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Mechanistic Insights for the Formation of Organometallic Co-C Bond in the Methyl Transfer Reaction Catalyzed by Methionine Synthase

Neeraj Kumar[1] *and Pawel M. Kozlowski*[1,*]

[1]Department of Chemistry, University of Louisville, Louisville, Kentucky 40292, USA,

AUTHOR EMAIL ADDRESS: Pawel@louisville.edu

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[*] Corresponding author: Pawel M. Kozlowski, Department of chemistry, 2320 South Brook Street, Louisville, KY 40208; Phone, (502) 852-6609; Fax, (502) 852-8149.

ABSTRACT: Methionine synthase (MetH) catalyzes the transfer of a methyl group from methyltetrahydrofolate (CH_3-H_4Folate) to the cob(I)alamin intermediate to form an organometallic Co-C bond, a reaction similar to that of CH_3-H_4Folate:corrinoid/iron-sulfur protein (CFeSP) methyltransferase (MeTr). How precisely it is formed remains elusive because the displacement of a methyl group from tertiary amine is not a facile reaction. To understand the electronic structure and mechanistic details of the MetH-cob(I)alamin:CH_3-H_4Folate reaction complex, we applied quantum mechanics/molecular mechanics (QM/MM) computations. The hybrid QM/MM calculations reveal the traditionally assumed S_N2 mechanism for the formation CH_3-cob(III)alamin resting state where the activation energy barrier for the S_N2 reaction was found to be ~ 8-9 kcal/mol which is comparable with respect to the determined experimental rate constant. However, the possibility of an electron transfer (ET)-based radical mechanism consistent with the close-lying diradical states observed from triplet and open-shell singlet states has also been suggested, where first an electron transfer from His-on cob(I)alamin to pterin ring of the protonated CH_3-H_4Folate takes place, forming $Co^{II}(d^7)$-pterin radical $(\pi^*)^1$ diradical state, followed by a methyl radical transfer. Although, the predicted energy barrier for the ET-mediated radical reaction is comparable to that of S_N2 pathway, the major advantage of ET is that a methyl radical can be transferred at a longer distance, which does not require the close proximity of two binding modules of MetH as does the S_N2-type. In addition, based on the energy barrier of the transition state (TS) in both the protonated (~ 8-9 kcal/mol) and unprotonated N5 (39 kcal/mol) species of the CH_3-H_4Folate, it can be inferred that the protonation event must takes place, either prior to or during the methyl transfer reaction in a ternary complex. The results of the present study including mechanistic insights can have implications to a broad class of corrinoid-methyltransferases, which utilize a CH_3-H_4Folate substrate or its related analogues as methyl donor.

Keywords: QM/MM, DFT, Methionine Synthase, Methyltetrahydrofolate, Methyltransferases, Acetyl Coenzyme A, S_N2 and Radical Mechanism.

1. Introduction

B_{12} (cobalamin)-dependent methyltransferases play a critical role in one-carbon metabolism and CO_2 fixation in anaerobic microbes, as well as in amino acid metabolism in several organisms including mammals and bacteria.[1, 2, 3, 4] The corrinoid-dependent methyltransferases usually catalyze the transfer of a methyl group from a methyl donor to a methyl acceptor in multi domains or/and multi proteins. One of the least understood reaction is the transfer of a methyl group from a methyltetrahydrofolate (CH_3-H_4Folate or CH_3-THF) substrate to a cob(I)alamin/cob(I)amide intermediate. This particular reaction is common in a wide range of methyltransferases, namely, cobalamin-dependent methionine synthase (MetH) from *E. coli*,[5, 6, 7, 8, 9, 10] CH_3-H_4Folate:corrinoid-iron/sulfur protein (CFeSP) methyltransferase (MeTr) in the Acetyl-CoA pathway from *C. thermoaceticum*,[11, 12, 13, 14, 15, 16, 17, 18] and bacterial mercury methylation (HgcAB) from *D. desulfuricans ND132*.[19] Among all the known corrionoid proteins, cobalamin-dependent MetH (Figure 1) is one of the best studied enzymes that catalyzes the transfer of a methyl group from CH_3-H_4Folate to homocysteine (Hcy) to form a methionine, in which cobalamin cofactor serves as an intermediate methyl carrier oscillating between methylcobalamin (MeCbl) and cob(I)alamin.[20, 21, 22]

MetH is a single polypeptide (136 kDa and 1227 amino acids) and has a modular architecture with four different binding modules including the CH_3-H_4Folate-binding donor domain, Hcy-binding acceptor domain, and MeCbl-binding domain, which are required for the main catalytic cycle and the adenosylmethionine (AdoMet) domain that is essential for the reactivation cycle (Figure 1).[6, 23, 24, 25] During the course of catalytic turnover, each substrate-binding domain interacts with the β-face of the enzyme-bound cobalamin prosthetic group, which requires various conformational changes to position the donor and acceptor domains in an alternate manner.[23] Unfortunately, the crystal structure of full length of MetH enzyme has not been solved yet due to high degree of conformational flexibility but the crystal structure of each individual domain has been successfully characterized.[23, 26, 27, 28]

The methyl transfer reactions to and from the cobalamin are extremely challenging and the way in which MetH activates the methyl donors such as MeCbl and the CH_3-H_4Folate through conformational changes remains ambiguous. In the first-half catalytic reaction (Figure 1), the cob(I)alamin intermediate is methylated by CH_3-H_4Folate, thus forming MeCbl and

tetrahydrofolate (H_4Folate or THF). During the second-half catalytic reaction, the resulting MeCbl form of the cofactor is demethylated by Hcy substrate forming cob(I)alamin and methionine amino acid (Figure 1). The key step in the MetH catalytic reaction is the formation of organometallic cobalt-carbon (Co-C) bond, i.e., Me-cob(III)alamin where the CH_3-H_4Folate donates a methyl group to exogenous cob(I)alamin. Precisely, how it is formed in the enzymatic reaction remains an open question. Moreover, it has been estimated that the methyl transfer from enzyme-bound CH_3-H_4Folate to cob(I)alamin is accelerated by 35-million-fold as compared to the similar reaction in the model complexes.[29] Therefore, the challenge is to determine the enzymatic mechanism(s) and the source of activation of an unreactive methyl donor CH_3-H_4Folate by modeling the intermodular methyl transfer reactions.

On the other hand, the displacement of a methyl group (as cation/radical) attached to a tertiary amine (i.e., CH_3-H_4Folate) is not a facile reaction in bioorganic chemistry even in the presence of strong nucleophile such as cob(I)alamin because the H_4Folate anion is a very poor leaving group. It has been demonstrated by Pratt and coworkers that 5,5-dimethyltetrahydropterin (structural analogue of protonated CH_3-H_4Folate) can serve as a methyl donor to cob(I)alamin.[30] The electrophilic activation of the CH_3-H_4Folate is required for the methyl transfer reaction, most probably by protonation at N5 position that will form an ammonium complex. In addition, Smith and Matthews have revealed using ^{13}C distortionless enhancement by polarization transfer (DEPT) NMR spectroscopy that the CH_3-H_4Folate protonates the N5 position but not the conjugated carbon next to it.[20] Thus, it is of great interest to understand the source of a protonation at N5 position of the CH_3-H_4Folate in order to investigate the catalytic methyl transfer reaction.

The X-ray structure of CH_3-H_4Folate-binding domain of the MetH (PDB ID: 1Q8J at 1.9 Å resolution, Figure 2) shows that the pterin ring of the CH_3-H_4Folate is interacting with the hydrophilic protein residues such as Asn508, Asn473 and Asp390 via H-bond network, indicating the polar environment might be involved to increase the p*K*a at N5 position.[26] Most importantly, Asn508 interacts from 2.8 Å distance with the N5 of the CH_3-H_4Folate substrate and this H-bond interaction could be the key for the stabilization of protonation state. Interestingly, the active site residues near the pterin ring of the CH_3-H_4Folate are conserved in both MetH and MeTr[17] including the Asparagine (Asn508 in PDB ID: 1Q8J and Asn199 in PDB ID: 2E7F, respectively) which is H-bonded to the N5 position. However, it has been shown that H_4Folate

which is the product of the methyl transfer reaction contains a proton at N5 position.[20] The most critical question that arise here is the timing of the proton uptake with respect to the methyl group transfer; nonetheless, the crystal structure of the CH_3-H_4Folate binding domain of the MetH shows no general acid (proton donor, BH) other than a H-bond network with the Asn508 (Figure 2), indicating an unprotonated CH_3-H_4Folate bound by the protein.[20] In addition, it has been further postulated by Matthews et al. that the N5 atom of the CH_3-H_4Folate cannot be protonated in the binary MetH.CH_3-H_4Folate complex. The protonation event occurs in a later stage instead, i.e., in the ternary MetH.Cob(I)alamin.CH_3-H_4Folate complex.[29] In the case of MeTr, the kinetic studies have shown that the CH_3-H_4Folate binds to the AcsE, a separate protein of MeTr, in an unprotonated form and then undergoes rapid protonation, thus the protonation occur in the binary complex.[12, 31]

Although several mechanistic pathways have been suggested for the methyl transfer reactions catalyzed by MetH,[3, 29] an exact route remains the subject of ongoing debate. It is generally believed that the methyl transfer reaction from CH_3-H_4Folate to cob(I)alamin proceeds via an S_N2-type nucleophilic displacement, nevertheless, alternative pathways including single electron transfer (SET) as well as oxidative addition (reductive elimination in the second-half catalytic reaction) have also been proposed by Matthews et al.[29] The major limitation of the oxidative addition mechanism is a three-centered bond requirement between the C_{Me}-N5 bond of the CH_3-H_4Folate and the Co of the cob(I)alamin intermediate, which might not be possible from enzymatic point of view due to steric interactions in the protein backbone of two reacting modules. In addition, the S_N2-type mechanism was assumed in the model complexes using DFT calculations and the activation energy of the transition state was found to be 13 kcal/mol.[32] However, this computational study lacks the crucial role of the MetH enzyme, particularly the cap subdomain that packs over the β-face of the cofactor, which could be involved in stabilizing the CH_3-H_4Folate substrate and subsequently help in facilitating the methyl transfer reaction. Despite the extensive studies of the MetH, an important role of enzyme-bound cob(I)alamin (containing cap and Rossman subdomain) for the methyl transfer reaction from CH_3-H_4Folate is poorly understood. In addition, there is no structural information available with regard to the reaction complex of MetH catalytic reactions, therefore the details of the reaction mechanism involving the methylation of cob(I)alamin by CH_3-H_4Folate substrate remain unclear.

In the present computational studies, we applied ONIOM-based quantum mechanics/molecular mechanics (QM/MM) calculations to investigate the E.S reaction complex, which was prepared by docking a CH_3-H_4Folate substrate in the β-face of the enzyme-bound cob(I)alamin. Based on the electronic structure and coordination of the cob(I)alamin intermediate in the cob(I)alamin:CH_3-H_4Folate reaction complex, two different mechanistic pathways were explored: the S_N2-type nucleophillic displacement and the possibility of an alternative ET-based radical mechanism where the electron is transferred from His-on conformation of cob(I)alamin to pterin ring of the CH_3-H_4Folate followed by methyl radical transfer. The results of this study including mechanistic details and the protonation process were further discussed, as they can be applicable to a broad class of corrinoid-methyltransferases.

2. Computational Methods

2.1. Model Preparation and ONIOM-based QM/MM Calculations: To investigate the electronic structure and mechanistic details of the cob(I)alamin:CH_3-H_4Folate reaction complex, ONIOM-based QM/MM analysis was first carried out for the enzyme-bound cob(I)alamin intermediate. Since there is no X-ray crystal structure available for enzyme-bound cob(I)alamin intermediate due to its high reactivity, a crystal structure of enzyme-bound MeCbl was utilized to generate the cob(I)alamin intermediate. The initial structure of the MeCbl-binding domain of the MetH was obtained from the Protein Data Bank (PDB ID: 3BMT at 3.0 Å resolution)[23] which was then used to build a computational model. At first, the experimental artifacts were removed from the crystal structure and therefore, no counterions were included in the structural model. Subsequently, the hydrogen atoms were incorporated using VMD[33] and GaussView[34] assuming the normal protonation state of all titratable residues except histidine. The protonation state of histidine residue that can be protonated at δ or/and ε was determined based on the local H-bonding network via visual inspection and using PROPOKA suite of program.[35, 36] The lower axial His759 residue was treated as a HID in which N_δ atom was protonated and the resulting protonated state of all the histidine residues are given in the Supporting Information. Consequently, AMBER parameters were assigned for the B_{12} cofactor as developed by Marques et al.[37] The model structure was then subjected to geometry optimization of all the hydrogens while keeping the heavy atoms fixed using AMBER force field as implemented in Gaussian09 program.[38] Subsequently, AMBER[39] optimization was performed on the side chains of the

protein residues except lower axial HIS759 residue. Furthermore, we carried out ONIOM (BP86/6-31G(d):AMBER)[39, 40, 41, 42, 43] Mechanical Embedding (ME) single-point calculations to determine Merz–Kollman[44] electrostatic potential (ESP) atomic charges for the Quantum Mechanical (QM) region (using +3 oxidation state of the cobalt). In this ONIOM-ME calculation, the cofactor was used as a part of the QM model system and the radius of the Co was set at 0.79 Å. After the preparatory calculations, the entire B_{12} cofactor, as well as His759 was included in the QM region (contains 194 atoms) where His759 was capped as an H-link atom. The protein residues within 20 Å of the Co center were used for geometry optimization first by the ONIOM-ME method followed by ONIOM-electronic embedding (EE) formalism. The overall geometrical parameters obtained from the QM(BP86)/MM-EE geometry optimization of the MeCbl-binding domain of MetH agree well with the experiments X-ray structure (Table 1, column 2 and 3). As a next step, we removed the methyl group of MeCbl and adjusted the number of electron of the QM system consistent with the Co(I) oxidation state to prepare a structure of enzyme-bound cob(I)alamin intermediate and then optimized the resulting structure with QM/MM calculations. Upon geometry optimization, the α-axial His759 ligand was found to be detached from the Co(I) center as expected. The calculated distance between the Co and N of the His759 was 3.02 Å, which clearly indicated the Co(I) is four coordinated inside the enzyme.

2.2. Enzyme-Substrate Reaction Complex: In order to analyze the structure of the cob(I)alamin:CH_3-H_4Folate reaction complex, the CH_3-H_4Folate substrate was docked on the β-face of the cofactor in cob(I)alamin-binding module using Maestro graphical user interface from Schrödinger suite of program.[45] The truncated CH_3-H_4Folate was used in which the long side chain was replaced with a methyl group to decrease the size of the QM model. We also placed a proton at the N5 position of the CH_3-H_4Folate substrate, which is referred to as a N5H protonation state in this article. Subsequently, Merz-Kollman[44] electrostatic potential atomic charges were determined for the CH_3-H_4Folate substrate (first for the stationary point of each model and then same charges were used in all optimizations). In the Enzyme.Substrate (E.S) complex with N5H protonation state, the total number of QM atoms was 218 including the entire cofactor, lower axial His759 ligand, and protonated CH_3-H_4Folate (Figure 3). To attain more reasonable structure, we used a number of different starting conformations of the CH_3-H_4Folate relative to cob(I)alamin intermediate. We found that after the QM(BP86/6-31G(d)/MM(AMBER)-ME optimization, all the conformations converge to a point where the

orientation of methyl group is pointing towards the cob(I)alamin (Figure 3) and energy of all these conformations were almost comparable. In all the conformations, the distance between the Co and the C_{Me} of CH_3-H_4Folate was never less than 3.6 Å. In addition, the propanamide side chain of the B_{12} cofactor interacts with -HN3 of the pterin ring and this intermolecular interactions act as an anchor, which would stabilize the pterin ring in the reaction complex. Therefore, we selected the final reaction model complex which has Co-C distance 3.59 Å that is shown in Figure 4a (hydrophobic surface is shown in Figure S1). All the resulting structures and transition states were optimized by QM/MM-ME in which the QM and MM parts were treated by the BP86/6-31G(d) method[42, 43] and AMBER96 force field,[39] respectively. The QM/MM-ME vibrational frequency calculation at the same level of theory was further performed to characterize and verify the key transition states and intermediates. Since the ME scheme does not include polarization of MM region into a QM Hamiltonian, the additional single point energy calculations using QM/MM-EE scheme were further carried out based on the QM/MM-ME optimized geometry.

3. Results and Discussion

3.1. Chemical Models and Enzyme-Substrate Reaction Complex: MetH enzyme is a modular protein containing four different functional domains.[23] During the course of catalytic methyl transfer, both substrate-binding domains (Hcy and CH_3-H_4Folate) alternatively come closer to the cobalamin-binding domain through conformational changes and form a reaction complex from the upper(β)-face of the cofactor in which cobalt center cycles between a Co(III) and a Co(I) oxidation states. Due to high conformational motion of the MetH, there is no X-ray crystal structure available for the enzyme-substrate reaction complex enclosing the cob(I)alamin intermediate and the CH_3-H_4Folate substrate. We applied our chemical intuition to study the cob(I)alamin:CH_3-H_4Folate reaction complex in terms of its electronic structure and mechanistic details of the methyl transfer. A reasonable chemical model was thus prepared based on the available X-ray structure of MeCbl-binding domain of the MetH (PDB code: 1BMT, at 3.0 Å resolution)[23] by docking the isolated CH_3-H_4Folate on its β-face.

As a first step, we optimized the geometry of Me-cob(III)alamin-MetH resting state by QM/MM-ME computations that is shown in the Supporting Information (Figure S2) and their main structural parameters are listed in Table 1 (column 2 and 3) for the direct comparison with

the experimental structure. The fact that the key structural parameters of the MeCbl are correctly reproduced by hybrid QM/MM-ME calculations strengthened our confidence that a cob(I)alamin intermediate could be well characterized by the same level of theory. The structure of the cob(I)alamin intermediate was subsequently prepared from enzyme-bound MeCbl by removing the methyl group and keeping the number of electrons of the QM (entire cofactor + His759) system consistent with a Co(I) oxidation state. Most interestingly, the α-axial His759 residue found to be displaced from the Co(I) center (Figure S3) upon QM/MM-ME geometry optimization of the cob(I)alamin-MetH. The QM/MM-ME simulations show that the Co-N(His759) distance elongated to 3.02 Å in Co(I) from 2.14 Å in Co(III) oxidation state. The axial His759 ligand displaced due to the change in oxidation state of the Co which is in agreement with the X-ray absorption spectroscopic studies,[46] indicating cob(I)alamin form of the cofactor is not axially coordinated in solution. The displacement of the axial His759 residue from the Co(I) center is also in agreement with the previous Car Parinello Molecular Dynamics (CPMD) QM/MM computational studies.[47] It is important to further mention that a short Co(I)-N(His759) axial distance may be advantageous from the enzymatic point of view, since it would allow the axial His759 to re-coordinate easily to the cobalt center when the cofactor is remethylated by CH_3-H_4Folate substrate.

To further investigate the electronic structure and mechanistic details of the MetH-cob(I)alamin:CH_3-H_4Folate reaction complex, we subsequently docked the CH_3-H_4Folate substrate on the β-face of the QM/MM optimized enzyme-bound cob(I)alamin intermediate. It has been suggested that an activation of CH_3-H_4Folate takes place by protonation (from general acid, BH)[29] at N5 position; therefore, we utilized the protonated CH_3-H_4Folate referred as a N5H protonation state. We first optimized N5H protonated E.S reactant complex for the methyl transfer (Figure 4a) without applying any structural and/or geometrical constraints either on the cofactor or on the CH_3-H_4Folate in order to attain a more reasonable structure of the model complex. We tested a number of different starting conformations of the CH_3-H_4Folate on the upper of face the cofactor, but it appears that all the conformations converge to a point (after the optimization) where methyl group of N5 is perpendicular to the Co(I)corrin. In addition, an O-end of propanamide side chain of the Co(I)corrin cofactor is interacting with the -HN3 of the pterin ring and such an intermolecular interaction serves as an anchor between E.S complex. The

communication between the CH_3-H_4Folate substrate and the cob(I)alamin through H-bond interactions could be the key in stabilizing the orientation of the pterin ring for a methyl transfer.

The optimized geometry of the N5H protonated cob(I)alamin:CH_3-H_4Folate reaction complex (RC_{N5H}) is shown in Figure 4a, and the corresponding key structural parameters are given in Table 1 (column 5, Co(I):[CH_3-H_4Folate]). Interestingly, the overall parameters of CH_3-H_4Folate substrate-bound cob(I)alamin (column 5 in Table 1) and substrate-free cob(I)alamin (column 4 in Table 1) are comparable. The distance between the Co and the carbon of the transferred methyl (C_{Me}) group is found to be 3.59 Å. On the other hand, the Co-N_{ax}(His759) distance increased to 3.17 Å which is referred as a His-off conformation of the cofactor because His759 is not bound to the Co center. It should be noted that axial His759 residue in RC_{N5H} cannot move further because it is H-bonded with Asp757 and Ser810 protein residues that act as catalytic triad (His-Asp-Ser) for a methyl transfer. Both His759 and Asp757 residues are tethered in a loop, conferring them a certain degree of flexibility that allows the axial base to be displaced during the catalytic cycle without breaking the hydrogen bond network between them. However, this flexibility is limited because Asp757 is also interacting with a Ser810 of a α-helix and this secondary structure is not as flexible as the loop. As a result, the Co-N(His759) cannot increase beyond the 3.17 Å in RC_{N5H} without disrupting the hydrogen bond network, something that probably might have a high energy cost.

The electronic structure of the RC_{N5H} was initially optimized considering a closed-shell singlet electronic configuration (Figure 4a). However, it has been noticed using hybrid B3LYP functional[48] that the open-shell singlet (oss) state of the free cob(I)alamin intermediate is energetically lower (1-2 kcal/mol) than the closed-shell singlet state (css) indicating a singlet instability of the ground state wave function.[47, 49, 50] The spin polarized solution of Co(I)corrin obtained from open-shell singlet calculations shows an unpaired electron on the cobalt metal coupled antiferromagnetically with an unpaired electron on the corrin ring, which was consistent with a $Co^{II}(d^7)$-corrin radical $(\pi^*)^1$ diradical state. Moreover, the ferromagnetic counterpart of the open-shell singlet state, a triplet $Co^{II}(d^7)$-radical corrin $(\pi^*)^1$ state, was also found to be lower in energy (3 kcal/mol) than that of the closed-shell singlet, further validating the diradical contribution to the Co(I)corrin.[47, 49, 50] Although the small energy difference between the states might be within the error of the hybrid B3LYP functional, nevertheless, it signifies that the

closed-shell and the diradical states are close in energy, suggesting that the electron transfer from the $Co^{I}(d^{8})$ to the corrin(π) to originate the $Co^{II}(d^{7})$-corrin radical $(\pi^{*})^{1}$ may be possible. The multiconfigurational character of the cob(I)alamin intermediate has been confirmed by the high level CASSCF calculations, consistent with that of a closed-shell $Co^{I}(d^{8})$ configuration (67%) and a diradical $Co^{II}(d^{7})$-corrin radical $(\pi^{*})^{1}$ configuration (23%).[47, 50] The formation of such unusual oxidation state at ground state was due to the overlap of low-lying metal d-orbitals with the corrin ligand orbitals that allow the electron transfer from the Co(I) to the corrin ring. Therefore, a singlet instability of the wave function can be tested either with triplet state (ferromagnetic coupling) or with an open-shell singlet state (antiferromagnetic coupling) calculations of the RC_{N5H}.

Subsequently, we checked the possibility of having a diradical state i.e., electron transfer at ground state for the protonated cob(I)alamin:CH_3-H_4Folate complex (RC_{N5H}) using triplet (T) as well as open-shell singlet (oss) state calculations. We employed BP86 functional instead of hybrid B3LYP for the QM/MM calculations because the BP86 functional produced correct Co-C bond dissociation energy (BDE) for both MeCbl and AdoCbl while the B3LYP underestimates the BDE.[51, 52, 53, 54, 55, 56, 57] Consequently, we first optimized the triplet state of the RC_{N5H} (T) using QM(BP86)/MM-ME calculations and found that the triplet state was 2.32 kcal/mol higher in energy than the corresponding closed-shell singlet. The QM/MM-ME optimized geometry of the RC_{N5H} (T) is shown in Figure 5a and the key structural parameters are listed in Table 1 (column 6, Co(II):[CH_3-Folate]$^{\bullet}$). Most interestingly, we noticed that the α-axial His759 ligand displaced and coordinated to the Co center during the triplet state optimization (Figure 5a). The QM/MM calculations of the RC_{N5H} reveal that the Co-N(His759) distance decreased from 3.17Å in the closed-shell singlet state to 2.07 Å in the triplet state. In other words, the axial ligand moved from four coordinated $Co^{I}(d^{8})$ closed-shell singlet state to five coordinated $Co^{II}(d^{7})$-pterin radical $(\pi^{*})^{1}$ diradical state. Since the cofactor is in a His-on conformation in triplet state, so it is referred as the RC_{N5H} (His-on). On the other hand, the distance between the Co and C_{Me} of the methyl group of CH_3-H_4Folate to be transferred is elongated to 4.16 Å as compared to the same distance in closed-shell singlet state (3.59 Å). The spin density distribution shows that there is one unpaired electron on the Co coupled ferromagnetically with an unpaired electron on the pterin ring of the CH_3-H_4Folate, consistent with the triplet $Co^{II}(d^{7})$-pterin radical $(\pi^{*})^{1}$ state.

Furthermore, we optimized the open-shell singlet (oss) state to investigate the possibility of a spin-polarized solution for the cob(I)alamin:CH_3-H_4folate reaction complex (RC_{N5H} (oss)). The optimized structure (Figure 5b) certainly shows the spin polarization as expected, with an unpaired electron on Co coupled antiferromagnetically with an unpaired electron delocalized on the pterin ring as well as small spin density on the corrin ring, consistent with a $Co^{II}(d^7)$-pterin/corrin radical $(\pi^*)^1$ diradical state. The energy of the open-shell singlet state was found to be 3.18 kcal/mol higher than the closed-shell singlet state, further validating the diradical contributions in RC_{N5H} (oss). It is important to mention that the optimized geometry of the open-shell singlet state is similar to the triplet state (as two spins are separated) but there is very small energy difference (~ 0.8 kcal/mol) between these two states. It has been shown previously for the bioinorganic system such as cob(I)alamin[47, 50] and heme-based compound 1 intermediates[58, 59] that energies and geometries of the open-shell singlet and triplet states are generally similar. Both these intermediates have a singlet instability and a non-innocent macrocycle (corrin and porphyrin) that can exchange electrons with the metal. As a consequence, the QM/MM-ME calculations of the RC_{N5H} indicate that the closed-shell and diradical states (both open-shell singlet and triplet) are close in energy with His-off and His-on conformation, respectively, suggesting that electron transfer from $Co^{I}(d^8)$ to the pterin (π) to originate the $Co^{II}(d^7)$-pterin radical $(\pi^*)^1$ may be feasible.

The methyl transfer from the CH_3-H_4Folate substrate to the cob(I)alamin to generate a Me-cob(III)alamin (Scheme 1) is generally assumed to proceeds via an S_N2-type displacement. In the present work, S_N2-type mechanism is consistent with the closed-shell singlet state (His-off) transferring methyl cation in a rate-limiting step. However, in view of the energetically close-lying diradical contribution as indicated by triplet and open-shell singlet states (His-on), it can be suggested that an alternative radical mechanism, consisting of an electron transfer (ET) from cob(I)alamin to CH_3-H_4Folate followed by a methyl radical transfer, is also possible. For the ET-based radical mechanism, the axial His759 residue plays a crucial role to enhance the electron transfer step. Based on the electronic structure of the RC_{N5H} as well as the coordination between the Co center and His759 residue, two possible mechanistic pathways for the methyl transfer reaction catalyzed by MetH can be envisioned (Scheme 1): I) S_N2-type pathway i.e., a methyl cation (formally) transfer, II) ET-based methyl radical transfer.

$$Co(I)Cbl + CH_3\text{-}H_4Folate + H^+ \rightarrow [CH_3\text{-}Co(III)Cbl]^+ + H_4Folate \quad (I)$$

$$Co(I)Cbl + CH_3\text{-}H_4Folate + H^+ \rightarrow [Co(II)Cbl]^+ + [CH_3 - H_4Folate]^{\bullet -}$$
$$\rightarrow [CH_3\text{-}Co(III)Cbl]^+ + H_4Folate \quad (II)$$

In the next two sections, we will focus on these two suggested mechanistic routes, complementing the previously reported experimental study.

3.2. S_N2-type Nucleophilic Displacement (His-off): The S_N2 pathway proceed in a His-off conformation of the cofactor consistent with closed shell singlet (css) state (Scheme 1). As a next step, a transition state (TS_{N5H}) for the methyl transfer from protonated CH_3-H_4Folate to the Co(I) center has been located using QM/MM-ME framework. The identity of the TS_{N5H} (css) has been confirmed by the frequency calculations displaying only one imaginary frequency (329*i* cm^{-1}). The vibration associated with an imaginary frequency corresponds to the axially directed S_N2 reaction coordinate containing N5 of the CH_3-H_4Folate, the carbon (C_{Me}) of the methyl group, and the Co metal. The TS_{N5H} was located along the S_N2 pathway, and can be described as early with respect to N5-C_{Me} bond (See in Figure 4b and Table 2). In the TS_{N5H} (css), the key entities (N5, C_{Me} and Co atom) were confined strictly in a linear geometry (∠N5-C-Co=174°) and both axial (N5-C_{Me}= 1.75 Å and C_{Me}-Co=2.81 Å) bonds were stretched out along the reaction coordinates. The distance between the Co center and the lower axial His759 ligand thus decreased from 3.17 Å in the RC_{N5H} to 2.58 Å in the TS_{N5H}. Note that we did not apply any structural constraints on the lower axial His759 residue during the TS_{N5H} geometry optimization. The shortening of Co-N(His759) distance in the TS_{N5H} could be advantageous from enzymatic point of view as it would facilitate the lower axial protein residue to re-coordinate with the Co center. Alternatively, the movements of lower axial His759 further corroborate that the proposed S_N2 pathway for the first-half MetH reaction will lead to the experimentally observed octahedral (d^6) Me-Cob(III)alamin species.

The activation energy barrier for the S_N2-type methyl cation transfer was found to be 9.01 kcal/mol with the QM/MM-ME calculation (Table 2 and Figure 6). In addition, we applied single point energy calculations with electronic embedding scheme (EE) referred as a QM/MM-EE using the QM/MM-ME optimized geometry, which show the energy barrier of 7.62 kcal/mol. This difference from the two types of schemes (ME and EE) is consistent with the previous studies,[59] and due to the fact that the electrostatic interactions between two level i.e., QM and MM part are treated differently. The main advantage of the QM/MM-EE calculations is that it

includes the electrostatic interaction between the QM and MM part correctly by incorporating the MM charges into a QM Hamiltonian. Most importantly, an approximate ~ 8-9 kcal/mol energy barrier for the methyl transfer from protonated CH_3-H_4Folate to the cob(I)alamin intermediate, are well in line with the experimental rate constant (~7 X 10^4 $mol^{-1}s^{-1}$ at 37 °C),[29, 60] which indicate that the QM/MM calculations reproduced the enzymatic effect of reaction acceleration by the methionine synthase (MetH). The Mullikan charges computed from the QM/MM-EE simulations (Table 2) show that the charge on the C_{Me} (-0.41) in TS_{5NH} increased as compared to the $RC_{N5H,}$ indicating methyl group transferred as a cation. Note that the predicted energy barrier in the present QM/MM calculations is lower than that of the reported for small model complex (13 kcal/mol), signifying the crucial role of the MetH.[32]

Upon methyl transfer to the β-face of Co center and further optimizing Co(III)-based resulting species with the lower axial His759 near to the Co consequently leading to hexacoordinated His-on Me-Cob(III)alamin and a protonated H_4-Folate in the product complex (PC_{N5H}). It is important to mention that we did not apply any constrains to the His759 ligand, therefore, the present QM/MM calculations validate the consensus that the ligation of the His759 residue with a Co center is certainly controlled by the oxidation state of the Co (Co(I) to Co(III)) as has been concluded by Matthews and coworkers.[61] Moreover, the key structural parameters of the MeCbl in the PC_{N5H} (css) are comparable with the available crystal structure of MeCbl (Table 1, column 3 and 7). In other words, our QM/MM calculations clearly proved that the Rossman subdomain (α/β-fold) containing catalytic triad (His-Asp-Ser) is communicating with cobalamin through a Co-N(His759) bond during the methyl transfer reaction in the first-half catalytic cycle of MetH. Thus, the role of the MetH is to stabilize the product complex using the Rossman subdomain that certainly plays a critical role in catalysis. An interesting observation here is that, in going from RC_{N5H} to PC_{N5H} via TS_{N5H}, the distance between the -HN3 of the CH_3-H_4Folate and O-end of the propinamide side chain of the corrin ring is elongated from 1.98 Å to 2.68 Å (2.01 Å in TS_{N5H}). This indicates that the side chain of corrin ring plays a crucial role in stabilizing the RC_{N5H} and TS_{N5H} for the methyl transfer reactions which were missing in the previous model studies.[32] As a result, the displacement of the His759 with respect to Co center and the H-bond communication between the cofactor and the CH_3-H_4Folate substrate are the key components in order to understand the catalytic methyl transfer reactions.

The QM/MM-ME optimizations from the TS_{N5H} to the product complex (PC_{N5H}) lead to the formation of six-coordinated MeCbl which is found to be -36.74 kcal/mol (-37.94 kcal/mol with QM/MM-EE) lower in energy than the RC_{N5H} (Figure 6 and Table 2). The energies of the product complex relative to the RC_{N5H} indicate that the reaction is strongly exothermic as would be expected from charge neutralization reaction (protonation at N5 in the product complex). However, such a highly exothermic nature of the reaction (35.95 kcal/mol in a proteinlike environment (ε=4)) has been also noticed for the model complex that mimics the second-half MetH reaction in which the S_N2 methyl transfer takes place from MeCbl to unprotonated methylthiolate (CH_3S^-).[62] Alternatively, the methyl transfer in the backward direction (first-half MetH reaction) i.e., from MeCbl to protonated H_4Folate requires a high-energy barrier for the reaction to proceed as an S_N2-type, which is energetically unreachable. This is due to the fact that the protonated H_4Folate product includes a proton at N5 and the methyl transfer to a protonated N5 species makes this reaction highly uphill. As discussed, the active site polar residues (Figure 2) such as Asn503 near the pterin ring of the CH_3-H_4Folate could be important for the stabilization of protonation state in reverse methyl transfer.[29]

The most important and interesting question that arises here (to understand the reverse methyl transfer) is the timing of proton uptake with respect to the methyl transfer and this information is unclear from experimental point of view. Although we have explored the feasibility of the S_N2 methyl transfer in N5H protonation state, it is worth investigating the activation energy and structural parameters of the reaction complex in unprotonated N5 species of the CH_3-H_4Folate i.e., N5 state, and that would further provide understanding of the protonation process. As a next step, we removed the proton of the N5H protonation state and reoptimized the unprotonated reactant complex (RC_{N5}). The QM/MM-ME optimized geometry is shown in the Supporting Information (Figure S4a) which revealed that the Co-C_{Me} and Co-N(His759) distances elongated to 4.92 Å and 4.02 Å, respectively as compared to protonated reactant complex (RC_{N5H}). This is due to the fact that the QM part of the QM/MM-ME simulation is relatively neutral because there is no proton at N5, which means neutral CH_3-H_4Folate is not activated for the methyl transfer reaction and keeps the E.S complex apart. Further, the optimization of the TS_{N5} (Figure S4b) for the methyl transfer reaction shows that the activation energy barrier is 39.07 kcal/mol (with negative frequency of 417i cm^{-1}), which is energetically very high. The predicted energy barrier for the RC_{N5} is almost four times higher

than that of the protonated reaction complex (RC_{N5H}). As a result, our QM/MM calculations discard the possibility of methyl transfer reaction from unprotonated CH_3-H_4Folate to Co(I). On the other hand, the interesting observation is that the optimized TS_{N5} is rather late as compared to the TS_{N5H} found in the protonated complex with respect to the Co-C_{Me} distance of 2.32 Å (Figure S4b). It is important to mention that we were unable to optimize the product complex in an unprotonated N5 state due to the formation of the H_4folate anion which is an unfavorable leaving group, though several attempts were made to optimize it. This can be accounted to the fact that H_4Folate anion is the conjugate base of an acid (formed by methyl transfer from CH_3-H_4Folate) that is expected to have a very high p*K*a because the p*K*a of ammonia dissociating to form the ammonia anion is 38.[63]

Our computational results indicate that without the protonation at the N5 of the CH_3-H_4Folate substrate, the S_N2 methyl transfer to Co(I) intermediate is unreachable because the barrier for the rate-limiting step is very high. Moreover, the H_4Folate anion is very poor leaving group. It is important to mention that the methyl transfer from aromatic tertiary amine is not a facile reaction even in the presence of strong nucleophile such as cob(I)alamin and it requires electrophilic activation of the methyl group probably by protonation to facilitate the methyl transfer. This has been pointed out by Pratt et al. based on their model studies where they demonstrated that a quaternary ammonium salts can function as a methyl donors to cob(I)alamin but not corresponding tertiary amines.[30] As a result, our QM/MM calculations of the E.S complex (both protonated and unprotonated) suggest that protonation at N5 position of the CH_3-H_4Folate must take place, either simultaneously with the methyl transfer reaction, or prior to the methyl transfer.

3.3. ET-based Methyl Radical Transfer (His-on): As discussed above in section 3.1, the ET-mediated radical mechanism (See equation II) has been suggested for the RC_{N5H} to form a CH_3-cob(III)alamin based on the energetically close-lying diradical states calculated from the triplet and open-shell singlet state calculations (Scheme 1 and Figure 5). The ET-based radical pathway proceed via a five coordinated His-on conformation (Scheme 1), where an electron transfer first takes place from cob(I)alamin to pterin ring of the CH_3-H_4Folate, consistent with a $Co^{II}(d^7)$-pterin radical $(\pi^*)^I$ diradical state (unpaired electrons coupled both ferromagnetically and antiferromagnetically). The reduction of the CH_3-H_4Folate substrate, thus induces the homolytic cleavage of N5-C_{Me} bond followed by methyl radical to Co(II) in order to generate

back Me-cob(III)alamin resting state consistent with the ET-mediated reductive cleavage of the N5-C_{Me}. Similar ET-based reductive cleavage mechanism has been suggested for the second-half MetH cycle,[52, 64, 65] where the electron transfer first takes places from unprotonated methylthiolate to antibonding π orbital of the corrin ring followed by the methyl radical transfer. In QM/MM-ME optimized structure of RC_{N5H} (T/oss), the electron transfer takes place to an antibonding π orbital of the pterin ring with spin density on the terminal and bridging carbon atoms but not directly on the N5 or C_{Me} atoms.

In order to investigate a complete reaction of the methyl transfer from the CH_3-H_4Folate substrate to His-on conformation of the Co center, N5-C_{Me} bond was systematically elongated (with an increment of 0.2Å) towards the Co metal and the Co-C_{Me} was shortened in such a way so that the Co-N5 equilibrium distance of 5.68 Å is fixed (See Scheme 2). Since there is no structural information available from the existing X-ray data regarding how the two modules containing the cofactor and CH_3-H_4Folate substrate interact during the methyl transfer, the distance (Co-N5) between substrate and the cofactor was kept fixed for all the Co-C_{Me} distances to avoid steric clashes between these two (Scheme 2). The QM/MM-ME potential energy profile associated with the cleavage of the N5-C_{Me} bond along the Co-C distances are shown in Figure 7. It should be noted that all degrees of freedom were fully optimized in ONIOM-based QM/MM-ME calculations for the every constrained Co-C_{Me} distances both in open shell singlet (oss) state as well as in triplet state optimizations. In addition, we performed single point QM/MM-EE calculations based on the previously optimized QM/MM-ME geometry for all distances to incorporate the electrostatic interaction between the QM and MM parts more accurately. The single point QM/MM-EE calculations provide a potential energy profile similar to that of the ME scheme (See Table 2 and Figure 7).

The energy barrier for the methyl transfer reaction in the open shell singlet (oss) calculations appears to be 8.05 kcal/mol and 8.68 kcal/mol with QM/MM-ME and QM/MM-EE scheme, respectively (Table 2 and Figure 7). The evolution of spin density distribution for the formation of the Co-C_{Me} bond is also plotted that is embedded in the lower part of Figure 7, and these spin density numbers are extracted from the open-shell singlet state QM/MM-EE calculations. In the equilibrium geometry of the RC_{N5H} with a N5-C_{Me} bond length of 1.52 Å, the α spin density is centered on the Co metal, while the β spin density is delocalized on π^* pterin orbital with small contribution of π^* corrin orbital (Figure 7). Therefore, one electron transfer

from the HOMO of the Co(I) metal to the LUMO of the pterin/corrin takes place, leading to a $Co^{II}(d^7)$-pterin/corrin radical $(\pi^*)^I$ diradical configuration. Note that the spin density distribution on the pterin ring of the CH_3-H_4Folate substrate calculated from open-shell singlet QM/MM calculations is the same that can be observed if we reduced the isolated CH_3-H_4Folate substrate by one electron. As the N5-C_{Me} bond is stretched or the Co-C bond is shortened, the β spin density shift from the pterin ring of the CH_3-H_4Folate to the N-C_{Me} bond. At a Co-C distance of 3.8 Å (which display energy barrier for the methyl transfer reaction), a crossing between the β spin densities on the CH_3-H_4Folate and the C_{Me} takes place with only residual spin density on the corrin ring (Figure 7). However, the C_{Me} atom attains maximum β-spin density at a Co-C_{Me} distance of 3.2 Å, consistent with the Co(II)--$C^{\bullet}_{Me}$--N5-(H_4Folate). In other words, the N5-C_{Me} bond cleaved homolytically leading to the formation of a methyl radical. Furthermore, an elongation of the N5-C_{Me} bond leads to the formation of a closed-shell configuration with no spin density distribution around Co-C_{Me} distance of 2.8 Å. This is due to the fact that the Co(III)-C_{Me} bond formation take place. Moreover, it is well known that the ground state of Me-cob(III)alamin is closed-shell singlet. Therefore, at a Co-C of distance of 2.0 Å the methyl group is completely transferred to the cofactor, giving the product of the reaction that is six coordinated Me-cob(III)alamin and protonated H_4Folate. The potential energy profile (Figure 7) further indicates the methyl transfer reaction is highly exothermic by -38.77 kcal/mol calculated with QM/MM-ME (-37.95 kcal/mol with QM/MM-EE) which is in agreement with the S_N2-type mechanism (~ 37 kcal/mol) from energetic point of view.

The energy barrier for the methyl transfer reaction in the triplet state QM/MM(T) calculations turns out to be 8.05 kcal/mol and 8.68 kcal/mol with QM/MM-ME and QM/MM-EE calculations, respectively (Table 2 and Figure 7) which is comparable to the one calculated with the open-shell singlet state calculations. All these energy values shown in Figure 7 should be considered as an upper bound because zero point energy corrections were not included in the QM/MM calculations. It is important to mention that we were unable to optimize the triplet state QM/MM-ME(T) calculations after the Co-C_{Me} distance 2.6 Å owing to the fact that the lowest state of the MeCbl is the closed shell singlet. In Figure 7, we also plotted the QM energy contribution to the overall QM/MM energy in order to understand the role of protein (MM part), which were extracted from the QM/MM-ME calculation. Although, the actual trend of QM energy profile for the Co-C_{Me} bond formation is similar to that of the QM/MM energy in both

open-shell singlet and triplet calculations, but there is small contributions from the MM part. In particular, the protein effect (MM energy components of the combined QM/MM-ME calculations) on the energy barrier (at Co-C_{Me} distance 3.8 Å) for the methyl transfer reaction is 0.45 kcal/mol and 0.67 kcal/mol in triplet and open shell singlet state calculations, respectively.

As discussed in section 3.1, the QM/MM optimized structures of the triplet and open-shell singlet (oss) states are energetically ~ 2-3 kcal/mol higher than that of the closed-shell singlet (css) state (Figure 6). However, such a small energy difference may reflects the fact that single-reference DFT-based wavefunction is not capable to describe complex electronic structure of the reactant complex where a multi-reference description would be required. Therefore, it can be concluded that the calculated energy barrier (i.e., in total ~10-11 kcal/mol) for the methyl radical transfer (with His-on conformation) in both triplet and open-shell singlet state calculations is quantitatively comparable to that of the previously calculated activation energy (~ 8-9 kcal/mol) for the S_N2 pathway. The main advantage of ET-based radical mechanism from enzymatic point of view is that a methyl radical can be transferred at a longer distance (such as 4.16Å in RC_{N5H}), which does not require the close proximity of the two binding modules of MetH, as would be required in an S_N2-type. It should be noted that the proposal based on the ET-based radical mechanism is similar to the one suggested by the Matthews et al. described as a single electron transfer (SET).[29] The difference lies in the initial step of the electron transfer from cob(I)alamin to the CH_3-H_4Folate substrate. Our theoretical study shows that the electron is being transferred to π^* orbital of pterin ring of N5H protonated CH_3-H_4Folate, whereas they suggested that the electron is transferred to unprotonated CH_3-H_4Folate followed by the proton transfer to initiate the homolytic cleavage of the N5-C_{Me} bond.

3.4. Understanding the Protonation Step of the CH_3-H_4Folate Substrate: As it has been shown based on the QM/MM calculations, if the methyl transfer from the CH_3-H_4Folate to cob(I)alamin intermediate proceeds via an S_N2 or an ET-based mechanism then one might expect the activation of the CH_3-H_4Folate to transfer the methyl group, presumably by a protonation at the N5 position. Therefore, it is important to understand and investigate the protonation event at N5 position when the CH_3-H_4Folate fragment is bound to its MetH domain.

The p*K*a of the free CH_3-H_4Folate substrate in aqueous solution was determined to be 5.05 which is associated with the protonation at N5 as detected by the measurement of fluorescence changes when the pH is lowered.[66] This p*K*a value of the CH_3-H_4Folate is likely to

be increased (~ 2 units) by the hydrophilic protein residues such as aspartatic acid (Asp) and aspargine (Asn) which are usually found to be near the pterin ring (Figure 2, PDB ID: 1Q8J at 1.9 Å resolution).[26] Among these residues, O-end side chain of the Asn508 residue is H-bonded with the N5 group of pterin ring and such an H-bonding interaction has been suggested to be involved in the stabilization of the protonation state and high energy intermediates (such as TS),[26] but this H-bonded interaction is not chemically suitable to act as a proton donor. Moreover, the crystal structure of the MeH-CH_3-H_4Folate shows there is no general acid catalyst (BH) near N5 position and the CH_3-H_4Folate has been suggested to be bound with the MetH in an unprotonated form.[20] On the other hand, it has been shown that product H_4Folate contains proton at N5 when the methyl group is transferred to cob(I)alamin/cob(I)amide.[29] As a result, the timing of proton uptake relative to methyl group transfer is very crucial but remains unclear from experimental point of view. Thus, the presence of cob(I)alamin cofactor may be important for the substrate activation, and the proton uptake could take place in a ternary MetH.cob(I)alamin.CH_3-H_4Folate complex, either prior to or during the methyl transfer reaction. On the other hand, it has been shown experimentally in the case CH_3-H_4Folate:CFeSP methyltransferase (MeTr) that the binding of CH_3-H_4Folate to AcsE protein of MeTr is associated with a p*K*a increase and a proton uptake in a binary E.S complex.[17]

In addition, it has been suggested by Matthews et al. using pH dependent and rate constant studies of a methyl transfer reaction from the CH_3-H_4Folate to exogenous cob(I)alamin that a general acid/base analysis could be involved in the ternary MetH-cob(I)alamin.CH_3-H_4Folate complex.[29, 31, 67] Therefore**,** based on the present QM/MM calculations of the MetH-cob(I)alamin:CH_3-H_4Folate complex and the analysis of the X-ray structure of CH_3-H_4Folate binding module (Figure 2) it can be inferred that the proton transfer and methyl transfer steps are somehow coupled, probably with the general acid catalyst (BH) that is initially hydrogen bonded to the N5 of the CH_3-H_4Folate. As the N-C_{Me} bond is weakened in the ternary complex (via either an S_N2 or ET-based mechanism), the N5 of the H_4Folate anion leaving group becomes increasingly more basic, and the proton transfer takes place when the p*K*a of N5 is more than that of the general acid catalyst. Alternatively, the presence of general acid catalyst would decrease the QM/MM energy barrier for the reverse methyl transfer i.e., from MeCbl to protonated H_4Folate which has been predicted to be very high in the present QM/MM calculations (~37-39 kcal/mol) of the N5 protonated E.S complex (Figure 6 and Table 2). Therefore, a reverse methyl

transfer from MeCbl to protonated H_4Folate will simultaneously facilitate the proton transfer back to the general base (B^-) to stabilize the neutral CH_3-H_4Folate. A full active site CH_3-H_4Folate substrate need to be considered which is thus beyond the present study due to the fact that the β-site (cap subdomain) of the corrinoid-protein is not wide enough to accommodate all the active site residues of the CH_3-H_4Folate binding domain. Moreover, there is no available X-ray structure of the reaction complex containing cob(I)alamin- and CH_3-H_4Folate- binding modules owing to high conformational flexibility of MetH.

3.5. Implications for the Remethylation of the cob(I)alamin/cob(I)amide intermediate to other Methyltransferases: In the present theoretical account, we suggested that the electrophilic activation of very difficult methyl donors such as CH_3-H_4Folate takes place by the protonation at methyl-bound N5, either simultaneously with the methyl transfer or prior to methyl transfer. The results of the present study including mechanistic details of methyl transfer reaction and protonation process can be applied to broad class of corrinoid-methyltransferases, such CH_3-H_4Folate: corrinoid/Fe-S protein (CFeSP) methyltransferase (MeTr) [68] and bacterial mercury methylation (HgcAB).[19] The CH_3-H_4Folate binding module of MetH shows significant sequence homology to AcsE, a separate binding protein of MeTr from *C. thermoaceticum* in which AcsE binds the CH_3-H_4Folate and catalyzes methyl transfer from CH_3-H_4Folate to the Co(I) of corrinoid/Fe-S protein in the Acetyl CoA pathway. In addition, MtrH,[69] an enzyme that catalyzes transfer of a methyl group from N5-methyltetrahydromethanopterin (structural analogue of CH_3-H_4Folate) to a corrinoid protein. All these enzymes are able to catalyze methyl transfer to exogenous cob(I)alamin/cob(I)amide intermediate. However, both MetH and MtrH bind the corrinoid cofactor with a histidine ligand from the protein serving as the lower axial ligand while corrinoid proteins of the HgcAB recently found to be bound with a cysteine residue.[19] On the other hand, a distinguished feature of the CFeSP is that it serves as a methyl acceptor from AcsE in its base-off and His-off conformation, which means no protein-derived His759 residue bound to the cobalt center. In addition, a methyl donors to cob(I)alamin or to structurally related corrinoid derivatives (cob(I)amide) include not only CH_3-H_4Folate but also methanol,[68] chloromethane,[70] mono-, di- and tri-methylamines.[70, 71] However, an activation of these challenging methyl donors is less understood and thus, a little is known in terms of the chemical mechanisms. One possibility is the protonation of the methyl donors while other is the involvement of the Zn(II) ion for the electrophilic activation. In the case of methanol coenzyme

M methyltransferase (MtaABC),[72, 73] a Zn(II) is found near the methanol binding module which is believed to be involved for the activation. Most interestingly, the crystal structure of the Methanol:cob(I)amide (MtaBC) reaction complex reveals the Co-N(His136) distance 2.51Å,[72] which clearly indicates the possibility of ET-mechanism as an alternative to S_N2 pathway. As a result, the attractive feature of the present computational study is that it can be applied to a wide range of methyltransferases to understand the activation process of the methyl donors and their subsequent enzymatic mechanisms.

4. Conclusions

In the present computational study, the electronic structure and mechanistic details of the first-half reaction of MetH involving methylation of cob(I)alamin has been investigated using QM/MM calculations. The structural model was prepared using X-ray crystal structure of the cobalamin binding domain[23] by docking the CH_3-H_4Folate substrate on the β-face of the cofactor. The possibilities of two different reaction pathways for the methyl transfer from CH_3-H_4Folate to the enzyme-bound cob(I)alamin intermediate based on the quantum mechanical calculations have been explored; the S_N2-type nucleophilic displacement and the electron transfer (ET)-based radical mechanism. The hybrid QM/MM calculations of the CH_3-H_4Folate:cob(I)alamin reaction complex reveal that the formation of organometallic Co-C bond, i.e., a six coordinated CH_3-cob(III)alamin, takes place via an S_N2-type nucleophilic route. The S_N2-type mechanism is consistent with the closed-shell singlet ground state (His-off conformation) transferring a methyl cation in a rate-limiting step followed by the displacement of the lower axial His759 residue. The activation energy barrier in the protonated CH_3-H_4Folate:cob(I)alamin complex was found to be ~ 8-9 kcal/mol that leads to a rate constant which is comparable to the estimated experimental rate constant.[29, 60] On the other hand, our QM/MM calculations of unprotonated CH_3-H_4Folate:cob(I)alamin complex predict a very high activation energy barrier (~ 39 kcal/mol) which is almost four times higher than that of found in its protonated analogue. This enormous energy barrier is attributed to the fact that the product of this reaction i.e., H_4Folate anion, is a very poor leaving group. Thus, these key kinetic parameters indicate that the methyl transfer reaction is inaccessible without the protonation step; the protonation of the CH_3-H_4Folate must takes place at the methyl-bound N5 species that leads to the formation of quaternary ammonium complex. As a result, our QM/MM calculations

discard the possibility of a methyl transfer from unprotonated CH_3-H_4Folate, thus complementing the previous experimental studies.[29, 60]

However, we also suggested a radical mechanism based on the energetically close-lying diradical states as indicated by the triplet and open-shell singlet states, consisting of an electron transfer from cob(I)alamin to protonated CH_3-H_4Folate followed by a methyl radical transfer. The ET-based radical pathway proceeds in a five coordinated His-on conformation, where an electron transfer first takes place from Co(I) to pterin ring of the CH_3-H_4Folate, consistent with $Co^{II}(d^7)$-pterin radical $(\pi^*)^I$ diradical state. The one electron reduction of the CH_3-H_4Folate thus induces the homolytic cleavage of the N5-C_{Me} bond followed by the transfer of methyl radical to Co(II) to form a six coordinated CH_3-cob(III)alamin and a neutral H_4Folate. It is important to mention that an alternative ET-based radical mechanism is similar to the single electron transfer (SET) mechanism suggested by Mathews et al. [29] but there is difference in the initial step of electron transfer. Most importantly, the predicted energy barrier (~ 8 kcal/mol) for the ET-mediated radical reaction is comparable with that of the S_N2 reaction pathway. However, the primary difference between the S_N2-type and the ET-based radical mechanisms is the initial step of the reaction complex that involves the one-electron reduction of the CH_3-H_4Folate. The other difference is the coordination of the lower axial His759 residue to the Co center. The ET-based radical mechanism takes place in His-on conformation where the His759 plays a crucial role in promoting the electron from $Co^I(d^8)$ metal to the CH_3-H_4Folate substrate, whereas the S_N2-type mechanism occurs in His-off conformation where the formation of the Co-C_{Me} bond takes place first, followed by the displacement of His759 ligand. The main advantage of the ET-based radical mechanism from enzymatic point of view is that a methyl radical can be transferred at a longer distance, which does not require the close proximity of two binding modules of MetH, as would be required for the S_N2-type.

The crystal structure of CH_3-H_4Folate binding module of MetH shows no obvious acid catalyst (BH) near the N5 position, but the product H_4Folate contains a proton at N5 when the methyl group is transferred to cob(I)alamin. Thus, the timing of proton uptake relative to methyl group transfer is very crucial but remains unclear from experimental point of view. Based on the activation energy of the TS in both the protonated and unprotonated N5 species, it can be suggested that the protonation event must takes place, either prior to or during the methyl

transfer reaction in a ternary complex. The results of the present study including enzymatic mechanisms and implications of the protonation process can be applied to a broad class of corrinoid-methyltransferases, which employ CH_3-H_4Folate substrate or its related structures as methyl donors.

ASSOCIATED CONTENT

Supporting Information: Details of the ONIOM-based QM/MM computational methodology. Figures containing key structural features of the QM/MM optimized models methionine synthase (MetH)-bound methylcobalamin (MeCbl) and cob(I)alamin. ONIOM-based QM/MM optimized structure of the unprotonated (N5) MetH-cob(I)alamin:CH_3-H_4Folate reaction complex of reactant complex and transition state. "This material is available free of charge via the internet at http://pubs.acs.org."

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TABLES AND FIGURES

Table 1. Key Structural Parameters of the Optimized Systems of MeCbl-MetH Binding Domain and protonated MetH-cobalamin:CH_3-H_4Folate Reaction Complex (RC_{N5H}) in Comparison with the Experimental Data[a]

	MeCbl-MetH		Co(I)-MetH	Co(I):[CH_3-H_4Folate] (RC_{N5H}(css))	Co(II):[CH_3-H_4Folate]$^{\bullet}$ (RC_{N5H}(T/oss))	Me-Co(III) :H_4Folate (PC_{N5H}(css))
	Calcd	X-ray[6a]	calcd	calcd	calcd	Calcd
Co-C	1.97	1.96				1.97
Co-N_{ax}(His)	2.14	2.24	3.17	3.02	2.07	2.11
Co-Nc	1.87-1.95	1.91-2.02	1.82-1.90	1.82-1.91	1.87-1.93	1.87-1.94
Nc-C	1.32-1.51	1.23-1.44	1.37-1.51	1.33-1.51	1.33-1.50	1.32-1.51
C1-C2	1.56	1.46	1.56	1.55	1.56	1.55
<C-Co-N_{ax}	174.6	173.9				174
<Nc-Co-N_{ax}	84.5-95.0	83.4-99.5	73.9-103.6	75.1-100.13	86.9-98.4	83.9-93.8
<C-Co-Nc	88.6-91.3	82.1-90.8				88.3-92.7

[a] Distances and angles are given in angstroms and degrees, respectively. T and oss stands for the triplet and open-shell singlet states, respectively.

Table 2. Relative Energies (in kcal/mol), Mulliken Charges and Spin Densities of MetH-cobalamin:CH_3-H_4Folate Complexes Calculated with QM/MM calculations.

		Energy (kcal/mol)		Mulliken charge (Spin density)		
		QM/MM-ME	QM/MM-EE	Co	C_{Me}	N_{5H}
S_N2	RC_{N5H} (css)	0.00	0.00	0.59 (0.00)	-0.41(0.00)	-0.54 (0.00)
	TS_{N5H} (css)	9.01	7.62	0.72 (0.00)	-0.49 (0.00)	-0.57 (0.00)
	PC_{N5H} (css)	-36.74	-37.94	0.88 (0.00)	-0.63 (0.00)	-0.63 (0.00)
ET (T)	RC_{N5H} (T)	0.00	0.00	0.88 (1.08)	-0.40 (0.01)	-0.55 (0.00)
	Barrier (T)	8.05	8.68	0.88 (1.06)	-0.49 (0.29)	-0.54 (0.034)
ET (oss)	RC_{N5H} (oss)	0.00	0.00	0.87 (0.98)	-0.40 (0.00)	-0.55 (0.00)
	Barrier (oss)	7.72	8.72	0.78 (-0.75)	- 0.49 (-0.3)	- 0.54 (-0.05)
	PC_{N5H} (oss)	-38.77	-37.95	0.86 (0.00)	-0.61 (0.00)	-0.62 (0.00)

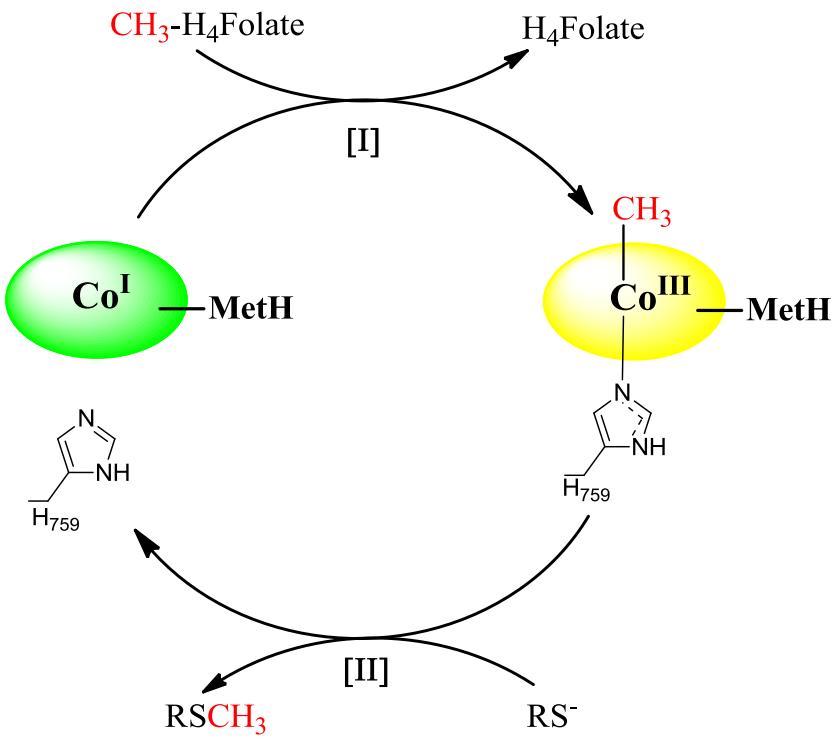
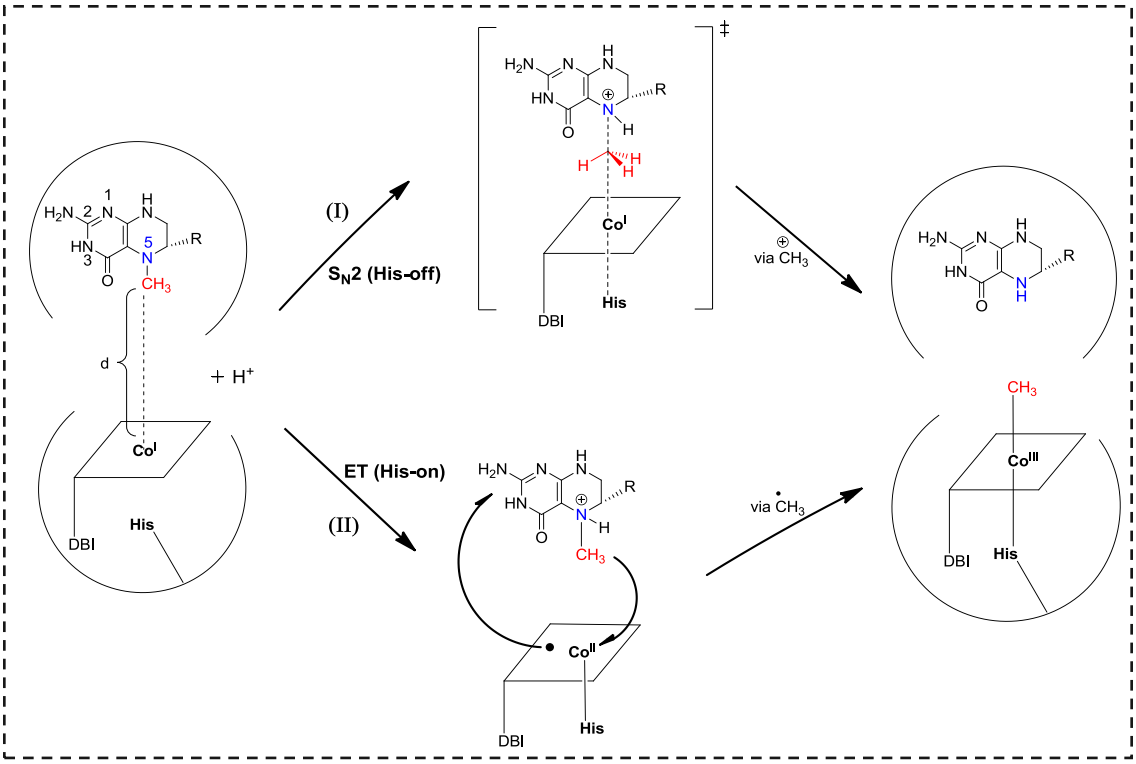


Figure 1. The catalytic cycle of methionine synthase (MetH): first-half catalytic reaction [I] and second-half catalytic reaction [II].



Scheme 1. (I) S_N2-type, and (II) ET-based radical mechanistic pathways for the methyl transfer reaction from a CH_3-H_4Folate substrate to cob(I)alamin intermediate catalyzed by methionine synthase (MetH).

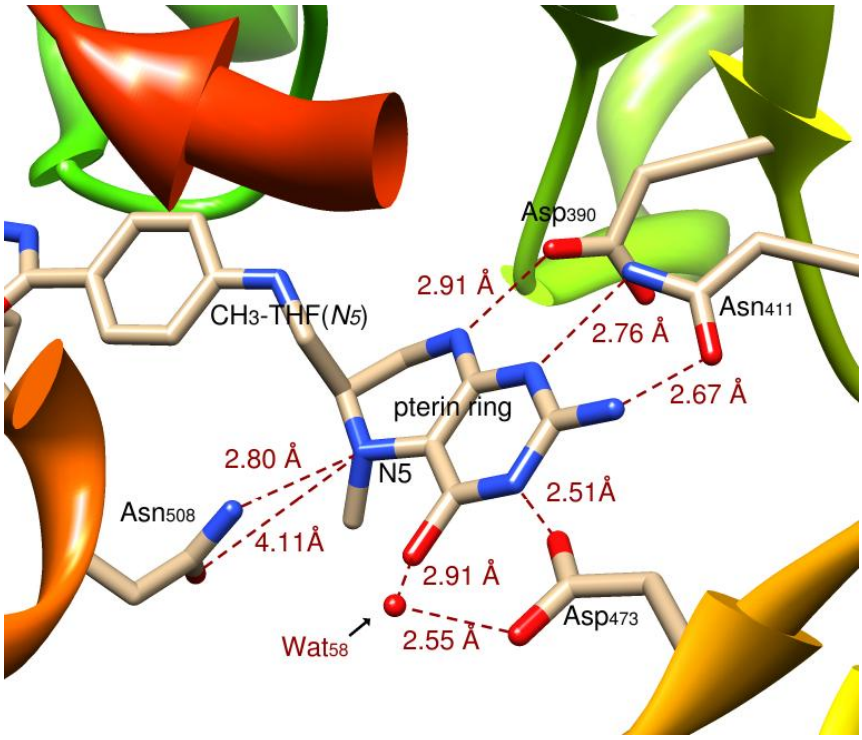


Figure 2. The active site of CH_3-H_4Folate substrate (PDB ID: 1Q8J at 1.9 Å resolution) showing the polar environment near the pterin ring of the CH_3-H_4Folate. The pterin ring is positioned by hydrogen bonds with four important protein residues: Asn508, Asp473, Asp390, and Asn411 and out of these, the N and O of the Asn508 are directly interacting via H-bond with the N5 of pterin ring.

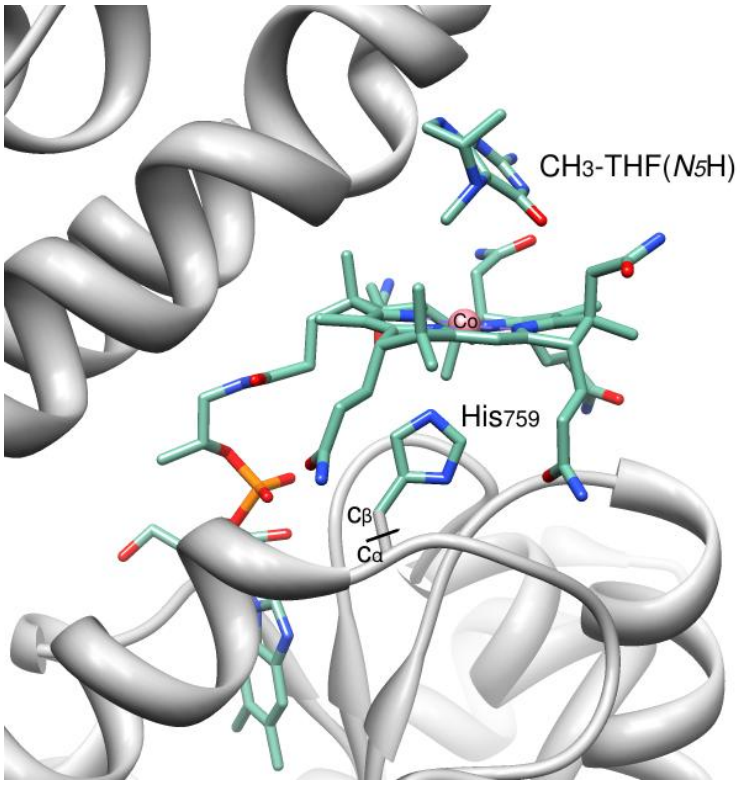


Figure 3. The quantum mechanical region of the MetH-cob(I)alamin:CH_3-H_4Folate(THF) reaction complex used in ONIOM-based quantum mechanics/molecular mechanics (QM/MM) calculations is shown in sticks framework while rest of the protein is treated as part of the MM region.

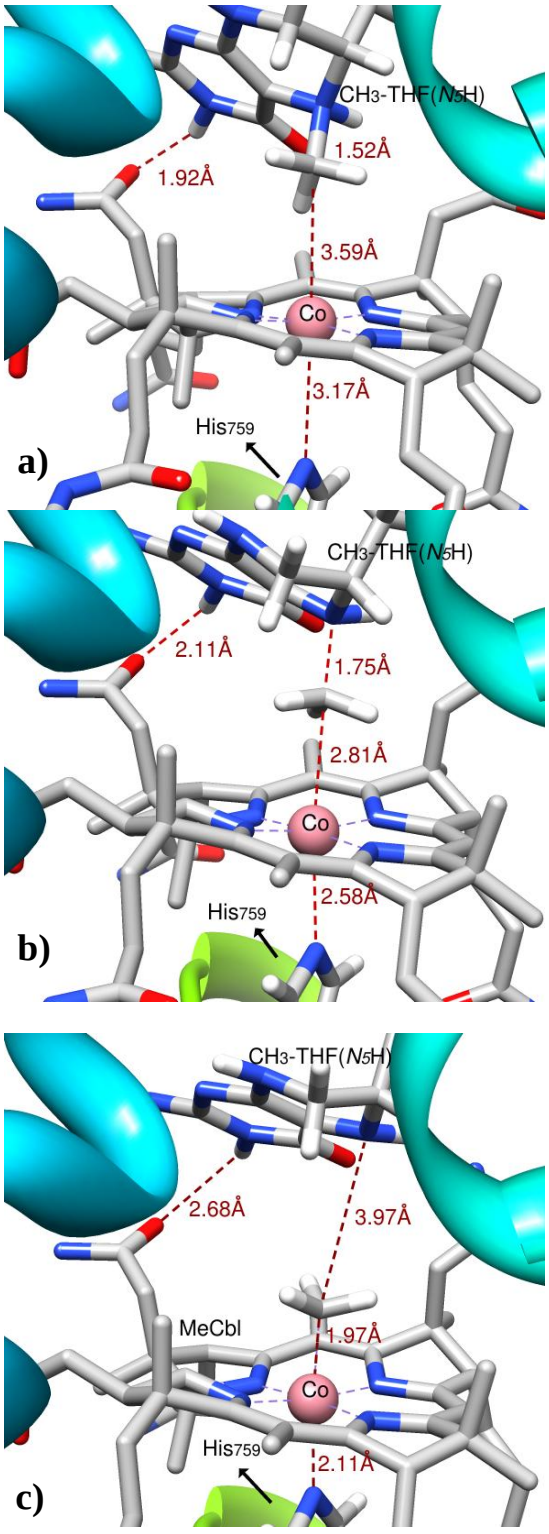


Figure 4. The QM/MM-ME(css) optimized geometries of N5H protonated cob(I)alamin:CH_3-H_4Folate complex: (a) reactant complex (RC_{N5H}) with His-off conformation of Co, (b) transition state (TS_{N5H}), and (c) product complex (PC_{N5H}) in closed shell singlet (css) state calculations.

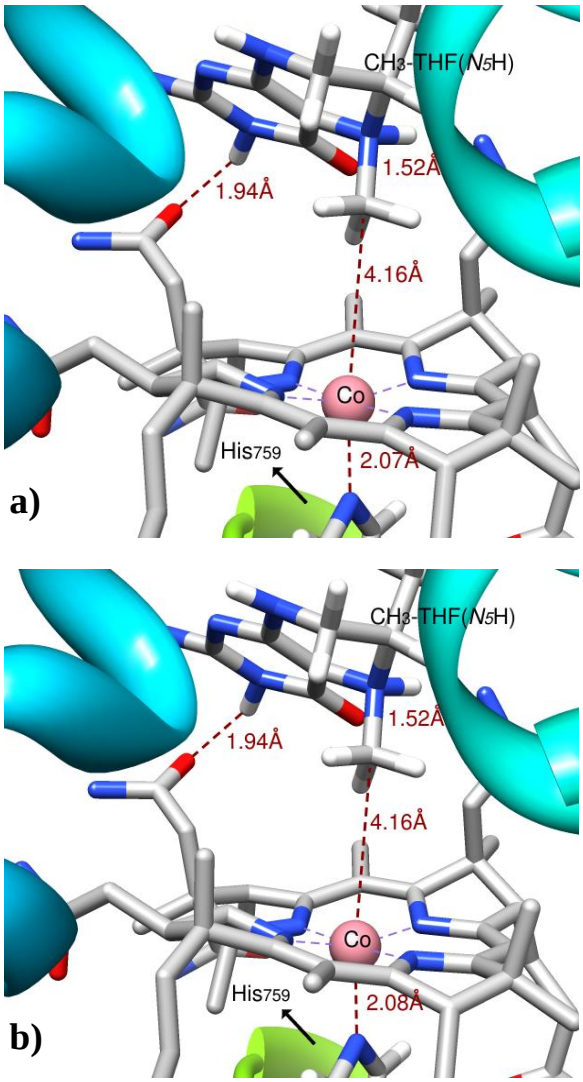


Figure 5. The QM/MM-ME optimized geometries of the N5H protonated cob(I)alamin:CH_3-H_4Folate reactant complex (RC_{N5H}) in a) triplet (T) state, b) open-shell singlet (oss) state calculations which shows His-on conformation of Co, consistent with Co^{II}(d7)-pterin radical (π^*) diradical configuration.

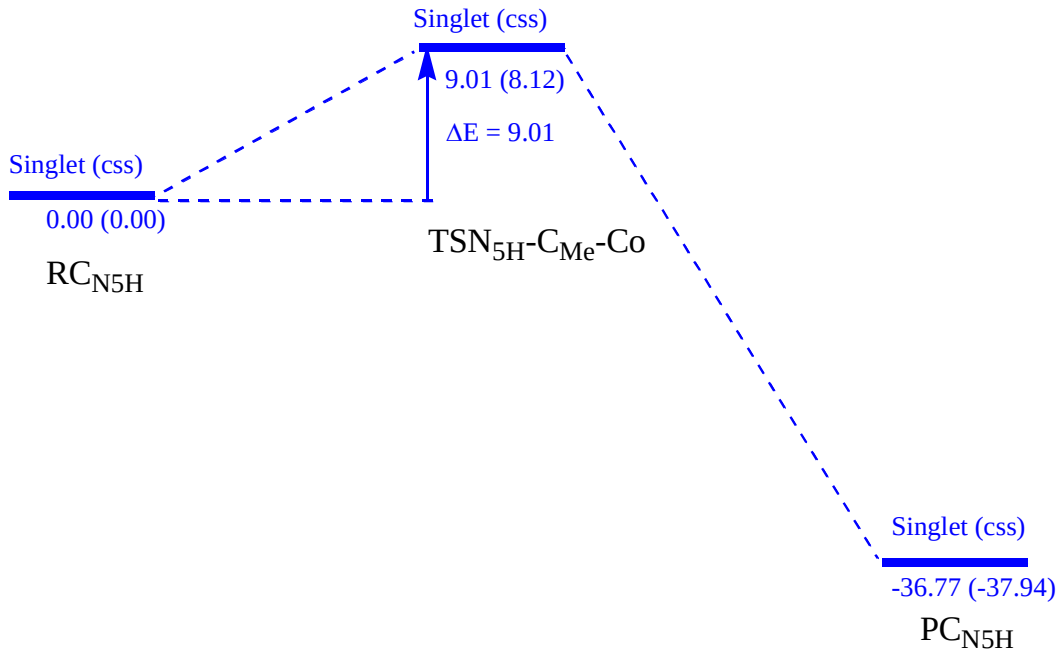


Figure 6. Potential energy profile (kcal/mol) for the Co-C_{Me} bond formation in the N5H protonated state based on closed-shell singlet (css) state QM/MM-ME calculations where the QM/MM-EE energies are given in the brackets and corresponding optimized geometries are shown in Figure 4.

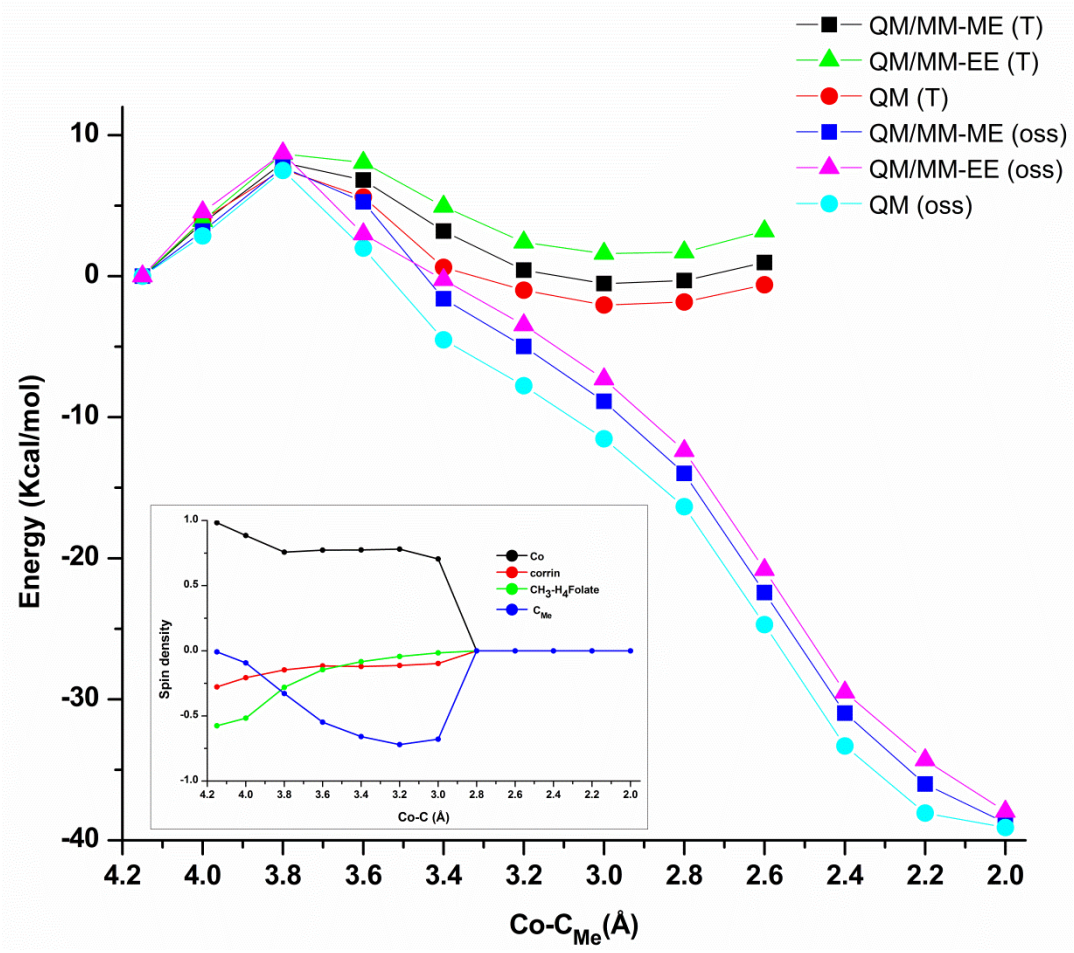
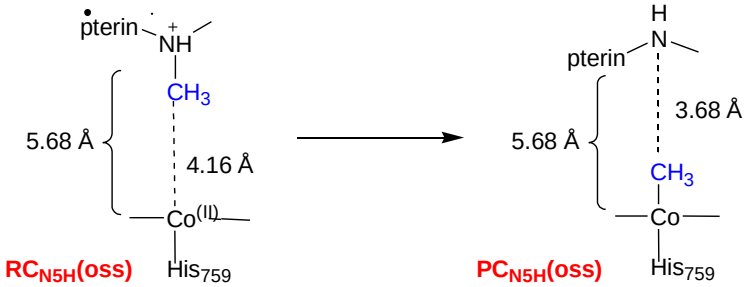


Figure 7. Potential energy profiles with energy barrier for the formation of the Co-C_{Me} bond in N5H protonated reaction complex, calculated with the triplet (T) as well as open shell singlet (oss) state QM/MM calculations. The energies are given relative to the RC_{N5H} where the Co-C_{Me} is 4.16 Å shown in Figure 5. The embedded plot is the evolution of spin density distribution along the Co-C_{Me} distance extracted from open shell singlet QM/MM-EE calculations.



Scheme 2. The schematic representation of the ET-mediated radical mechanism where the N5-C_{Me} bond is elongated and Co-C_{Me} bond is systematically shortened so that the Co-N5 distance (5.68 Å) is fixed.

Table of Contents Figure