (Supplementary Fig. 1b), Linker II is found as a structured and flexible helix. Even so, the Cap-on states reveal that the helix of Linker II is more structured in the Hcy-gate conformation and slightly less structured in the Fol-gate conformation, shortening the helix by 11.2 Å and shifting the N-terminal portion of the Fol:Cap linker 18.8 Å laterally (Supplementary Fig. 2). This partial melting of the helix in the Fol-gate state allows Linker II to act as a hinge, as the domains themselves maintain their topology and move as rigid bodies (Supplementary Fig. 2). The hinge contracts and rotates the Fol domain 120° to orient the folate binding site toward and above the Cap:Cob

domain, as opposed to its orientation in the Hcy-gate state, where the active site is facing away (Supplementary Fig. 2).

Consequently, these Cap-on structures demonstrate the importance of Linker II in allowing for proper and guided transitioning between Cap-on states. The cofactor status (hexacoordinate, His-on, methylcob(III)alamin) is a key factor, as it bridges the catalytic and reactivation cycles. Conformational morphing indicates that each state can interchange freely via a lasso-like movement of Linker II (Supplementary Movie 1). This enables MS to 'reset' and cycle between Cap-on gateway conformations which can then transition to catalytic ones depending on substrate binding. As such, the status of Linker II is essential, with a loop-helix transition allowing for facile conversion between Cap-on states.

Visualizing methyl-transfer in *t*MS

Folate demethylation (Fol-on) conformation

The use of truncated constructs with methylcobalamin and even propylcobalamin was not enough to yield the Cap-off states. The inability of propylcobalamin to yield a Cap-off state was particularly surprising, given that there is literature precedence for its use in preferentially binding cobalamin in a His-off, Cap-off state; these studies demonstrated that MS loaded with propylcobalamin was trapped in the reactivation conformation (Act-on) and was found to be catalytically inactive[13,14]. This is especially surprising when considering that it was coupled with a tetradomain construct (tMS^{HcyFolCapCob}) lacking the Act domain, removing its ability to sample said reactivation conformation. For this reason, we next sought to exploit the robustness of our *t*MS model to protein engineering by introducing mutations (namely D762G) designed to steer the conformational ensemble away from the His-on, Cap-on state and explored the use of bulkier, non-alkyl upper axial ligands that would further destabilize the Cap-on conformation(s) [8,12,15,16,17]. To that end, we selected γ-carboxypropyl cobalamin (cpCbl) (Fig. 1b), a synthetic cobalamin that does not support catalysis; using this analog, we were able to crystallize tMS$^{\text{FolCapCob•D762G}}$, solving the structure to 2.87 Å (Figs. 2b, 3b, and Supplementary Table 1, 9CBQ).

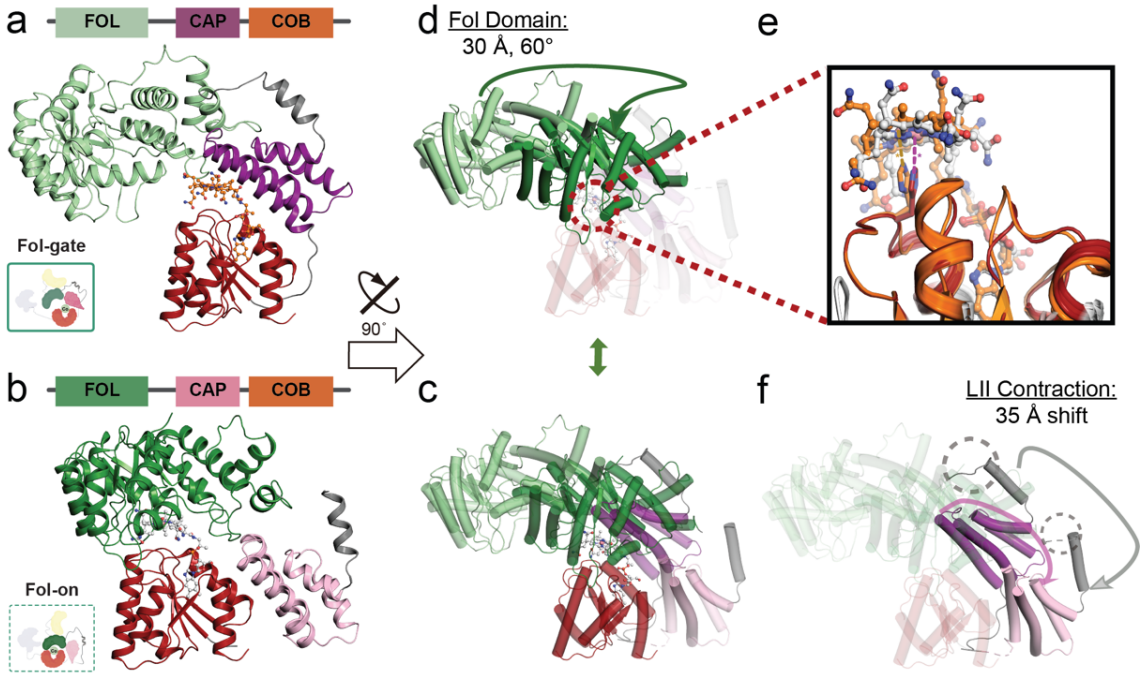

Figure 3. Structural comparison of gateway states and catalytic states (Folate-gate and Folate-on). a Structure of tMS^{FolCapCob} captured in the Fol-gate conformation colored by domain (Fol domain, pale-green; Cap, dark purple; Cob, orange) with the Fol:Cap linker shown in grey. **b** Structure of tMS^{FolCapCob} captured in a catalytic conformation (Fol, forest green; Cob, dark red; Cap, light pink) with the Fol:Cap linker shown in grey. Superposition of those structures are illustrated in **c**. The Cob domain was used as the reference for the structure alignment. The cobalamin

cofactor must traverse 25 Å to form the Fol-on ternary complex. **d** Superposition using the Cob domain as a reference of the Fol domain *t*MS^{FolCapCob} structures using the same coloring scheme as **a** and **b**. Contraction of the Fol:Cap linker causes a 60° rotation coupled with a 30 Å translation of the Fol domain, while simultaneously uncapping the cobalamin cofactor bound in the Cob domain (**f**). **e** His761 distance between gateway and catalytic states in *t*MS^{FolCapCob}. The interactions between His761-Nε2 and the Co center are shown in dark orange (2.6 Å) and magenta (3.4 Å) dotted lines for Fol-gate and Fol-on, respectively.

In the captured conformation (Fol-on), the Fol-domain is positioned over the Cob-domain, placing the MTF-binding site directly above cobalamin cofactor (Fig. 3b). The cofactor is bound in the His-on state, where His761 is coordinated through its imidazole side chain 3.4 Å from Co (Fig. 3e). The Cap domain, destabilized from its protective position, is displaced ~25 Å from the Cap-on state conformations[10,11] in a manner virtually identical in form and position to previously captured Cap-off structures, lying to the periphery of the Cob domain (8SSC, 8SSD, 8SSE, Supplementary Fig. 3)[9]. This suggests that the position of the displaced Cap domain is programmed, rather than random. The dramatic rearrangement of the cobalamin cofactor and its associated domain (Cob), transversing 25 Å after uncapping (Supplementary Fig. 2), is mediated by the restructuring of Linker II in lieu of intradomain interactions, validating our previously proposed hypothesis that the flexibility of the linkers not observed in our previous structures must be key in orchestrating these domain movements[9].

The Fol:Cap linker, previously found as an unstructured loop in the full-length apo structure (8SSC)[9], is now found as a partially structured helix, demonstrating that the Fol:Cap linker contributes to the complexity and versatility of the MS structural ensemble. Starting from a Cap-on state (Fol-gate), this hinge must undergo a loop-helix transition to form the Fol-on ternary catalytic complex. In this case, the N-terminal portion of the linker is disordered (4 residues unmodeled); again, this small change results in a corresponding 34.5 Å shift of the linker and 60° rotation of the Fol domain relative to the Cob domain (Fig. 3d and f, Supplementary Fig. 2). Combined with the Cap-off transition, this allows for the cobalamin cofactor to be completely buried by the new Fol:Cob intradomain interface/surface (Supplementary Fig. 3). The gateway to catalytic transition also results in repositioning of the cobalamin cofactor: the Co center is shifted ~1.5 Å laterally with an associated downward tilt of ~3°. The corrin ring, which experiences significant distortion, is more planar in the captured Fol-on state (Supplementary Fig. 4), due to an increase in the ligating His761 distance (2.2-2.6 Å from the Co center in Fol-gate versus 3.1 Å in the Fol-on state), relieving downwards "doming" towards the ligating His. In total, this structure represents the Fol-on (Fol:Cob) state, adopted for the methyl transfer from MTF to Cob(I). As the only/first catalytic structure visualized for any corrinoid folate enzyme, this structure gives unique insight into the required orientation of the Fol and Cob domains and informs on how the initial methyl transfer to form methylcobalamin can proceed.

Previous structures of MTF-bound Corrinoid iron-sulfur protein/Methyltransferase (CFeSp/MeTr) were captured in an *en route* conformation, as the distance between the cofactor and MTF was too large to support catalysis (~8 Å) and further motions are required to bring the Co center closer to the methyl group[18]. SN_2 nucleophilic displacement, favored as the mechanism of methyl transfer in cobalamin-dependent methyltransferases[19,20], requires an expected reaction distance of 3-4 Å between the cobalt center and the MTF-methyl group. To determine if the orientation captured in the Fol-on structure could support catalysis, we modeled MTF into the active site and found that the N5-methyl of MTF is positioned directly above and in-line with the cofactor, ~3.2 Å from Co-center (Supplementary Figs. 4 and 5)[21]. Both the arrangement and proximity of the reacting centers indicate the captured state represents a catalytically competent structure, or a 'right-after-catalysis' state.

Encouraged by the ability to capture transient conformations using crystallography, we next sought to structurally interrogate the homocysteine methyltransfer reaction (Reaction II, Fig. 1).

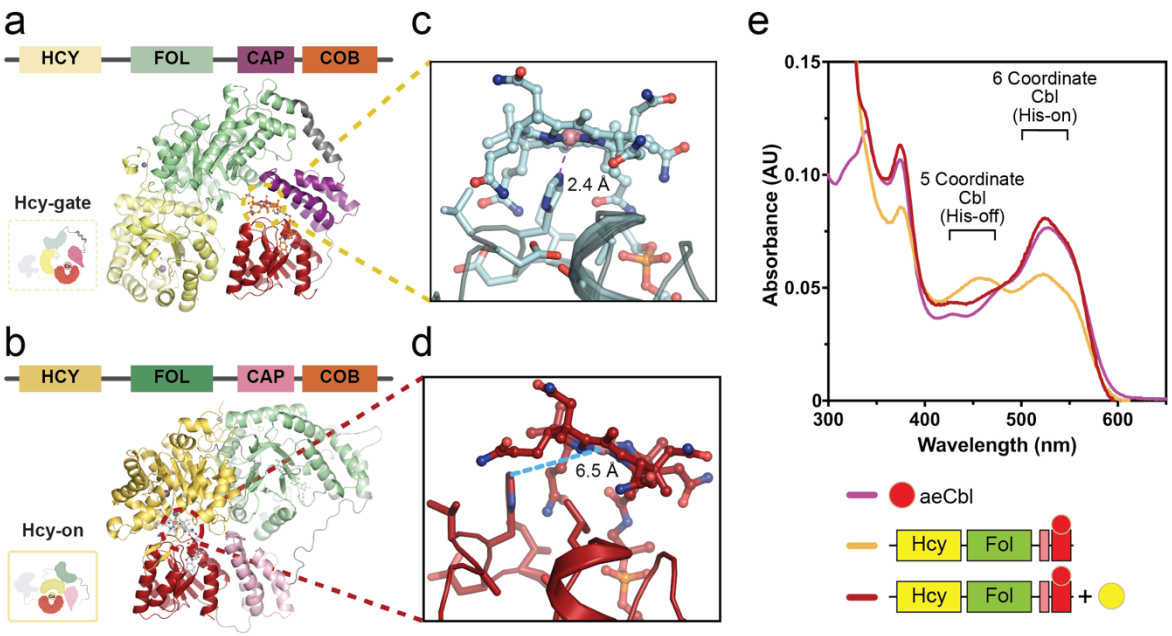

Figure 4. Structural comparison of gateway states and catalytic states (Homocysteine-gate and Homocysteine-on). a Structure of *t*MS^{HcyFolCapCob} captured in in the Fol-gate conformation colored by domain (Hcy domain, pale yellow; Fol domain, pale-green; Cap, dark purple; Cob, orange) with the Fol:Cap linker shown in grey. **b** Structure of *t*MS^{HcyFolCapCob} captured in a catalytic conformation (Hcy, yellow orange, Fol, forest green; Cob, dark red; Cap, light pink) with the Fol:Cap linker shown in grey. Superposition of those structures are illustrated in **c**. The Cob domain was used as the reference for the structure alignment. **d** His761 distance between gateway (light cyan, Hcy-gate) and catalytic states in *t*MS^{HcyFolCapCob} (brick red, Hcy-on). The cobalamin cofactor is displaced 3.8 Å laterally and is bound His-off in the catalytic conformation. **e** Free aminoethylcobalamin (aeCbl) shows a peak at approximately 530 nm, characteristic of six-coordinate alkyl-cobalamin (magenta). Upon binding to *t*MS, the aeCbl spectrum exhibits an additional peak at around 460 nm, suggesting that a fraction of aeCbl binds in the His-off mode (five-coordinate) (golden yellow). Furthermore, the addition of excess exogenous homocysteine to the sample (tMS^{HcyFolCobmut2}•aeCbl) induces changes in the spectrum (brick red), shifting the equilibrium back to the His-on mode, presumably due to the induction of steric clashes; its non-reactive nature could likewise cause a shift in the conformational equilibrium towards a His-on, Cap-on state to protect the cofactor. Given that cobalamin in the Cap-on state is known to adopt a His-on, six-coordinate state, it is proposed that cobalamin in the Hcy-on conformation adopts a His-off five-coordinate state. Data in panel **e** is of a representative experiment, which has been repeated ≥3 times.

Homocysteine methylation (Hcy-on) conformation

Having capitalized on the tridentate approach of rational non-native cofactor, truncated construct, and protein engineering use to capture transient conformations via crystallography, we next sought to capture the homocysteine methylation conformation (Hcy-on). Given that *t*MS^{HcyFolCapCob} was used, the sample space was inherently more expansive than that of the tridomain construct used to capture the Fol-on conformation. Consequently, the biochemical features associated with the Hcy-on conformation were explored via the use of non-native cobalamins and mutations designed to shift the conformational ensemble from the captured Cap-on states (Hcy-gate, Fol-gate) towards Cap-off states. The three Cap-on, gateway structures, namely the Fol-gate and Hcy-gate states, provided molecular level insights into the structural features that could gate entry to the Hcy-on state, particularly the role the Fol domain and Linker II play in structural transitions. The helical nature of Linker II in the Hcy-gate state, along with the helix-loop transition associated with the gateway-to-Fol-on transition, provided the tantalizing prospect that capitalizing on/exploiting the flexibility of the linker by its partial melting could preferentially steer the ensemble towards catalytic states.

As such, the nature of the non-native cobalamin coordination bound to several *t*MS^{HcyFolCapCob} mutants was studied via UV-Vis spectroscopy, confirming that the constructs adopt both His-on and His-off binding modes (Fig. 4e, golden yellow line). Addition of homocysteine shifts this equilibrium to the His-off state, despite using non-

reactive cobalamins, presumably due to a steric clash between HCY and the cobalamin cofactor, or to protect the cofactor from sampling a non-reactive/productive conformation. The use of aminoethylcobalamin (aeCbl) resulted in the most pronounced effects, yielding a visible color change from yellow (His-off, 5 coordinate, Co(III)) (Supplementary Fig. 6) to red/pink (presumably Cap-on, His-on, 6 coordinate, Co(III), Fig. 4e, brick red line). The absence of the Act domain precluded sampling of the reactivation state (Act-on), and the intriguing change to a yellow color, normally associated with cob(II)alamin (and thus inactivation), implied another set of states were being sampled in solution; His-off, pentacoordinate cob(III)alamin, which also presents as a yellow color, could thus be the key cofactor state associated with the elusive Hcy-on conformation.

In light of these findings, mutations designed to disrupt the helical nature of the Fol:Cap linker and favor His-off cobalamin binding were introduced (Supplementary Fig. 7), with aeCbl used as the non-reactive cobalamin of choice. Using this mutated *t*MS construct and alternate cofactor coupling, we were able to crystallize $tMS^{HcyFolCapCob}$, solving the structure to 2.38 Å (Fig. 4 and Supplementary Table 1).

In the Hcy-on conformation, the Hcy-domain is positioned over the Cob-domain, placing the HCY-binding site directly above the β-face of the cobalamin cofactor (Fig. 4b). The cobalamin cofactor is bound in the His-off state, where His761 is ~6.3 Å from Co (Fig. 4d). Indeed, crystals were found to be yellow in color, as compared to the red/pink color observed for the Hcy-gate crystals (Supplementary Fig. 6). Akin to the Fol-on structure, there is a dramatic rearrangement of the cobalamin cofactor and its associated domain Cob, transversing ~45 Å after uncapping (Fig. 5, Supplementary Fig. 2). The Fol:Cap linker is found to undergo a helix-loop transition, confirming its role in facilitating transitions within the catalytic ensemble (Fig. 5).

The cobalamin cofactor in the catalytic structure is laterally shifted ~3.8 Å relative to its gateway state with a concurrent ~20° tilt, culminating in a change from a distorted to more planar corrin ring (Supplementary Figs. 8, 9). The corresponding changes associated with the cobalamin cofactor are analogous to those observed in previous MS structures in the reactivation conformation (Act-on), particularly the His-on versus His-off states/transition[6,9,22,23]. In total, these structures represent the Hcy-on (Hcy:Cob) state, which is adopted for the methyl transfer from methylcobalamin to homocysteine to yield methionine and Cob(I) (Reaction II, Fig. 1a).

Homocysteine methylation/methionine formation has been postulated to occur via an SN_2 mechanism whereby Zn acts as a Lewis acid to prime homocysteine for nucleophilic abstraction of the methyl group from methylcobalamin[19,24,25]. To determine if the orientation captured in the Hcy-on structures could support catalysis, we modeled HCY into the active site and found that the sulfur moiety of HCY is positioned directly above and in-line with the cofactor, ~4.4 Å from Co (Supplementary Fig. 9). Modeling methylcobalamin in the active site shows that the distance between the sulfur/thiolate moiety and the methyl group of methylcobalamin is ~2.1 Å. As such, this structure represents one that is catalytically competent for HCY methylation, indicating that the combination of non-native cofactors and rational protein engineering allowed for the crystallographic snapshot of a transient complex.

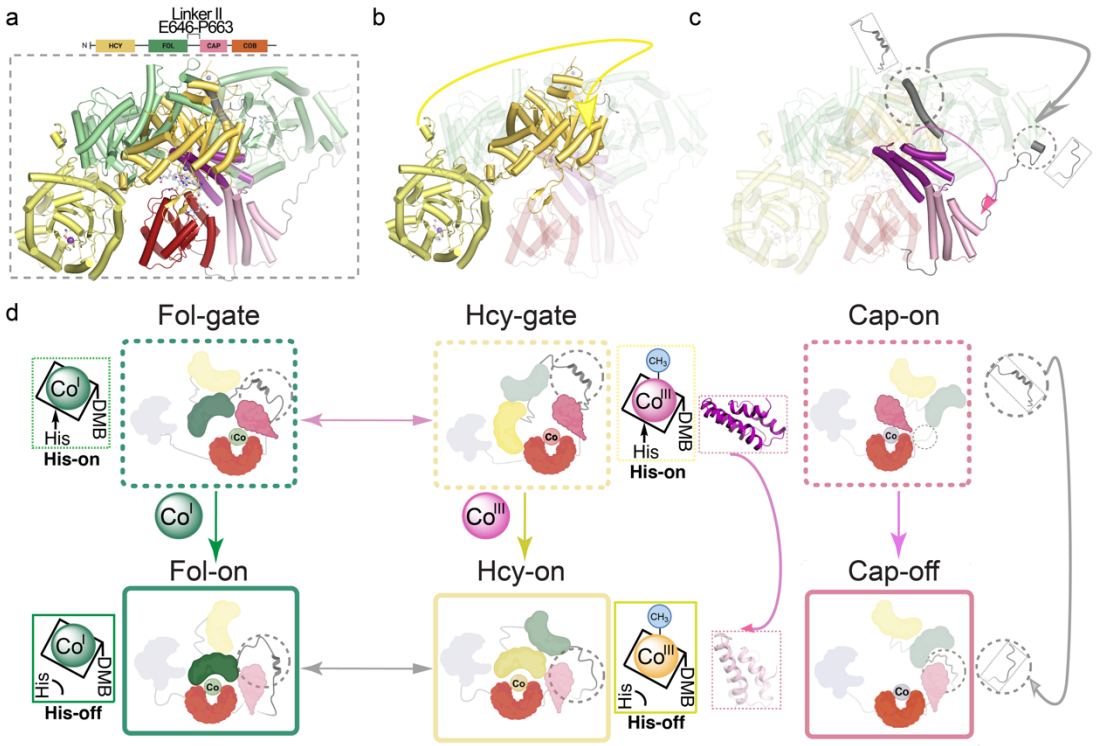

Figure 5. Fol:Cap linker helical melting defines structural transition associated with homocysteine methylation (Homocysteine-gate, Cap-on to Homocysteine-on, Cap-off). a Superposition of the Hcy and Fol domains using the Cob domain as a reference. *t*MS^{HcyFolCapCob} structures are shown as cartoons use the same coloring scheme as in Figure 3. **b** Contraction of the Fol:Cap linker causes a dramatic 40 Å repositioning of the Cob domain with the Hcy domain to form the Hcy-on ternary complex. **c** The Fol:Cap linker undergoes a helix-loop transition. The near complete melting of the helix enables the Hcy:Fol domains to move as rigid bodies after uncapping, repositioning the Hcy binding site and domain directly above the Cob domain and its bound cobalamin cofactor. **d** A revised cartoon model representing the transitions between Cap-on (gateway) and Cap-off (ternary, catalytic states). In all Cap-on structures, cobalamin is bound His-on. The Cap domain is placed in an identical position in all Cap-off structures, guided by Linker III, while Linker II helix-loop transitions mediate Hcy:Fol domain motions.

The Fol domain is required for Homocysteine Methylation

To interrogate the biochemical basis of the role of the Fol domain, particularly in the homocysteine methyltransferase reaction, we employed a UV-Vis assay using the Hcy, Hcy:Fol, and Cap:Cob domains in trans[26]. The excised domains were chosen to provide a simpler, minimal model sufficient to study the ability of MS to perform homocysteine methylation[27]. Methylcobalamin consumption was monitored using UV-Vis to quantify the activity of various kinds of *t*MS domains (Fig. 6). The Hcy:Fol didomain and the isolated Hcy domain of tMS (designated *t*MS^{HcyFol} and *t*MS^{Hcy}, respectively) contain the homocysteine-binding sites. Methylcobalamin was prepared and used in its free form and complexed with the Cap:Cob didomain (*t*MS^{CapCob}).

Consumption of exogenous methylcobalamin was detected in the presence of both *t*MS^{HcyFol} and *t*MS^{Hcy} (Fig. 6) but not in their absence (Supplementary Fig. 10). The consumption of methylcobalamin bound to *t*MS^{CapCob} was observed with *t*MS^{HcyFol} (Fig. 6c) but not with *t*MS^{Hcy} (Fig. 6d). These data suggest that the Fol domain is important for the formation of the Hcy-on conformation, and without it, a catalytically competent Hcy-on ternary complex cannot be formed[7,8,11,28].

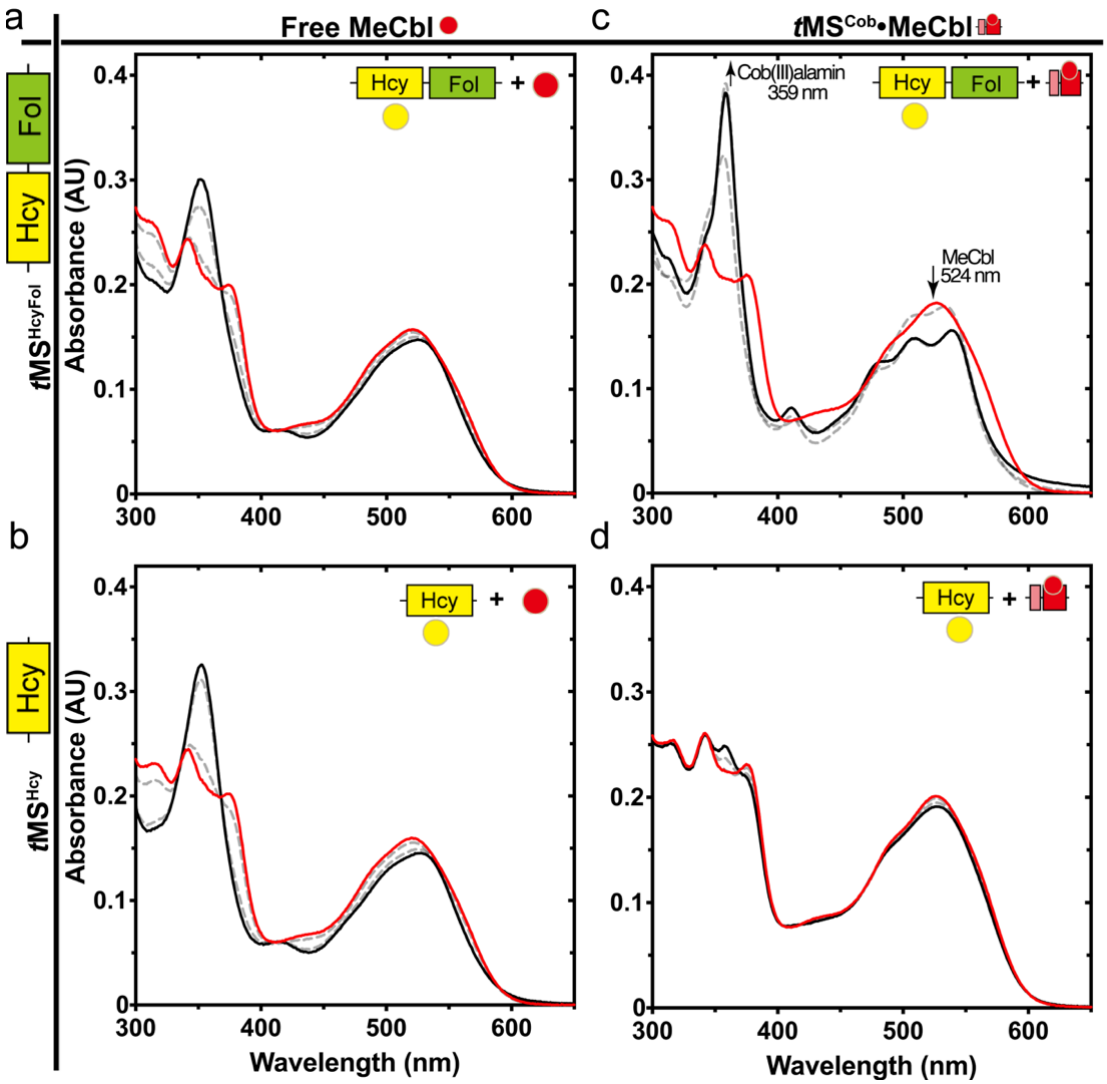

Figure 6. **Homocysteine methylation requires the Fol domain. a** Effects of *t*MS domain fragments on methylcobalamin:homocysteine methyltransferase reaction. The time-dependent spectral changes of methylcobalamin (MeCbl) in the presence of homocysteine were monitored at 50 ˚C for up to 10 minutes after adding homocysteine (final concentration of 100 µM) using 20 µM of free (non-protein bound) MeCbl incubated in the presence of 2 µM *t*MS constructs (**a** and **b**) or MeCbl (23 µM) was bound to the isolated *t*MS^{CapCob} domains first (**c** and **d**). Red lines represent the initial time point, with black lines representing the endpoint (final 10 min); gray lines represent intermediary time points (2, 5 min). Note that the presence of the Fol domain is required for activity when methylcobalamin is protein bound (**c**). Free homocysteine and methylcobalamin did not react (Supplementary Fig. 10), similar to *t*MS bound cobalamin (**d**). Free cobalamin can react minimally with *t*MS bound homocysteine, presumably due to the lack of the Cap domain and the ability of the Hcy domain to activate homocysteine. Data are of representative experiments, which have been repeated ≥2 times.

Proposed mechanism for MS catalysis

The Fol domain governs access to catalytic conformations

Our previously captured *t*MS structures (8SSC, 8SSD, and 8SSE) highlighted the preformed cobalamin pocket/cleft, which is solvent-exposed and thus readily accessible even *in crystallo*[9]. Similarly, previous structures of MS excised domains revealed that the Hcy and Fol domains were preformed, with minimal microenvironment changes in the substrate binding pocket induced upon ligand/substrate binding[21,29,30]. Even so, MS must exert global control over the conformational rearrangements that would allow access to the cobalamin cofactor (i.e. the formation of a ternary complex with the Cob domain and a substrate domain) following substrate binding[28].

The first step in the MS catalytic cycle is the methylation of HCY using methylcob(III)alamin to form methionine and Co(I), with the Co(I) being used to demethylate MTF and regenerate methylcob(III)alamin in the second step. Intriguingly, while purification of *t*MS routinely results in copurification with MTF[21], use of the *t*MS^{FolCapCob} tridomain did not yield any MTF-bound structure. MS that proceeds with homocysteine methylation in the absence bound MTF could undergo futile cycling, with the generated Co(I) cofactor being prone to oxidative inactivation. As such, the importance of MTF-binding, and thus of the Fol domain, makes intuitive sense. As a whole, the finding that only *t*MS^{HcyFol} was active in the trans assay using methylcobalamin loaded *t*MS^{CapCob} indicates that the Fol domain plays a pivotal and decisive role in guiding the conformation rearrangement necessary for the formation of the Hcy-on ternary complex (Fig. 6), likely through its role in destabilizing the Cap-on state (uncapping activity)[11,21]. Whether the Hcy domain itself is necessary for proper MTF-binding and reactivity remains unknown. The flexible interdomain linker region connecting Fol:Cap (Linker II) must be considered as an additional, significant factor. The presented structures indicate that while said linker is flexible, it can become ordered, forming a structured helix. Linker II can, putatively, act as a hinge. This coil-helix transition is a tantalizing explanation for why the Fol domain and Linker II are necessary for the formation of the Hcy-on conformation and provides a guided mechanism by which MS integrates substrate-binding information with cofactor status to undergo the pre-requisite rearrangements necessary for catalysis[7,8,11,28].

Linker regions guide domain rearrangements centered on the cobalamin cofactor

The captured structures provide the first experimental validation that interdomain linkers play an important role in facilitating the formation of specific conformations. The Hcy:Fol linker (Linker I), being rigid, is not found to change significantly[29]. As such, the Fol:Cap linker (Linker II) seems to play an outsized role in dictating and influencing the formation of catalytically-competent ternary complexes in the MS catalytic cycle, along with intermediates contained therein (Supplementary Figs. 1, 2, and 8). Alignment of the catalytic structures, including the previously obtained Act-on reactivation structures[9], indicate that the Cap-off Cob domain interface remains static. As such, the structural rearrangements observed center on the Cap:Cob domains, with substrate-binding domains vying for access to the protected (Cap-on) Cob domain (Supplementary Figs. 1, 2, and 3). Concurrently, the Cap:Cob linker (Linker III) could play a role in mediating the uncapping motion required for cobalamin access that is interdependent on the cofactor redox state and coordinate number, placing the Cap domain in a predetermined position. Morphing of gateway structures (Cap-on states, Fol-gate, Hcy-gate, Supplementary Movie 2) and catalytic structures (Cap-off, Fol-on, Hcy-on, Supplementary Movies 3 and 4) show that the Fol:Cap linker acts as a swiveling point (hinge) around which the Hcy:Fol domains, presumably, use the Fol domain to initiate “uncapping” of the Cob domain. Even in the Cap-on structures, the Cap:Cob domain remains relatively static (Supplementary Figs. 1, Supplementary Fig. 12, Supplementary Movie 1). It seems that the Fol domain, in conjunction with its associated Fol:Cap linker, plays a critical role in initiating and thereafter guiding the conformational ensemble during catalysis, while the Cap:Cob linker allows for proper repositioning of the Cap domain in one of two states (Cap-on or Cap-off).

Discussion

While the conformational plasticity of MS in solution is well-established, the precise regulatory mechanisms governing these domain arrangements remain elusive[5,6,7,8]. In this work, we have leveraged our previously discovered thermophilic methionine synthase homolog (*t*MS)[9] to structurally probe the conformations that underpin MS catalysis. This work has expanded the known conformational landscape of MS, revealing Cap-on states that function as gatekeepers to the catalytic cycle. Furthermore, we have captured the first catalytic structures of MS poised for MTF demethylation (Fol-on) and HCY methylation (Hcy-on), providing novel insights into the residues and global rearrangements that dictate catalytic activity. This study addresses long-

standing questions in the field that have been the subject of research for decades and contributes to a deeper understanding of MS, potentially leading to its exploitation as a biocatalytic tool.

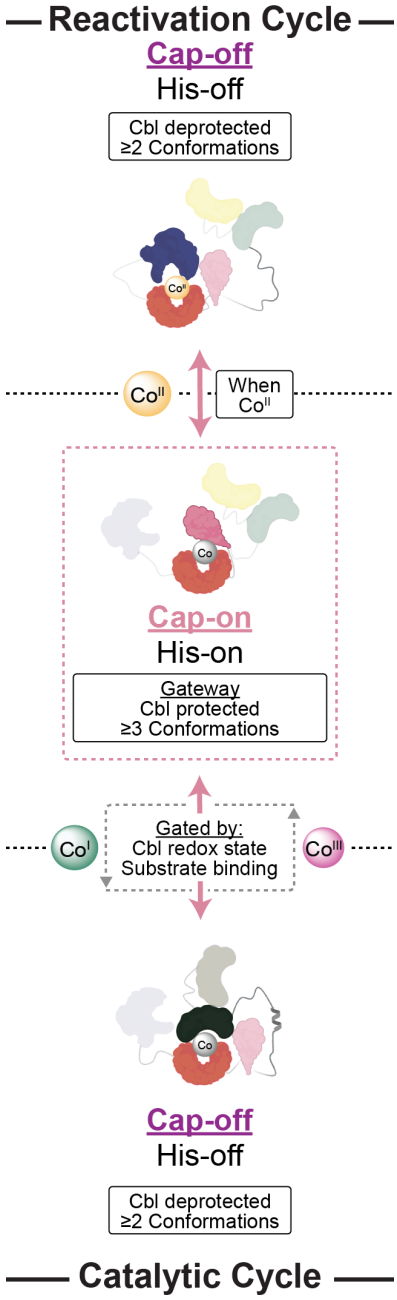

Figure 7. Conformational transitions during catalysis are mediated by the Hcy:Fol linker and signaled by His761. Methionine synthase can cycle through Cap-on, His-on states that function as gateway conformations, obviating the need for uncapping. Linker II (Fol:Cap linker) allows for facile transition between these gateway states, what we term 'resetting'. In our gateway conformations, hexacoordinate, His-on methylcobalamin (Co(III)) highlights its role as a bridge between the primary catalytic and reactivation cycles. Depending on the cobalamin cofactor's status (redox state, coordinate number) and substrate presence, the appropriate gateway state can grant entry to reactive conformations by uncapping and allowing for cobalamin flexibility by binding in the His-off state. Notably, the active methylating agent for methionine formation has been revised from hexa- to penta-coordinate His-off methylcob(III)alamin. Each methylation is associated with a distinct cobalamin redox state (Co(III) for homocysteine methylation, Reaction I; Co(I) for methyltetrahydrofolate demethylation, Reaction II; Co(II) for reductive methylation via SAM/AdoMet, Reaction III). The primary catalytic cycle of methionine synthase involves cycling between Co(III) and Co(I) states; Co(I) production acts to lock MS to either the catalytic cycle (homocysteine methylation) or the reactivation cycle (Co(II) reductive alkylation) until methylcobalamin is regenerated or Co(I) undergoes oxidative inactivation.

Our unified structural and functional model proposes that Cap-on states act as regulatory checkpoints, controlling access to the catalytically active Cap-off conformations. The precise mechanisms underlying transitions between catalytic states, however, warrants further investigation. Our model outlines two potential pathways: direct transitions between Hcy-on and Fol-on states (Supplementary Fig. 13, Model 1, Supplementary Fig. 14, Supplementary Movie 5) or recapping-dependent transitions involving multiple Cap-on states (Supplementary Fig. 13, Model 2, Supplementary Movies 6 and 7). In both scenarios, the Fol:Cap (Linker II) orchestrates the necessary domain rearrangements. If transitions between Hcy-on and Fol-on states can occur without recapping, a single Cap-on to Cap-off transition would be sufficient for catalytic entry. Thereafter, the Hcy:Fol domains can use rigid body motions as guided by Linker II to form their respective ternary complexes (Fig. 7, Supplementary Fig. 13, Model 1, Supplementary Fig. 14, Supplementary Movie 5). Conversely, if recapping is obligatory after catalysis, multiple Cap-on transitions, also orchestrated by Linker II, would be necessary for gated re-entry into the catalytic cycle (Fig. 7, Supplementary Fig. 13, Model 2, Supplementary Fig. 14, Supplementary Movies 6 and 7). The contrasting features of these models offer a valuable framework that facilitates hypothesis-driven experimentation and iterative model refinement, allowing for a more comprehensive understanding of the intricate structural ensemble that governs MS's multifaceted functionality. Currently, the catalytic efficiency of MS leads us to favor Model 1, where transitions between catalytic conformations are allowed, as seminal biochemical studies have shown that the rate limiting steps in MS are associated with conformational changes, and not its chemistry[7,28,29].

Our findings reveal a five-coordinate His-off methylcob(III)alamin intermediate during catalysis (Hcy-on, Figs. 4, and 5, Supplementary Figs. 6, 8, and 9)[28] and suggest the captured hexacoordinate His-on Co(III) state in the Fol-on complex represents a post-catalytic configuration, where cpCbl is in the Co(III) state and thus mimics methylcob(III)alamin (Supplementary Fig. 4). While a four-coordinate Co(I) species is implicated in folate demethylation[28], the role of the five-coordinate His-off cobalamin post-demethylation warrants further study. Although pentacoordinate His-on cob(I)alamin has not been directly observed, there is literature precedence for its potential role in enhancing folate demethylation, presumably through transition state stabilization (mimicking hexacoordinate methylcobalamin)[20,31]. Biochemical studies of cobalamin-dependent methyltransferases indicate His-on ligation (hexacoordinate) stabilizes methylcobalamin rather than being crucial for methyl transfer, while His-off, pentacoordinate methylcobalamin would promote homocysteine methyl transfer[18,20,31,32,33,34,35,36,37].

Significantly, all Cap-on structures observed to date exhibit alkylcob(III)alamin bound in the His-on state. Using rigorous biochemical studies, Bandarian *et al.* (Fig. 1b) introduced a model where a minimal set of four conformations was proposed along with the notion that methylcobalamin serves as a key branching point in both the catalytic and reactivation cycles[7]; while His-off cobalamin was emphasized for its importance in steering the conformational ensemble towards reactivation, we note that His-off methylcob(III)alamin is the active methylating agent during homocysteine methylation, and that His-off binding of cobalamin is associated with Cap-off transitions. In a separate study, Bandarian *et al.* demonstrated that the catalytic cycle of methionine synthase could be reconstituted *in trans* using two mutants that uncoupled homocysteine methylation and folate demethylation, positing 'turnover in two encounters', i.e. multiple uncapping events (Model 2)[31]. Using our structural data, we offer a nuanced revision to the model introduced in both seminal works[7,31]. Methylcobalamin generated from folate demethylation can either proceed to the Hcy-on state in the presence of homocysteine (Fig. 7, Supplementary Fig. 13, Model 1, Supplementary Fig. 14) or 'recap' to safeguard the cofactor (Fig. 7, Supplementary Fig. 13, Model 2, Supplementary Fig. 14, Supplementary Movie 8). Similarly, methylcobalamin produced during reductive methylation can exit the reactivation cycle, likely through recapping, before entering the catalytic cycle. In contrast, cob(I)alamin is exclusively generated during homocysteine methylation or

cob(II)alamin reduction. Once formed, cob(I)alamin is committed to its respective cycle (catalytic or reactivation) until methylcobalamin is regenerated or oxidative inactivation to cob(II)alamin occurs. This could explain the observed specificity of cob(I)alamin reactivity towards MTF in the catalytic cycle and AdoMet in the reactivation cycle[8]. Our models highlight the central role of hexcoordinate His-on methylcobalamin as a bridge between the catalytic and reactivation cycles, with the generation of Co(I) representing a commitment step to either cycle. Future work will focus on elucidating the fate of Co(I) during catalysis and further characterizing the dynamic interplay between His ligation, cobalamin reactivity, and structural transitions; said approach will also aid in characterizing the nature of the cobalamin cofactor during holoenzyme formation/cofactor loading and the conformation(s) associated therein.

Overall, this revised mechanistic and structural model synthesizes the wealth of biochemical data with unprecedented molecular level structural insights, providing a new unified framework with which to understand cobalamin-dependent methyltranferases such as methionine synthase (Fig. 7, Supplementary Figs. 11, 13, and 14). Starting from one of three Cap-on, His-on states (Fig. 7), MS can alternate between these three gateway conformations through rigid body motion of the Hcy:Fol domain towards the Cob domain (Fol-gate, Hcy-gate) or away from it (Act-gate/resting), all mediated by the remarkable polymorphism of Linker II (Supplementary Fig. 2, Supplementary Movie 1). Linker II is a versatile element capable of adopting at least three distinct structural conformations. This flexibility enables it to rearrange and reposition the domains attached to its termini. His-off ligation of cobalamin acts as a switch to allow for gated entry from gateway to catalytic transitions and vice-versa (Fig. 7). Structural transitions to catalytic states are primarily cofactor dependent, signaled by His ligation and the coordination number/environment, mediated by the Fol:Cap hinge (Linker II), programmed rigid body movement of the Cap:Cob domain via the Cap:Cob linker (Linker III), and substrate-bound domain association to form a ternary complex ready for catalysis. In this manner, MS harnesses the catalytic potential of cobalamin while avoiding futile cycling, leveraging unheralded structural motifs to orchestrate the dramatic rearrangements required to perform three improbable chemistries.

Methods

Cobalamin Analogue Synthesis

Cyanocobalamin (CNCbl) was purchased from Sigma/MilliporeSigma. cobalamin analogues were synthesized following previously established methods[38,39,40]. Briefly, CNCbl reduced by $NaBH_4$ was mixed with 4-chlorobutyric acid (Sigma/MilliporeSigma), 2-chloroethylamine hydrochloride (Sigma/MilliporeSigma), or n-propyl iodide (Sigma/MilliporeSigma) to yield γ-carboxypropyl-cobalamin (cpCbl), aminoethyl-cobalamin (aeCbl), and propyl-cobalamin (prCbl), respectively.

Construction of *t*MS Expression Vectors

Expression vectors for truncated *t*MS expression were generated using the ligation-independent cloning (LIC). Molecular cloning was conducted using pMCSG7 as the expression plasmid DNA vector and *E. coli* XL1-Blue as the host cell. All constructed bacterial expression vectors were designed to produce truncated *t*MS with a hexa-histidine tag and a Tobacco Etch Virus (TEV) protease cleavable sequence at the N-terminus.

The genes encoding truncated *t*MS were amplified by PCR, using pET(tMS^{wt}) as the template, obtained from the Riken BioResource Center[41]. Subsequently, these amplicons were integrated into a pMCSG7 vector using the ligation-independent cloning technique to generate truncated *t*MS expression vectors. To express tridomain *t*MS, pMCSG7($tMS^{FolCapCob}$) was constructed, encompassing the Fol, Cap, and Cob domains; pMCSG7($tMS^{HcyFolCapCob}$) was prepared in a similar fashion to express tetradomain *t*MS, containing all domains except for the Act domain. Didomain and single domain vectors to express the constructs used for assays were similarly generated, designated pMCSG7(tMS^{CapCob}), pMCSG7(tMS^{HcyFol}), and pMCSG7($tMS^{\Delta N35Hcy}$).

Mutant constructs were made by site-directed mutagenesis (QuikChange, Agilent). Mutant constructs of the tetradomain *t*MS used for crystallography include several mutations: pMCSG7($tMS^{HcyFolCapCobF110A,E123A,E124A,Y296A,D651A,P652A,G653A}$/$tMS^{HcyFolCapCobDPG2A\ dEE\ F110A,Y296A}$) used for crystallization of Hcy-

gate (mutant 1/construct 1) and pMCSG7(*t*MS^{HcyFolCapCobF110A,E123A,E124A,R268A,N283G,D651A,P652A,G653A,D759A,N774H,R826A}/*t*MS$^{HcyFolCapCobDPG2A\ dEE\ dRR\ F110A,N283G,D759A,N774H}$) used for crystallization of Hcy-on (mutant 2/construct 2), will be referred to as such in the methods for the sake of simplicity. A complete list of the bacterial strains, plasmids, and primers used in this study are listed in Supplementary Table 2.

Expression of *t*MS Constructs

E. coli BL21star(*DE3)* was transformed with the desired expression vectors for protein expression. *E. coli* transformed with the desired pMCSG7(*t*MS) constructs were propagated at 37 °C in Luria Broth containing 50 µg/mL ampicillin, and protein overexpression was induced using autoinduction media[42,43]. *E. coli BL21star(DE3)* transformed with pMCSG7(*t*MS^{FolCapCobD759A}) were propagated at 37 °C in Luria Broth containing 50 µg/mL ampicillin and protein overexpression was induced using Isopropyl β-D-1-thiogalactopyranoside (IPTG) (final concentration of 1 mM). In the case of *t*MS^{HcyFolCapCobmut1}, $ZnCl_2$ was added to the medium (0.5 mM) and protein overexpression was induced using autoinduction media. Cells were propagated at 30 °C with shaking at 250 rpm overnight prior to harvesting via centrifugation and stored at -80 °C.

*t*MS Protein Purification

Immobilized metal chelate affinity (IMAC) chromatography was employed to purify recombinant *t*MS (HisTrap Chelating HP, Cytvia, 5 mL), using a $NiSO_4$ charged column for all constructs except for *t*MS^{HcyFolCapCob}, where a $ZnSO_4$ charged column was used.

*t*MS$^{FolCapCobD759A•MeCbl}$ (Fol-gate)

For holoenzyme purification with Mecobalamin (*t*MS^{FolCapCobD759A}, *t*MS^{CapCob}), the harvested cell pellet was first resuspended in 50 mM potassium phosphate buffer (KPB) (pH 7.4), 0.3 M sodium chloride, (4-5 mL/1 g of pellet), to which lysozyme (0.1 mg/mL) and PMSF (1 mM) were added. The resuspended cell pellet was lysed via sonication (4 °C, 5 s on, 5 s off, 5 min total). The crude lysate was centrifuged (15 min × 3, 10,000 × g, 4 °C) decanting the supernatant to remove any cellular debris/pellet. Methylcobalamin (MeCbl) (MilliporeSigma) was added to the crude supernatant (20 mM) to form holo-*t*MS. The crude extract with Mecobalamin was incubated at 70 °C for 15 min, then centrifuged (15 min, 10,000 × g, 4 °C). The supernatant was pooled and filtered via syringe (0.45 µm), to which imidazole was added (20 mM) and the supernatant was applied/loaded onto a Ni-affinity column (His-trap Chelating HP, Cytvia, 5 mL) equilibrated with 50 mM KPB, pH 7.4, and 20 mM imidazole. The column was washed with 50 mM KPB, pH 7.4, 0.3 M NaCl, 20 mM Imidazole (5 CV), then 50 mM imidazole (5 CV), and the protein was eluted in bulk with buffer consisting of 50 mM KPB, pH 7.4, 0.3 M NaCl, and 80 mM imidazole (8 CV) and 150 mM Imidazole (3 CV). Red-colored fractions were collected and subjected to a TEV digest, with dialysis at 4 °C overnight (50 mM KPB, pH 7.4). The dialyzed sample was incubated at 70 °C for 15 min to quench the TEV digest, then centrifuged (15 min, 10,000 × g, 4 °C). The resulting supernatant was clarified via centrifugation and concentrated via centrifugation in 50 mM KPB, pH 7.4 to yield purified *t*MS$^{FolCapCobD759A•MeCbl}$ (~10 mg/mL), which was stored at 4 °C or flash-frozen for long-term storage at -80 °C.

*t*MS$^{CapCob•MeCbl}$

*t*MS^{CapCob} was prepared similarly, save for the use of a TEV digest. Following IMAC purification and pooling of red-colored fractions, the combined fractions were subject to dialysis at 4 °C overnight (50 mM KPB, pH 7.4). The dialyzed sample was concentrated via centrifugation in 50 mM KPB, pH 7.4 to yield purified *t*MS$^{CapCob•MeCbl}$ (~10 mg/mL), which was stored at 4 °C or flash-frozen for long-term storage at -80 °C.

*t*MS$^{FolCapCobD762G•cpCbl}$ (Fol-on)

Purification of *t*MS^{FolCapCobD762G} was conducted similarly, with no added cobalamin. The harvested cell pellet was resuspended in 50 mM KPB (pH 7.4) (~5 mL/1 g of the pellet), to which lysozyme (0.1 mg/mL) and PMSF (1 mM) were added. The resuspended cell pellet was lysed via sonication (4 °C, 5 s on, 5 s off, 5 min total). The crude lysate was centrifuged (45 min, 20,000 × g, 4 °C) decanting the supernatant to remove any cellular debris/pellet. To the crude supernatant was added Imidazole (final concentration, 20 mM) before purification using a modified IMAC bulk elution (equilibration buffer: 50 mM KPB, pH 7.4, 20 mM Imidazole, wash buffer: 50 mM KPB, pH 7.4, 0.3 M NaCl, 50 mM Imidazole, elution buffer A: 50 mM KPB, pH 7.4, 0.3 M NaCl, 80 mM Imidazole, elution buffer B: 50 mM KPB, pH 7.4, 0.3 M NaCl, 150 mM Imidazole), with the desired protein

eluting at 80 mM and 150 mM Imidazole. Fractions containing protein as confirmed by SDS-PAGE were then pooled, and an ammonium sulfate cut was used to remove any impurities, using an equal volume of saturated ammonium sulfate (~4 M) to achieve a final concentration of 50% (w/v). The mixture was centrifuged (10,000 × g, 10 min, 4 °C) to pellet any precipitated protein. The supernatant was carefully decanted to avoid disturbing the pellet; the pellet was resuspended in 0.1 M KPB, pH 7.4, 2 mM DTT (10 mL). A TEV digest and overnight dialysis (4 °C, 0.1 M KPB, pH 7.4, 2 mM DTT) followed. To the dialyzed sample containing ~135 µM apo-tMS$^{\text{FolCapCobD762G}}$ was added γ-carboxypropyl cobalamin (cpCbl, ~170 µM) and the TEV digest quenched by heating at 60 °C (15 min), then centrifuged (10 min, 4,000 × g, 4 °C). The resulting supernatant was loaded onto a pre-equilibrated desalting column (PD-10, GE/Cytvia, 50 mM KPB, pH 7.4) to remove excess cpCbl and concentrated via centrifugation in 50 mM KPB, pH 7.4 to yield purified holo-tMS$^{\text{FolCapCobD762G•cpCbl}}$ (~10 mg/mL), which was stored at 4 °C or flash-frozen for long-term storage at -80 °C.

Purification of apo constructs (tMS$^{\Delta\text{N35Hcy}}$, tMS^{HcyFol}) was conducted similarly, with no added cobalamin.

<u>tMS$^{\Delta\text{N35Hcy}}$</u>

The harvested cell pellet was resuspended in 50 mM Tris (pH 7.4), 0.1 M NaCl (~5 mL/1 g of the pellet), to which lysozyme (0.1 mg/mL) and PMSF (0.1 mM) were added. The resuspended cell pellet was lysed via sonication (4 °C, 5 s on, 5 s off, 5 min total). The crude lysate was centrifuged (10 min, 8,000 × g, 4 °C) decanting the supernatant to remove any cellular debris/pellet. The crude supernatant was subjected to a heat-treatment step at 60 °C (15 min), then centrifuged (10 min, 4,000 × g, 4 °C), decanting the supernatant to remove any cellular debris/pellet. To the crude supernatant was added Imidazole (final concentration, 50 mM) purification using a modified IMAC bulk elution (equilibration buffer: 50 mM Tris, pH 7.4, 0.1 M NaCl, 50 mM Imidazole, wash buffer: 50 mM Tris, pH 7.4, 0.3 M NaCl, 50 mM Imidazole, elution buffer: 50 mM Tris, pH 7.4, 0.3 M NaCl, 200 mM Imidazole) , with the desired protein eluting at 200 mM Imidazole. Fractions containing protein as confirmed by SDS-PAGE were then pooled. The combined fractions were subject to dialysis at 4 °C overnight (50 mM Tris, pH 7.4), to which TCEP was added (final concentration, 1 mM), followed by a second heat-treatment step at 60 °C (15 min) then centrifuged (10 min, 4,000 × g, 4 °C). The resulting supernatant was concentrated via centrifugation in 50 mM Tris, pH 7.4, 1 mM TCEP to yield purified tMS$^{\Delta\text{N35Hcy}}$ (~10 mg/mL), which was stored at 4 °C or flash-frozen for long-term storage at -80 °C.

<u>tMS^{HcyFol}</u>

The harvested cell pellet was resuspended in 50 mM KPB (pH 7.4) (~5 mL/1 g of the pellet), to which lysozyme (0.1 mg/mL) and PMSF (0.1 mM) were added. The resuspended cell pellet was lysed via sonication (4 °C, 5 s on, 5 s off, 5 min total). The crude lysate was centrifuged (10 min, 8,000 × g, 4 °C) decanting the supernatant to remove any cellular debris/pellet. The crude supernatant was subjected to a heat-treatment step at 70 °C (15 min), then centrifuged (10 min, 4,000 × g, 4 °C), decanting the supernatant to remove any cellular debris/pellet. To the crude supernatant was added Imidazole (final concentration, 20 mM) before purification using a modified IMAC bulk elution (equilibration buffer: 50 mM KPB, pH 7.4, 50 mM Imidazole, wash buffer: 50 mM KPB, pH 7.4, 0.3 M NaCl, 50 mM Imidazole, elution buffer: 50 mM Tris, pH 7.4, 0.3 M NaCl, 80 mM Imidazole), with the desired protein eluting at 80 mM Imidazole. Fractions containing protein as confirmed by SDS-PAGE were then pooled. The combined fractions were subject to dialysis at 4 °C overnight (50 mM KPB, pH 7.4). The resulting dialyzed sample was concentrated via centrifugation in 50 mM KPB, pH 7.4 to yield purified tMS^{HcyFol} (~10 mg/mL), which was stored at 4 °C or flash-frozen for long-term storage at -80 °C.

Purification of tMS^{HcyFolCapCob} constructs was conducted similarly, without added cobalamin, but using a $ZnSO_4$-loaded IMAC column (GE/Cytvia, 5 mL).

<u>tMS$^{\text{HcyFolCapCobmut1•prCbl}}$ (Hcy-gate)</u>

The harvested cell pellet was first resuspended in 50 mM potassium phosphate buffer (KPB) (pH 7.4) (4-5 mL/1 g of pellet), to which lysozyme (0.1 mg/mL) and PMSF (1 mM) were added. The resuspended cell pellet was lysed via sonication (4 °C, 5 s on, 5 s off, 5 min total). The crude lysate was centrifuged (45 min, 20,000 × g, 4 °C) decanting the supernatant to remove any cellular debris/pellet. The supernatant was pooled and syringe filtered (0.45 µm), to which imidazole was added (20 mM) and the supernatant was applied/loaded onto a Zn-affinity column (His-trap Chelating HP, Cytvia, 5 mL) equilibrated with 50 mM KPB, pH 7.4, and 20 mM imidazole. The column was washed with 50 mM KPB, pH 7.4, 0.3 M NaCl, 20 mM Imidazole (1 CV), then 50 mM imidazole (5 CV), and the protein was eluted in bulk with buffer consisting of 50 mM KPB, pH 7.4, 0.3 M NaCl, and 80 mM

imidazole (10 CV) and 150 mM Imidazole (3 CV). Fractions containing protein as confirmed by SDS-PAGE were then pooled and subjected to a TEV digest, with dialysis at 4 °C overnight (50 mM KPB, pH 7.4). The dialyzed sample was incubated at 50 °C for 15 min to quench the TEV digest. The supernatant was clarified via centrifugation (10,000 × g, 10 min, 4 °C), then loaded onto a pre-equilibrated HiLoad 16/600 Superdex 200 pg column (Cytvia) (2 CV of SEC Buffer: 25 mM Tris, pH 7.4, 0.1 M KCl, 1 mM TCEP), yielding the desired protein (~95 kDa). The protein was found to elute as a monomer, with minimal to no aggregation visible. Fractions containing the desired protein, as judged by SDS-PAGE, were pooled and concentrated via centrifugation in SEC buffer to yield purified apo-*t*MS^{HcyFolCapCobmut1}(~15 mg/mL). To the purified apo protein (~150 μM) was added propylcobalamin (prCbl, ~170 μM). The resulting mixture was loaded onto a pre-equilibrated desalting column (PD-10, GE/Cytvia, SEC buffer) to remove excess prCbl and concentrated via centrifugation in SEC buffer to yield purified holo-*t*MS$^{HcyFolCapCobmut1•prCbl}$ (~15 mg/mL), which was stored at 4 °C or flash-frozen for long-term storage at -80 °C.

*t*MS$^{HcyFolCapCobmut2•aeCbl}$ (Hcy-on)

Purification of *t*MS$^{HcyFolCobmut2•aeCbl}$ was conducted similarly, with no added cobalamin. Protein purification yielded purified apo- of *t*MS^{HcyFolCapCobmut2} (~20 mg/mL); to the purified apo protein (~150 μM) was added aminoethylcobalamin (aeCbl, ~170 μM). The resulting mixture was loaded onto a pre-equilibrated desalting column (PD-10, GE/Cytvia, 25 mM Tris, pH 7.4, 0.1 M KCl, 1 mM TCEP) to remove excess aeCbl and concentrated via centrifugation in 25 mM Tris, pH 7.4, 0.1 M KCl, 1 mM TCEP to yield purified holo-*t*MS$^{HcyFolCapCobmut2•aeCbl}$ (~18 mg/mL), which was stored at 4 °C or flash-frozen for long-term storage at -80 °C.

*t*MS Qualitative UV-Vis Assay

The methylcobalamain:homocysteine methyltransferase reaction was monitored in the presence or absence of *t*MS constructs spectrophotometrically in the dark, tracking the time-dependent spectral changes of methylcobalamin (MeCbl) in the presence of homocysteine at 50 ˚C for up to 10 minutes. Briefly, the reaction mixture (1 mL total volume) was carried out under aerobic conditions containing either free MeCbl (20 μM) or protein-bound MeCbl (*t*MS$^{CapCob•MeCbl}$, 23 μM) and homocysteine (100 μM) in 50 mM KPB, pH 7.4, 0.5 mM TCEP using *t*MS if applicable (tMS$^{\Delta N35Hcy}$ or *t*MS^{HcyFol}, 2 μM). Reactions were incubated with the reaction mixture in the absence of homocysteine at 50 ˚C, then initiated by adding homocysteine. Time-dependent spectral changes were recorded were recorded for 0-10 min using a Cary 300 Bio UV-Vis spectrophotometer (Varian, Inc.)

Crystallization of *t*MS Constructs

Crystals were grown via sitting drop vapor diffusion. *t*MS$^{FolCapCobD759A•MeCbl}$ (~10 mg/mL in 25 mM KPB, pH 7.4) was mixed with a reservoir solution containing 25% PEG 3350, 0.1 M Tris (pH 8.5), and 0.2 M ammonium acetate in a 1:1 ratio (0.4 μL each) and incubated at 4 ˚C. Crystals were briefly transferred to a cryoprotectant solution containing approximately 20% glycerol and 20% PEG 3350, 0.08 M Tris (pH 8.5), and 0.16 M ammonium acetate for 30 seconds prior to harvesting and flash freezing in liquid nitrogen.

*t*MS$^{FolCapCobD762G•cpCbl}$ (~10 mg/mL in 25 mM KPB, pH 7.4) was mixed with a reservoir solution containing 1.26 M $(NH_4)_2SO_4$, 0.1 M CHES-Na (pH 9.5), and 0.2 M NaCl in a 1:1 ratio (0.4 μL each) and incubated at 20 ˚C. Crystals were briefly transferred to a cryoprotectant solution containing approximately 20% glycerol and 1 M $(NH_4)_2SO_4$, 0.08 M CHES-Na (pH 9.5), and 0.16 M NaCl for 3 minutes prior to harvesting and flash freezing in liquid nitrogen.

*t*MS$^{HcyFolCapComut1•prCbl}$ (~15 mg/mL in 25 mM Tris, pH 7.5, 50 mM KCl, 1 mM TCEP) was mixed with a reservoir solution containing 30% PEG 3350, 0.1 M Bis-Tris (pH 6.5), and 0.2 M NaCl in a 1:1 ratio (0.4 μL each) and incubated at 20 ˚C. Crystals were briefly transferred to a cryoprotectant solution containing approximately 20% glycerol and 24% PEG 3350, 0.08 M Bis-Tris (pH 6.5), and 0.16 M NaCl for ~30 seconds prior to harvesting and flash freezing in liquid nitrogen.

*t*MS$^{HcyFolCapCobmut2•aeCbl}$ (~18 mg/mL in 25 mM Tris, pH 7.5, 50 mM KCl, 1 mM TCEP) was mixed with a reservoir solution containing 20% PEG 3350, 0.1 M Tris (pH 8.5), and 0.2 M NaCl in a 1:1 ratio (0.4 μL each) and incubated at 4 ˚C. Crystals were briefly harvested and flash frozen in liquid nitrogen.

Data collection and processing statistics are summarized in Supplementary Table 1. Data for *t*MS$^{FolCapCobD759A•MeCbl}$ were indexed to spacegroup $P4_12_12$ (unit-cell parameters a = b = 187.77, c = 325.55 Å) with six molecules in the

asymmetric unit (Matthew's coefficient VM = 4.22 $Å^3$ Da^{-1}, 71% solvent content). Data for $tMS^{FolCapCobD762G•cpCbl}$ were indexed to spacegroup $P6_122$ (unit-cell parameters a = b = 95.15, c = 274.29 Å) with one molecule in the asymmetric unit (Matthew's coefficient VM = 3.17 $Å^3$ Da^{-1}, 61% solvent content). Data for $tMS^{HcyFolCapCobmut1•prCbl}$ were indexed to spacegroup $P2_12_12_1$ (unit-cell parameters a = 69.95, b = 83.82, c = 163.72 Å) with one molecule in the asymmetric unit (Matthew's coefficient VM = 2.51 $Å^3$ Da^{-1}, 51% solvent content). Data for $tMS^{HcyFolCapCobmut2•aeCbl}$ were indexed to spacegroup $P2_12_12_1$ (unit-cell parameters a = 70.81, b = 115.10, c = 120.80 Å) with one molecule in the asymmetric unit (Matthew's coefficient VM = 2.58 $Å^3$ Da^{-1}, 52% solvent content).

Data Collection and Refinement

X-ray data sets were collected at 100 K on GM/CA beamline 23-ID-B at the Advanced Photon Source, Argonne National Laboratory (Argonne, IL) for $tMS^{FolCapCobD759A•MeCbl}$ and $tMS^{FolCapCobD762G•cpCbl}$, on GM/CA beamline 23-ID-D for $tMS^{HcyFolCapCobmut2•aeCbl}$, and on LS-CAT beamline 21-ID-D at the Advanced Photon Source, Argonne National Laboratory (Argonne, IL) for $tMS^{HcyFolCapCobmut1•prCbl}$. Data sets were processed using xia2/DIALS[44]. Initial phases were obtained using MOLREP[45]. Individual domains of the full-length *t*MS structure (8SSC) were used as search models. Iterative model building and corrections were performed manually using Coot[46] following molecular replacement and subsequent structure refinement was performed with CCP4 Refmac5[47]. Initial refinement was conducted using BUSTER[48] to rapidly fix Ramachandran, rotamer, and density fit outliers, refining to convergence and adding waters in the final automated round of refinement. Phenix eLBOW[49] was used to generate the initial ligand restraints using ligand ID "COB" or "B12". Phenix LigandFit[50] was used to provide initial fits and placements of the ligands. PDB-REDO[51] was used to assess the model quality in between refinements and to fix any rotamer and density fit outliers automatically. The model quality was evaluated using MolProbity[52]. The Hcy-on structure data set showed partial crystal twinning and final rounds of refinement were conducted using twin refinement, as suggested by PDB-REDO. Figures showing crystal structures were generated in PyMOL[53].

Statistical Analysis and Reproducibility

Unless otherwise stated, functional assays were conducted using n = 2 independent replicates. At least three independent experiments were conducted for each functional assay. All attempts at replication were successful. Analysis and curve-fitting was performed using Prism 10.2.3.

Data Availability

The structure coordinates and structure factors reported in this study have been deposited in the Protein Data Bank under accession codes 9CBO ($tMS^{FolCapCobD759A•MeCbl}$), 9CBP ($tMS^{HcyFolCapCobmut1•prCbl}$), 9CBQ ($tMS^{FolCapCobD762G•cpCbl}$), and 9CBR ($tMS^{HcyFolCapCobmut2•aeCbl}$). PDB codes of previously published structures used in this study are 8SSC, 8SSD, 8SSE, and 8G3H. All other data are available from the corresponding authors upon request. Source data are provided with this paper.

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Competing Interests

The authors declare no competing interests.