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The role of the conserved histidine-aspartate pair in the 'base-off' binding of cobalamins

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Abstract—The conserved cobalamin-binding domain of glutamate mutase exists as a separate dissociable subunit, MutS. The results obtained from BIAcore analysis indicate that MutS alone, in the absence of E component of glutamate mutase (MutE, catalytic subunit), is capable of binding hydroxocobinamide (OHCbi) with a K_d of $15.4 \pm 1.6\,\mu M$, but fails to bind adenosylcobalamin (AdoCbl). The UV–visible spectrum indicates that histidine ligation to the cobalt atom only occurs when both MutE and MutS are present in the solution. MutS mutants, MutS-D14N and MutS-H16G, are also capable of binding OHCbi, but their binding kinetics altered. Our experimental results show that the electrostatic interaction between histidine-aspartate pair is important in the binding of OHCbi or AdoCbl, no matter whether histidine coordinates to the cobalt atom or not. The catalytic subunit is also involved in histidine ligation to the cobalt atom. Meanwhile, mutation of either His16 or Asp14 significantly impairs the enzyme to cleave the cobalt–carbon bond of AdoCbl.

1. Introduction

Cobalamin is one of the most complicated naturally occurring coordination compounds. There are two biologically active forms, adenosylcobalamin and methylcobalamin, used in mammalian (shown in Fig. 1A). The recognition of cobalamin by proteins is, without a doubt, rather complicated. Earlier studies on the binding of cobalamin to intrinsic factor indicated that Coβ-ligand is not essential in the formation of the cobalamin-intrinsic factor complex.[1] One interesting hypothesis was proposed based on this observation: intrinsic factor uses a histidine residue to displace 5,6-dimethylbenzimidazole from the sixth coordination (α) position of cobalt upon binding cobalamin. This inference comes from CD spectral studies, photolysis data and the fact that intrinsic factor's cobalamin binding ability is significantly reduced under the exposure of UV light or iodine (histidine residue is sensitive to both reagents).

Evidence for a conserved B_{12}-binding motif, DXHXXG, came from a sequence alignment between glutamate mutase, methylmalonyl-CoA mutase and methionine synthase.[2] The structure of a proteolytic fragment of *Escherichia coli* methionine synthase consisting of the two methylcobalamin-binding domains was solved.[3] An unexpected finding was the replacement of the pseudo-nucleotide tail of methylcobalamin by the imidazole of histidine 759 as a ligand to cobalt. The conserved aspartate forms a hydrogen bond with the histidine residue. The structures of methylmalonyl-CoA mutase and glutamate mutase solved by X-ray crystallography show this mode of cobalamin-binding as well.[4,5] This base-off/histidine-on binding of cobalamin is subsequently found in isobutyryl-CoA mutase, lysine aminomutase and D-ornithine aminomutase, as demonstrated by the results of sequence alignment, EPR studies or UV–vis spectroscopy.[6–8] Despite the difference in the upper ligand, AdoCbl and MeCbl are bound in the same way. However, whether the cobalamin-binding domain alone is able to bind cobalamin molecules has not investigated yet.

Keywords: B_{12}; Glutamate mutase; Cobalamin; Adenosylcobalamin.
Abbreviations: AdoCbl, adenosylcobalamin; Cbl, cobalamin; AdoCbi, adenosylcobinamide; OHCbi, hydroxocobinamide; MeCbl, methylcobalamin.
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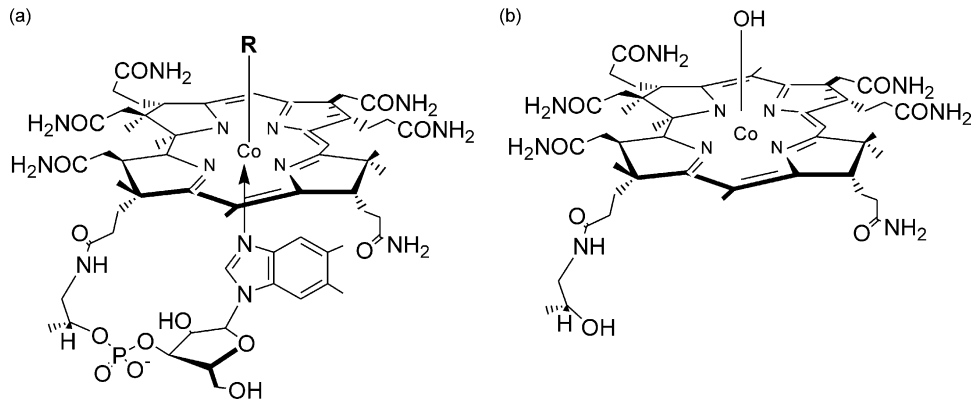

Figure 1. Chemical structure of (A) AdoCbl, R = adenosyl group; MeCbl, R = methyl group. (B) OHCbi.

MutS, the S component of glutamate mutase, is the smallest known protein subunit that carries Cbl-binding domain. The association of MutS, MutE, and AdoCbl is required to form the active holoenzyme (shown in Fig. 2). According to previous report, glutamate mutase binds cob(II)alamin, methylcobalamin, and AdoCbl with the same affinity. This result leads to the conclusion that the upper ligand of Cbl plays a minor role in Cbl-binding process in glutamate mutase system.[9] OHCbi, which lacks the upper ligand and ribonucleotide tail moiety of AdoCbl, was used to investigate this coenzyme-binding process (Fig. 1B). Because this coenzyme analogue doesn't possess lower axial ligand, the energy barrier for coenzyme-binding should be altered. Interactions between corrinoids and MutS were directly monitored on a BIAcore surface plasmon resonance system in this study. In addition, several experiments have been conducted with the use of MutS variants, MutS-D14N and MutS-H16G to characterize the mutational effect of His-Asp pair on coenzyme-binding and catalysis. A sensitive tritium exchange experiment was also employed to measure the cobalt–carbon cleavage rate in both mutants.

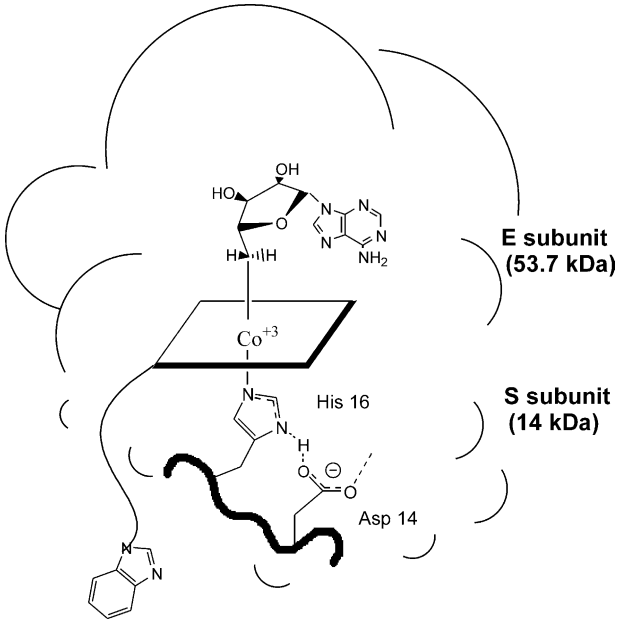

Figure 2. Model of glutamate mutase showing AdoCbl bound between MutS and MutE subunits. The substrate-binding site and catalytic domain is on the MutE subunit.

2. Experimental

2.1. Materials

AdoCbl was purchased from Sigma or Fluka. Crude HOCbi, the analogue of AdoCbl, was synthesized as described previously.[10] For the purpose of BIAcore binding analysis, OHCbi was further purified by reverse-phase HPLC on a 3 μm C_4 column (4.6×100 mm). The column was pre-equilibrated in 0.1% TFA and compounds were eluted with a linear gradient of acetonitrile containing 0.1% TFA. The flow rate was 1 mL/min and eluent was detected by monitoring absorbance at 460 nm. All chemicals and reagents were molecular biology grade or higher.

2.2. Protein expression and purification

MutE (the E component of glutamate mutase from *Clostridium tetanomorphum*) and MutS (the S component of glutamate mutase from *C. tetanomorphum*) were over-expressed in *E. coli* and purified as described previously.[11] The procedure for overexpression and purification of MutS mutants, MutS D-14N and MutS-H16G, was the same as that of MutS wild type protein.[12]

2.3. BIAcore binding analysis

A CM-5 Biosensor chip was activated according to manufacture's instructions. All surface plasmon resonance experiments were carried out on a BIAcore X instrument and done at a flow rate of 5 μL/min at 25 °C unless otherwise stated. To immobilize MutS or MutS mutant proteins on the sensor chip, 50 μM purified protein in 50 mM potassium phosphate buffer, pH 7.0 containing 1 mM DTT was injected onto the activated surface of a CM5 sensor chip for 10 min. The remaining active sites on the chip were blocked by 0.1 M ethanolamine hydrochloride. To perform binding assays, samples of various concentrations of AdoCbl or OHCbi were injected in 50 mM potassium phosphate buffer, pH 7.0 containing 1 mM DTT. The sensor chip was

regenerated by washing the protein-coated surface with 50 mM potassium phosphate buffer, pH 7.0 containing 1 mM DTT to the initial state. K_d values were calculated from the equilibrium resonance signal as a function of analyte concentration. Kalielida Graph™ program (Abelbeck Software) were used to fit binding data to eq 1 as appropriate,

$$[PL] = [P_T][L]/([L] + K_d) \quad (1)$$

where [L] is the free ligand concentration; [PL], the extent of binding at ligand concentration [L]; K_d, the dissociation constant; $[P_T]$, the maximal extent of binding.

2.4. UV–vis absorption spectra

To determine the coordination state of the cobalt atom of enzyme-bound OHCbi, 600 μL protein solutions containing 1 mM MutS, or 1 mM MutS/500 μM MutE in 0.1 M potassium phosphate buffer, pH 7.0 were mixed with 1.5 μL solution containing 2 mM AdoCbi. After mixing and incubating in the dark for 1 min, spectra were recorded using a Hitachi U-3000 spectrophotometer.

2.5. Tritium exchange experiments

To study the tritium transfer from AdoCbl to substrate analogue, 2-ketoglutarate, reactions were set up at 25 °C in the dark. The reaction mixture contained, in a total volume of 50 μL, MutE protein, 50 μM; MutS-D14N or MutS-H16G protein 250 μM, 5′-[^{3}H]AdoCbl (with specific activity of 6700 dpm/μM), 40 μM, and was buffered by 50 mM potassium phosphate, pH 7.0. Reactions were initiated by addition of 5 μL of 100 mM 2-ketoglutarate to give a final concentration of 10 mM. After incubation for 10 min in the dark, the reactions were quenched by addition of 50 μL of 5% trifluoroacetic acid solution. Proteins were removed by centrifugation at 14,000 *g* for 5 min and precipitated in the form of white pellet at the bottom tube. Reaction mixture (25 μL) was injected and separated on a C4 reverse phase column as described previously.[13] Fractions that contained 2-ketoglutarate and AdoCbl were collected by hand and the tritium content was determined by scintillation counting.

3. Results

3.1. Interactions between corrinoids and MutS

Previous equilibrium gel filtration studies have shown that neither MutS nor MutE binds AdoCbl on their own, implying that the assembly of MutE-MutS complex is essential for glutamate mutase to bind AdoCbl. In addition, MutS binds MutE cooperatively with a Hill coefficient of 1.3, and the binding of AdoCbl by glutamate mutase displays a protein concentration-dependent behavior. In the presence of equimolar MutS and MutE, the apparent K_d for AdoCbl was 5.4 μM, but this decreased to 1.8 μM when MutS was present in five-fold molar excess.[11]

The binding of AdoCbl and OHCbi to MutS was investigated using surface plasmon resonance. As shown in Figure 3A, MutS conjugated to the sensor chip is capable of binding OHCbi. By fitting the results into eq 1, the K_d for OHCbi was calculated at 15.4 ± 1.6 μM. The assay was performed at 25 °C and neutral pH. The binding of AdoCbl by MutS was also examined by the same sensor chip. The molecular weight of AdoCbl is higher than that of OHCbi, and therefore the response unit (RU) of the binding of AdoCbl is expected to be higher than that of OHCbi. However, no significant binding of AdoCbl could be observed even when the concentration of AdoCbl was increased to 400 μM (shown in Fig. 3B). This result is consistent with the conclusions obtained from equilibrium gel filtration. To confirm this finding, different batches of OHCbi and MutS protein are used to repeat this experiment and the results are reproducible.

3.2. UV–vis spectra of OHCbi-MutS complex

The UV–vis spectra of cobalamins provide a useful tool to examine the coordination state of cobalt. For example, the red-shift observed in UV–vis absorption spectrum when OHCbi was bound to methylmalonyl-CoA mutase was interpreted to mean that a protein residue coordinated to the cobalt atom, thereby altering the cobalamin spectrum from 'base-off' to 'base-on'.[14]

We have used UV–vis spectroscopy to examine the coordination state of the cobalt atom of OHCbi–MutS and OHCbi-MutS-MutE complexes. Based on the K_d for OHCbi measured by BIAcore binding assay, a sufficient excess of MutS protein was used in the measurement to ensure that almost all the OHCbi was bound by MutS. The spectra of OHCbi bound by MutS alone show that no evidence for a change in the coordination state of cobalt, suggesting that the histidine residue of DXHXXG motif does not coordinate to the cobalt atom (shown in Fig. 4A). However, a significant red-shifting of the spectrum is evident that OHCbi is bound in the 'His-on⟺base-off' mode when both the catalytic subunit, MutE, and the cobalamin-binding subunit, MutS, are present in the solution (shown in Fig. 4B). This suggests that a conformational change in MutS, effected by the E subunit may be necessary for the proper binding of AdoCbl.

3.3. Interaction between OHCbi and MutS mutants

To examine the role that the His-Asp pair in the DxHXXG motif play in coenzyme-binding, we measured the binding of OHCbi to MutS mutants, MutS-D14N and MutS-H16G, using surface plasmon resonance. The sensorgrams for MutS-D14N and MutS-H16G are shown in Fig. 5A and B, respectively. It is evident that both mutants were capable of binding OHCbi. However, two main differences in the OHCbi-binding behavior of the wild type and mutant proteins

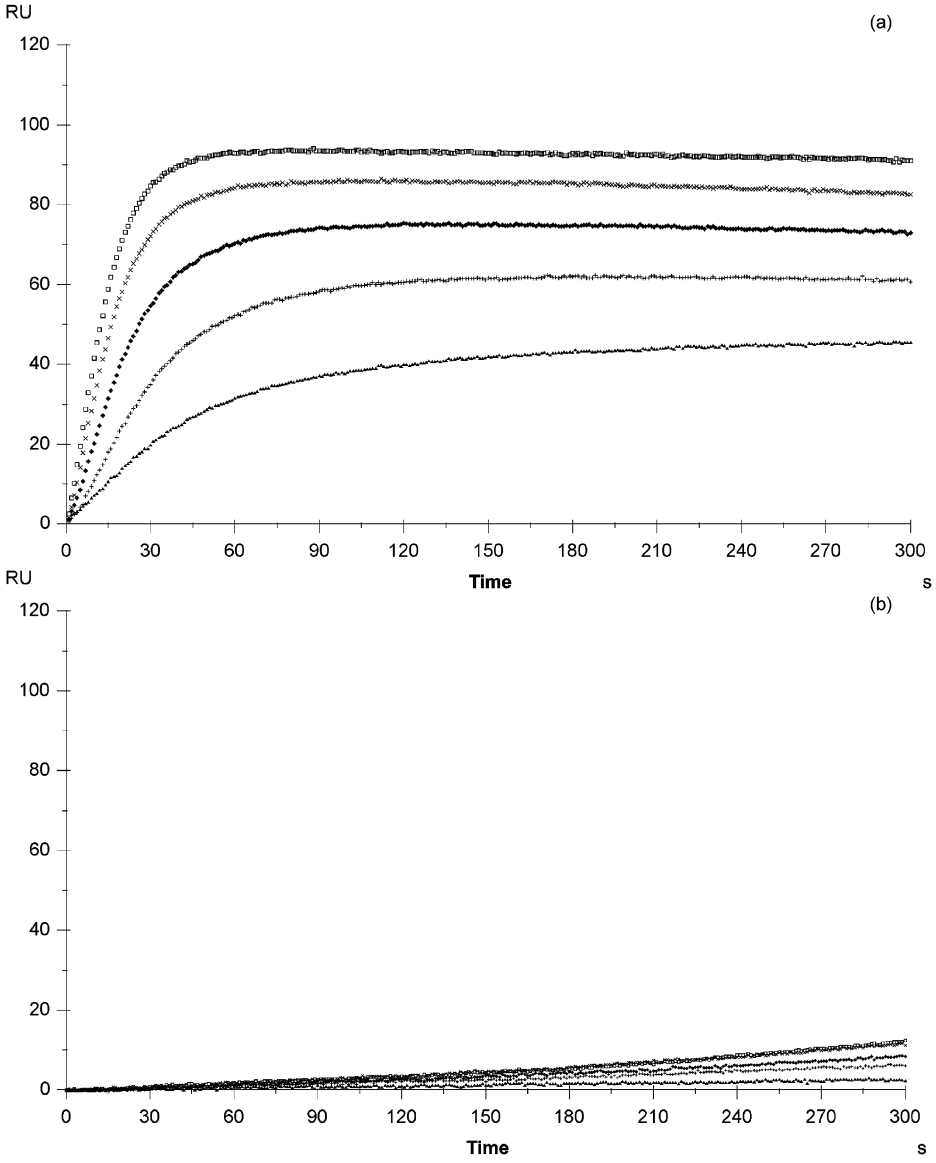

Figure 3. (A) Sensorgrams of OHCbi binding to MutS protein by BIACORE. The concentrations of OHCbi were 150 μM (□), 100 μM (×), 50 μM (◆), 25 μM (+), and 10 μM (▲). (B) Sensorgrams of AdoCbl binding to MutS protein by BIACORE. The concentrations of OHCbi were 500 μM (□), 400 μM (×), 300 μM (◆), 200 μM (+), and 100 μM (▲).

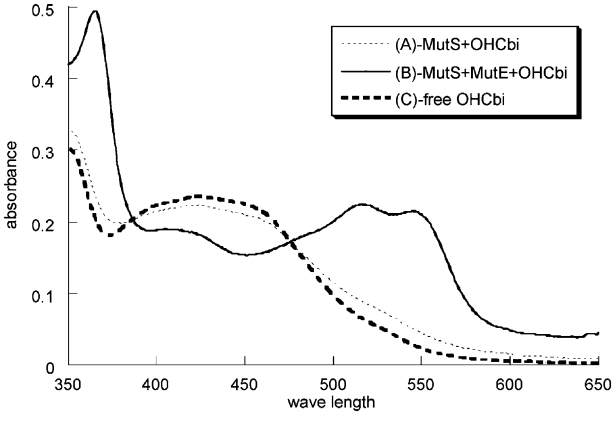

Figure 4. UV–visible spectra of (A) OHCbi bound by MutS, (B) OHCbi bound by MutS–MutE complex, (C) free OHCbi.

are observed. First, although increasing the concentration of OHCbi results in a large signal, suggesting a great fraction of the protein is binding the coenzyme, the data does not fit well to eq 1 and therefore give a K_d value with very large error range (data not shown). Second, it took much longer time for MutS-D14N mutant proteins to bind OHCbi to equilibrate. In addition the stability of the complex of OHCbi and MutS-H16G is weaker than that of wild type. Both results suggest that the mutation on either Asp14 or His16 somehow affect the enzyme's OHCbi-binding ability, although the reason is not clear at this moment.

3.4. The mutation effect of the His-Asp pair on the homolysis of the cobalt–carbon bond

2-Ketoglutarate is able to induce the cleavage of cobalt-carbon bond of AdoCbl, although it does not undergo

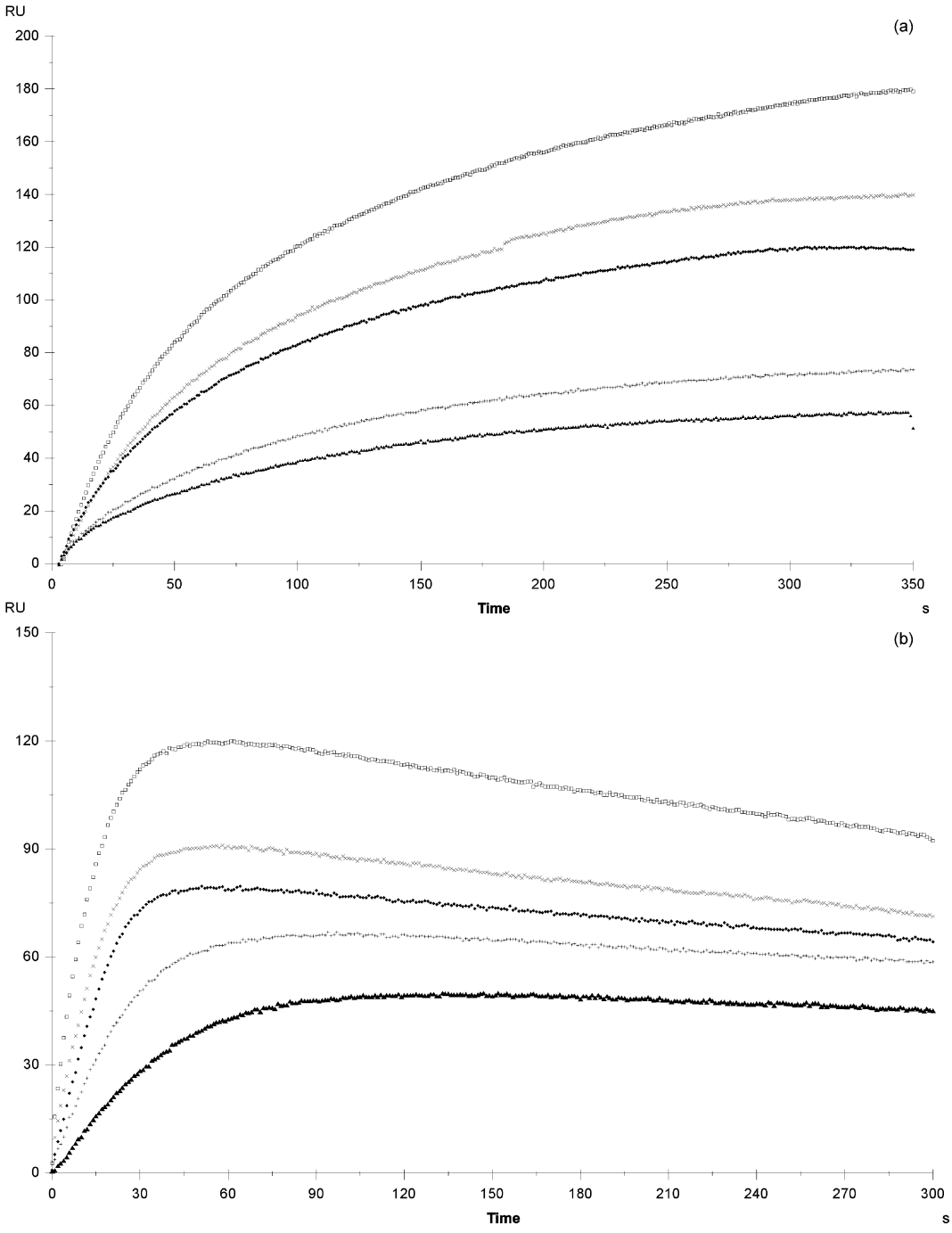

Figure 5. (A) Sensorgrams of OHCbi binding to MutS-D14N protein by BIACORE. The concentrations of OHCbi were 400 μM (□), 300 μM (×), 200 μM (◆), 100 μM (+), and 50 μM (▲). (B) Sensorgrams of OHCbi binding to MutS-H16G protein by BIACORE. The concentrations of OHCbi were 150 μM (□), 100 μM (×), 50 μM (◆), 25 μM (+), and 10 μM (▲).

rearrangement on enzyme. There is a rapid transfer of tritium between the 5′-carbon of AdoCbl and C-4 of 2-ketoglutarate. More than 50% of tritium labeled on the 5′-carbon of AdoCbl could be transferred into C-4 of 2-ketoglutarate within 10 seconds.[15] Tritium exchange therefore provides a sensitive test to detect the cleavage of the cobalt-carbon bond. After 10-min incubation, only 1.8% and 1.3% of tritium was transferred to 2-ketoglutarate in MutS-D14N and MutS-H16G, respectively. It is interesting to note that MutS-D14N variant binds AdoCbl in 'base-off/His-on' form, and MutS-H16G in 'base-off/His-off' form. Apparently, the ability to initiate the homolysis of the cobalt–carbon bond in both mutants was significantly impaired.

4. Discussion

The results obtained from BIAcore analysis in this study demonstrate that MutS alone is capable of binding OHCbi with a K_d of 15.4 ± 1.6 μM, but is unable to bind AdoCbl, suggesting that the catalytic subunit (MutE) is involved in replacing the lower axial ligand of AdoCbl. Interestingly, the histidine residue present in DXHXXG

motif doesn't coordinate to the cobalt atom of OHCbi, the cobalamin-binding domain alone still binds corrin ring reasonably tightly. The interactions between OHCbi and the cobalamin-binding domain seem to be attributable to hydrophobic interactions between the corrin ring and the protein.

The dissociation of lower axial ligand is a slow step in the docking of AdoCbl to the active site of methylmalonyl-CoA mutase.[16] The histidine residue, as suggested by previous mutational studies, is important in this ligand displacement process.[17] Given that MutS binds OHCbi in the 'His-off' mode, one might expect that the binding of OHCbi to MutS mutants, MutS-H16G and MutS-D14N, would display a similar binding behavior as wild type. However, mutations on D14 or H16 clearly alter the ability of the protein to bind OHCbi. This result suggests that the electrostatic interaction between histidine-aspartate pair is important in the binding of OHCbi or AdoCbl, no matter whether histidine coordinates to the cobalt atom or not. The structure of MutS determined by NMR shows the His-Asp motif and the α-helix following it adopt a random coil structure, implying that binding of cobalamin results in a conformational change.[18] Lack of His-coordination with OHCbi may suggest that DXHXXG motif and the α-helix remains unstructured until MutE is present.

An analogue of AdoCbl, AdoCbi-GDP, has been used to evaluate the lower axial ligand effect in the methylmalonyl-CoA mutase catalyzed reaction.[19] Interestingly, even though the cofactor analogue is bound in the 'histidine-off/base-off' mode, it still functions efficiently as a cofactor. This result suggests that histidine ligation to the cobalt atom plays a minor role in methylmalonyl-CoA mutase. The tritium exchange result is in accord with this finding. No significant difference in the cleavage rate of the cobalt-carbon bond could be observed between MutS-D14N and MutS-H16G, no matter whether the lower axial ligand is present or not. This result also clearly indicates that the His-Asp pair is important in breaking the cobalt–carbon of AdoCbl.

The ribonucleotide tail of the coenzyme is thought to provide binding energy to anchor to the coenzyme to the protein.[20] Previous NMR studies have shown that MutS alone can bind the ribonucleotide tail moiety of AdoCbl. However, the value of K_d, 5.6 ± 0.7 mM, is very large in contrast to the K_d for OHCbi.[5] In addition, methylmalonyl-CoA mutase still binds AdoCbi with high affinity.[14] The role of the ribonucleotide tail may therefore not be simply to facilitate the binding of the coenzyme; it might be important in other catalytic steps, such as the cleavage of cobalt–carbon bond or 1,2-rearrangement. Although the full detail of the mechanism by which glutamate mutase binds cobalamin remain to be elucidated, the results presented in this study shed some light on this complicated process.

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