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# Providing a Chemical Basis toward Understanding the Histidine Base-On Motif of Methylcobalamin-Dependent Methionine Synthase: An Improved Purification of Methylcobinamide, plus Thermodynamic Studies of Methylcobinamide Binding Exogenous Imidazole and Pyridine Bases

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Reported herein are the synthesis and improved purification of $MeCbi^+ \cdot BF_4^-$ leading to 95% pure product. The availability of this higher purity $MeCbi^+ \cdot BF_4^-$ has, in turn, allowed a study of the $K_{assoc}$, $\Delta H$, and $\Delta S$ for exogenous imidazole and pyridine bases binding to $MeCbi^+$ in ethylene glycol and buffered aqueous solution. The results show that (1) the bases studied have larger $K_{assoc}$ values (where measurable) when binding to $MeCbi^+$ than when binding to $AdoCbi^+$ under analogous conditions; (2) comparison of the thermodynamic binding parameters for py and *N*-MeIm show that these bases bind similarly, within experimental error to $MeCbi^+$, contrary to what was seen earlier with $AdoCbi^+$; (3) the bases follow the expected trend, with the base with the highest p$K_a$ of those studied, 4-$Me_2$Npy, exhibiting the highest $K_{assoc}$ value ($K_{assoc}$(25 °C) = 18.0 ± 0.3 $M^{-1}$) and the base of lowest p$K_a$, py, exhibiting the lowest detectable $K_{assoc}$ value ($K_{assoc}$ (25 °C) = 6.2 ± 0.4 $M^{-1}$); (4) there is no detectable binding ($K_{assoc}$ = 0.07 $M^{-1}$) for 2-Mepy or 2,6-$Me_2$py with $MeCbi^+$; and (5) the base that is closest to the biologically relevant axial His759 residue in methionine synthase, *N*-MeIm, exhibits an unusual $\Delta H$ value for the formation of $MeCbi^+ \cdot$*N*-MeIm, results interpreted as offering further support for the presence of $\sigma$ plus $\pi$ effects when imidazole bases bind to alkylcobinamides. The results of these studies allow the percentage of base-on methylcobinamide, $MeCbi^+ \cdot$base, to be calculated as a function of temperature and added base. As such, they provide necessary background information for $RS^-$ + $MeCbi^+ \cdot$base and other methionine synthase chemical precedent studies.

## Introduction

Methylcobalamin-dependent methionine synthase (MetH)[1,2] catalyzes two successive methyl transfer reactions[3] to form tetrahydrofolate and methionine from methyltetrahydrofolate and homocysteine. The reaction proceeds by heterolytic cleavage of the Me−Co bond of methylcobalamin (MeCbl, Figure 1), generating $Co^I$cobalamin ($Co^I$Cbl), and transferring the methyl carbocation to deprotonated homocysteine[4] to form methionine, eq 1. MeCbl is then regenerated by accepting a methyl carbocation from methyltetrahydrofolate concurrent with uptake of a proton,[5] generating tetrahydrofolate and completing the catalytic cycle, eq 2. The cob(I)alamin cofactor is occasionally oxidized to the inactive cob(II)alamin,[6] which then is reactivated by reductive methylation using reduced flavodoxin as an electron donor[7] and adeno-

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(1) Abbreviations: MetH (methionine synthase); MeCbl (methylcobalamin); $MeCbi^+$ ($MeCbi^+ \cdot BF_4^-$, methylcobinamide); AdoCbl (coenzyme $B_{12}$, adocobalamin, 5′-deoxy-5′-adenosylcobalamin); $AdoCbi^+$ ($AdoCbi^+ \cdot BF_4^-$, adocobinamide, 5′-deoxy-5′-adenosylcobinamide); $Co^I$Cbl ($Co^I$cobalamin); py (pyridine); 2-Mepy (2-methylpyridine, 2-picoline); 2,6-$Me_2$py (2,6-dimethylpyridine, 2,6-lutidine); 4-CNpy (4-cyanopyridine); 4-$Me_2$Npy (4-dimethylaminopyridine); *N*-MeIm (*N*-methylimidazole); 1,2-$Me_2$Im (1,2-dimethylimidazole); $Me_2$bzm (5,6-dimethylbenzimidazole); AU (absorbance units).

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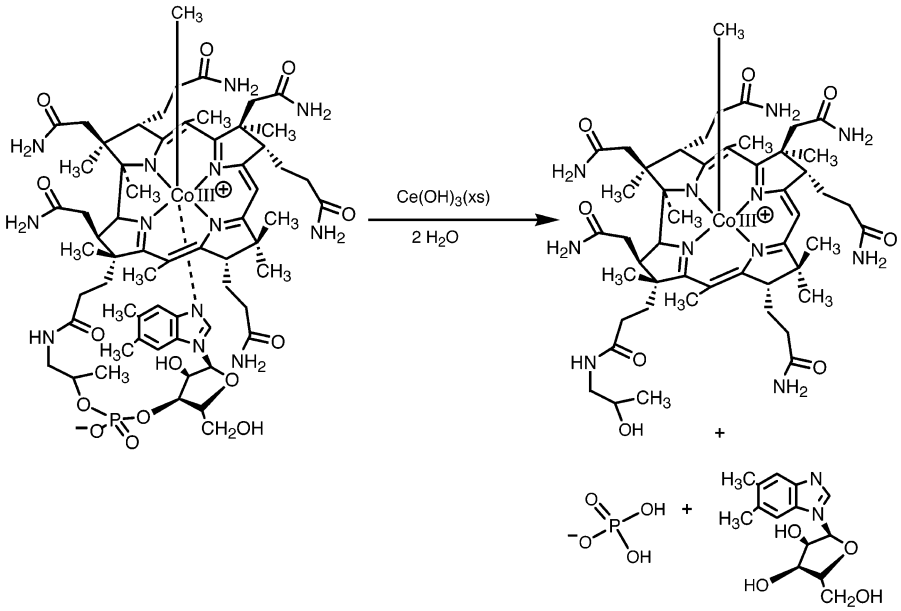
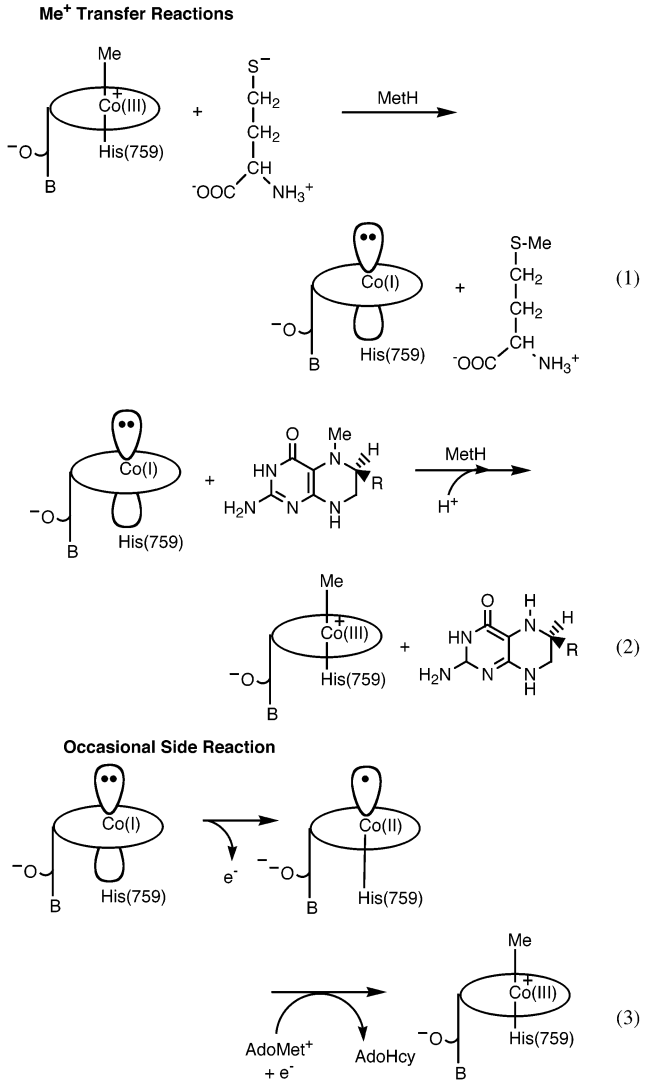



**Figure 1.** The stoichiometric reaction of MeCbl (left) reacting under aqueous conditions with $Ce(OH)_3$ to generate $MeCbi^+$, the axial-base free analogue of MeCbl. An unnaturally long $Co-CH_3$ bond is shown only to clearly depict the presence of this key $Co-CH_3$ bond.

sylmethionine (AdoMet) as a methyl donor,[8,9] eq 3.




MetH from *Escherichia coli* is unusual in that the large, 136.1 kDa monomeric enzyme[10] can be divided into four separate domains that independently fold and bind homocysteine, methyltetrahydrofolate, methylcobalamin, and adenosylmethionine, respectively.[6,11,12] The X-ray crystal structure of the 27 kDa cobalamin-binding fragment of *E. coli* methionine synthase was reported in 1994 at 3.0 Å resolution.[13] The crystal structure revealed an unexpected binding motif for MeCbl: the appended 5,6-dimethylbenzimidazole ($Me_2bzm$) base, which is coordinated to cobalt in neutral pH solution as well as in the $Me_2bzm$ base-on subclass of adenosylcobalamin (AdoCbl)-dependent enzymes,[14−19] is displaced from cobalt and is sequestered in a separate fold of the enzyme. The sequestered $Me_2bzm$ base appears to be important in organizing the cobalamin binding pocket of the enzymatic active site.[20,21] In its place, a histidine residue (His759) binds to cobalt as the sixth α-axial ligand. This 5,6-dimethylbenzimidazole base-off, histidine base-on binding motif defines a $Me_2bzm$ base-off subclass of AdoCbl-dependent enzymes,[22−24] now verified in the X-ray crystal structures of AdoCbl-dependent methylmalonyl-CoA mutase[25] and glutamate mutase.[26]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Since the publication of the methionine synthase X-ray crystal structure, enzymatic work has focused on probing the role of the imidazole/histidine ligand. In addition to ligating the cobalt atom of MeCbl, His759 is also hydrogen-bonded to Asp757, which is then hydrogen-bonded to Ser 810.[13] This trio of residues, the "ligand triad", affects the observed catalytic activity: mutation of His759 results in inactive enzyme,[27] and mutations of Asp757 and Ser 810[28] slightly reduce catalytic activity.[27,28] In addition, the Asp757 and Ser 810 mutations lead to decreased photostability of the cobalamin cofactor.[27] The full mechanistic implications of these mutations are currently under investigation.[29] Most intriguing is that in the reactivation step of cob(II)alamin in MetH, binding of flavodoxin to MetH changes cob(II)alamin from the base-on to base-off form,[30] concomitant with uptake of a proton within the ligand triad.[31] These results imply that the ligand triad regulates the Co−His bond and suggest that the ligand triad influences (or is an indicator of) the substantial conformational changes that the enzyme undergoes during catalysis and reactivation. Hence, it is important to understand the fundamental chemical properties of the Co−N(histidine/imidazole) bond to further deconvolute the role of the histidine ligand in MetH. In order to accomplish this, one must use an analogue of MeCbl that does not have the appended 5,6-dimethylbenzimidazole base, namely, the base-free analogue of MeCbl, methylcobinamide ($MeCbi^+$, Figure 1), thereby allowing studies of the binding and effects of exogenous bases.

**Initiation of Chemical Precedent Studies with High-Purity $MeCbi^+$.** By employing $MeCbi^+$, it is possible to study the binding of a variety of exogenous bases to cobalt without changing any of the other properties of the coenzyme, as has been done for AdoCbl using adenosylcobinamide ($AdoCbi^+$).[32−35] Ideally, one can then study the axial-base effect in model reactions of each enzymatic step in eqs 1−3. This first requires a clean, well-characterized sample of $MeCbi^+$ and a study of its axial-base binding $K_{assoc}$, $\Delta H$, and $\Delta S$ thermodynamic values, so that the percentage of base-on and base-off species is known at any desired temperature and for the desired range of specific bases.

**Table 1.** Literature Thermodynamic Parameters for $MeCbi^+$ plus Selected Bases in $H_2O$ (25 °C)

| base | p$K_a$ | $K_{assoc}$ (25 °C, $M^{-1}$) | $\Delta H$ (kcal/mol) | $\Delta S$ (eu) | ref |
|---|---|---|---|---|---|
| Im | 7.24 | 8.01 ± 0.13 | | | 42 |
| Im | 6.95 | 11[a] | | | 40 |
| Py | 5.19 | 7.32 ± 0.05 | −3.82 ± 0.15 | −8.8 ± 0.5 | 42 |
| Py | 5.19 | 6[a] | | | 40 |
| Py | 5.19 | 9[a] | | | 41 |
| 4-Mepy | 6.36 | 11.5 ± 0.2 | | | 42 |
| 4-$NH_2$py | 9.40 | 24.0 ± 0.1 | −5.27 ± 0.50 | −11.4 ±1.8 | 42 |
| *N*-MeIm | 7.33 | 5 | | | 40 |

[a] Temperature, error bars not stated.

There are two published syntheses of $MeCbi^+$: a $Ce(OH)_3$ catalyzed phosphodiester hydrolysis synthesis,[36] the "classic" method of preparing cobinamides,[37] and a trifluoromethane sulfonic acid nucleotide cleavage synthesis,[38] based on an earlier Factor B preparation by Brown and co-workers[39] (Factor B is a mixture of α- and $\beta$-$CNCbi^+$). However, neither preparation provides a quantitative assessment of the purity of the resultant $MeCbi^+$.

Previous thermodynamic studies of aromatic, nitrogenous bases binding to $MeCbi^+$ are limited;[40,41] the most recent work has been done by Brown's group,[42] Table 1. A summary of the available data in Table 1 reveals that (a) only a limited number of bases have been studied, (b) the $K_{assoc}$ values reported by four different laboratories vary significantly, and (c) previous equilibria were measured only in water, not in organic solvents (which might be better mimics of the interior of the folded enzyme),[32] or in buffered aqueous solutions (typical of enzymatic kinetic studies). In short, there is a need for a systematic study of imidazole and pyridine bases binding to pure, well-characterized $MeCbi^+$ in both nonaqueous solvents *and* buffered aqueous solutions. In addition, completion of these $MeCbi^+$ axial-base binding studies will allow a previously unavailable, interesting comparison to the earlier, analogous studies with $AdoCbi^+$.[32,34,35]

Herein we report (a) a reinvestigation of the synthesis and purification of $MeCbi^+ \cdot BF_4^-$, resulting in a repeatable synthesis of material proven to be ∼95% pure by both $^1$H NMR and HPLC; and (b) the $K_{assoc}$, $\Delta H$, and $\Delta S$ thermo-


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dynamic parameters for imidazole and pyridine bases, of varying basicity and steric bulk, binding to $MeCbi^+$ in both ethylene glycol and buffered aqueous solutions. The results reveal that (1) the bases studied have larger $K_{assoc}$ values (where measurable) when binding to $MeCbi^+$ than when binding to $AdoCbi^+$ under analogous conditions; (2) comparison of the thermodynamic parameters between py and *N*-MeIm show that the bases bind to $MeCbi^+$ similarly, within experimental error, contrary to what was seen for $AdoCbi^+$;[32] (3) the bases followed the expected trends, with the base with the highest p$K_a$ of those studied, 4-$Me_2$Npy, exhibiting the highest $K_{assoc}$ value and the base of lowest p$K_a$, py, exhibiting the lowest detectable $K_{assoc}$ value; and (4) there is no detectable binding ($K_{assoc} = 0.07\ M^{-1}$) for 2-Mepy or 2,6-$Me_2$py with $MeCbi^+$. This work provides the first, *proven* ≥95% purity $MeCbi^+$, as well as the axial-base $K_{assoc}$, $\Delta H$, and $\Delta S$, required before reliable chemical precedent kinetic studies of model reactions for eqs 1−3 employing $MeCbi^+$ can begin. Only when a comparison is complete of the axial-base effects within methionine synthase itself, to those in enzyme-free $MeCbi^+$, can the interesting $Me_2$bzm base-off, His759 base-on binding motif exhibited in methionine synthase be claimed to be understood.

## Experimental Section

**Chemicals.** MeCbl (Sigma; ≥98% pure by both $^1$H NMR and HPLC) was stored at 0 °C and used as received. All other chemicals were obtained from Aldrich. *N*-Methylimidazole (redistilled by the manufacturer), pyridine, 2,6-$Me_2$py, 2-Mepy, 4-CNpy, 4-$Me_2$Npy, NaOH, and ethylene glycol (redistilled by the manufacturer) were stored at room temperature and used as received. $^1$H NMR of 4-CNpy in $CD_3OD$: 7.76 (s, 2H), 8.79 (s, 2H), 97% pure. 1,2-$Me_2$Im (Aldrich) was used both commericially and after purification over a silica column (vide infra). $^1$H NMR of commercial 1,2-$Me_2$-Im in $CD_3OD$: 2.32 (s, 3H), 3.59 (s, 3H), 6.76 (s, 1H), 6.92 (s, 1H), 98% pure. $Ce(NO_3)_3 \cdot 6H_2O$ was purchased fresh, stored under argon at room temperature, and used within 3 months of purchase.

**Instrumentation.** The drybox, HPLC, and UV−visible instrumentation are exactly as previously described.[34] Proton NMR spectra were recorded in $D_2O$ solutions at room temperature on an Inova-300 MHz spectrometer with use of TSP (3-(trimethylsilyl)-propionic-2,2,3,3-$d_4$ acid, sodium salt) as an internal standard. Due to the light sensitivity of cobalamins and cobinamides, all work was done in a dark room illuminated only with dim (≤25 W) red light. Note that since cobalamins and cobinamides are yellow and red, respectively, all cobamide solutions appear effectively colorless to the eye under these conditions. Hence, all column fractions were identified by UV−visible spectroscopy of aliquots.

**Attempted Synthesis of Pure $MeCbi^+ \cdot BF_4^-$ Using the Trifluoromethane Sulfonic Acid Method.**[38,39] MeCbl (100 mg) was placed in a glass 2 dram vial equipped with a stir bar and covered with aluminum foil; the vial was then dried overnight under vacuum at room temperature. The vial was moved to the drybox, and 2 g of neat trifluoromethane sulfonic acid (from 2 × 1 g sealed glass ampules) was added to the MeCbl solid. The resulting mixture was immediately sealed with a screw-top lid, and the vial was completely covered with aluminum foil and then stirred at room temperature overnight under $N_2$ in the drybox. The reaction solution was taken out of the drybox and quenched by being poured into 100 mL of a 0.0665 M solution of $Na_2HPO_4 \cdot 7H_2O$ (1.786 g) in a 250 mL Erlenmeyer flask in a hood, all in a darkened lab. The product solution was then desalted on a 6 × 15 cm Amberlite XAD-2 column. After rotary evaporation of the product to dryness, the $BF_4^-$ counteranion salt, $MeCbi^+ BF_4^-$, was obtained by running the product down a 1 × 10 cm DEAE cellulose column in its $BF_4^-$ form. Complete details are given in the Supporting Information.

HPLC analysis of the $MeCbi^+$ product (C18 Versapack column, Aldrich; isocratic conditions, 0.01 M pH = 3.0 $NH_4H_2PO_4$ buffer, 0.8 mL/min, detected at 340 nm) gave two peaks at 15.2 and 15.7 min with an integrated area ratio of 1:2.3, respectively. $^1$H NMR of the mixture, aromatic region[43] ($D_2O$, referenced to internal TSP), yielded two doublets at 6.5 and 6.6 ppm in the aromatic region from 6 to 8 ppm with an area ratio of 1:2.5, respectively. The C10 proton is a singlet at 6.77 ppm in pure $MeCbi^+$, vide infra, indicating that a mixture of unknown products has been produced with this synthesis employing trifluoromethane sulfonic acid. L-SIMS, again of the mixture of as-obtained products (*m*-nitrobenzyl alcohol matrix, positive ion spectrum): calcd mass for $MeCbi^+$ $C_{49}H_{75}N_{11}O_8$-Co, 1005.1; found parent ion at 1005.17. UV−visible shows an apparently clean spectrum of $MeCbi^+$ with a $\lambda_{max}$ at 462 nm and despite the two doublets seen in the $^1$H NMR; this reveals the importance of especially $^1$H NMR,[43] as well as HPLC, in correctly judging the purity of cobamides. Although we were not able to baseline separate the compounds using HPLC, it is likely that the literature[38] FPLC method does obtain reasonably pure product; however, since that method did not quantify the purity of their $MeCbi^+$, and since we did not have available the exact FPLC system used in the literature, we did not pursue this synthesis further.

**$Ce(OH)_3$-Catalyzed Synthesis of $MeCbi^+ \cdot BF_4^-$.** This preparation was done analogously to the original $Ce(OH)_3$ preparation published by Brown and Peck;[36] note that, in order to obtain 95% pure $MeCbi^+$, it is necessary to run the SP Sephadex C25 column with water to elute unreacted cobalamins, and then elute the $MeCbi^+$ product with 0.01 M sodium phosphate buffer, pH = 7.0, rather than the 0.1 M acetate buffer, pH = 5, cited in the original preparation. Full details are given in the Supporting Information.

Final yield: 64 mg (35%). $^1$H NMR (taken of the aromatic region, ∼5.5−9.0 ppm): s (6.770 ppm), ≥95% pure, Figure S2, Supporting Information. HPLC (C18 Versapack column, Aldrich; isocratic conditions, 30% $CH_3CN$; 70% 0.001 M NaOAc buffer, pH = 4.5; 1 mL/min, detected at 260 nm) gave one major peak at 5.01 min, ≥95% pure, Figure S3, Supporting Information. L-SIMS (*m*-nitrobenzyl alcohol matrix): calcd mass for $MeCbi^+$ $C_{49}H_{75}N_{11}O_8$-Co, 1005.1; found parent ion at 1005.2. UV−visible: $\lambda_{max}$ = 462 nm ($\epsilon$ = 13400 ± 300 in ethylene glycol; $\epsilon$ = 11100 ± 300 in $H_2O$).

**$K_{assoc}$ Measurements and Calculations.** Analogous to our earlier work,[32,33] samples of 1 × $10^{-4}$ M $MeCbi^+$ in 2.0 mL of ethylene glycol, or in independent experiments in pH = 7.2 50 mM $KP_i$ buffered aqueous solution, were titrated with either neat base or, for bases that are solids, concentrated solutions (2−5 M) at 16, 25, 34, and 43 °C (±0.1 °C). (Note that, due to the viscosity of ethylene glycol, there is a 3−5 min mixing time in the cuvettes employed, even with vigorous shaking for 60 s before equilibration, during which a given spectrum's absorbance will vary by up to 0.1 AU. It is important to wait until the spectrum has stabilized, and equilibrium has been reached, for quantitative studies.) The absorbance data at 460 and 520 nm were worked up according to the appropriate equations, all exactly as described previously[32] (eq 5). The resulting temperature-dependent $K_{assoc}$ data were analyzed via the usual ln($K_{assoc}$) vs 1/$T$ plot to yield $\Delta H$ and $\Delta S$ values for base binding to $MeCbi^+$; the results are summarized in Table 2. A

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**Table 2.** Association Constants and Thermodynamic Parameters for $MeCbi^+$ plus Selected Bases in Ethylene Glycol or pH = 7.2 Buffered $H_2O$ (50 mM $KP_i$) (25 °C)

| base | p$K_a$ | solvent | $K_{assoc}$ (25 °C, $M^{-1}$) | $\Delta H$ (kcal/mol) | $\Delta S$ (eu) |
|---|---|---|---|---|---|
| 4-CNpy | 1.9 | $HOCH_2CH_2OH$ | nd[a] | | |
| Py | 5.19 | $HOCH_2CH_2OH$ | $6.2 \pm 0.4$ | $-6.2 \pm 0.6$ | $-17 \pm 2$ |
| Py | 5.19 | pH = 7.2 $H_2O$ | $6.9 \pm 0.1$ | $-3.0 \pm 0.5$ | $-7 \pm 2$ |
| 2-Mepy | 5.96 | $HOCH_2CH_2OH$ | $\leq 0.07$ | | |
| 2,6-$Me_2$py | 6.62 | $HOCH_2CH_2OH$ | $\leq 0.07$ | | |
| *N*-MeIm | 7.33 | $HOCH_2CH_2OH$ | $6.9 \pm 0.2$ | $-6.57 \pm 0.06$ | $-18.2 \pm 0.2$ |
| *N*-MeIm | 7.33 | pH = 7.2 $H_2O$ | $4.9 \pm 0.2$ | $-3.1 \pm 0.2$ | $-7.3 \pm 0.6$ |
| 1,2-$Me_2$Im | 7.85 | $HOCH_2CH_2OH$ | nd | | |
| 4-$Me_2$Npy | 9.7 | $HOCH_2CH_2OH$ | $18.0 \pm 0.3$ | $-7.4 \pm 0.5$ | $-30 \pm 2$ |

[a] Not determined (i.e., could not be determined).

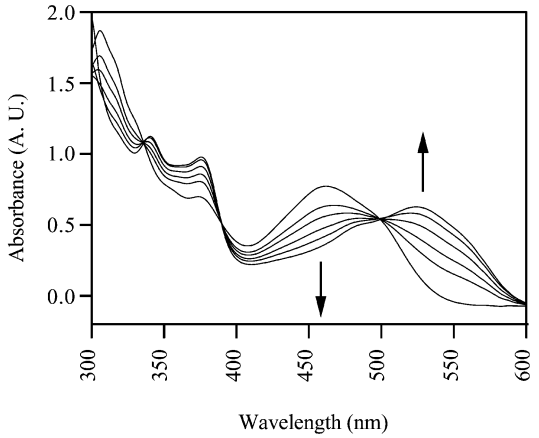



**Figure 2.** Titration of $\sim 1 \times 10^{-4}$ M $MeCbi^+ \cdot BF_4^-$ with neat *N*-MeIm (625, 1250, 2500, 5000, and 10000 equiv) in ethylene glycol at 25 °C. Final [*N*-MeIm] = 1.00 M; $K_{assoc}$ = 6.9(2) $M^{-1}$. Clean isosbestic points are seen at 330, 380, and 500 nm.

representative titration is given in Figure 2; see also Figures S4−S42 of the Supporting Information.

**Purification of 1,2-$Me_2$Im and Attempted $K_{assoc}$ Measurements.** It has been proposed in the literature[46] that some commercial, sterically hindered nitrogenous bases (such as 2-methylpyridine or 2,6-dimethylpyridine) have a low-level impurity that binds to cobinamides, resulting in false thermodynamic or kinetic data when using this base with cobinamides.[46] Hence, we wished to verify our results using 1,2-$Me_2$Im, since it is also a sterically hindered nitrogenous base (and even though this base is not discussed in the above-mentioned literature),[46] with both commercial and purified 1,2-$Me_2$Im. Commercial 1,2-$Me_2$Im is a yellow-brown, translucent crystalline substance, mp = 29−35 °C. We purified the commercial material by passing it down a silica gel column (1 × 7 cm, packed with diethyl ether). The yellow-brown impurity remained at the top of the column, while the clear 1,2-$Me_2$Im was collected off of the column in one large fraction. The fraction volume was reduced under vacuum through rotary evaporation to a clear oil, which solidified when placed in the refrigerator. The resulting clear, translucent, crystalline product has a mp of 36−38 °C.

Titrating the $\sim 1 \times 10^{-4}$ M $MeCbi^+$ solution with a concentrated, 5 M, solution of commercial 1,2-$Me_2$Im resulted in a slight increase at 520 nm (<0.05 AU), Figure S35, Supporting Information, but the change in absorbance, when evaluated using eq 5, did not yield a linear plot, nor, therefore, a $K_{assoc}$ value. The purified 1,2-$Me_2$Im is less soluble than the commercial product (the yellow-brown impurity is highly polar as demonstrated by the silica gel column, and must enhance solubility of 1,2-$Me_2$Im), and a concentrated, 5 M, solution cannot be made. An attempt was made to titrate a $MeCbi^+$ solution with a heated, 1.6 M 1,2-$Me_2$Im solution, but the concentrated solution would precipitate out once it was added to the 25 °C $MeCbi^+$ solution, and only very slowly (2−24 h, based on the size of the aliquot) dissolve into solution, resulting in absorbance increases that eliminated any quantifiable data, Figure S36, Supporting Information. Overall, the data for the purified 1,2-$Me_2$Im is qualitatively similar to that obtained with the unpurified, commercial product, neither set of data yielding a $K_{assoc}$ value.

## Results and Discussion

**Synthesis of $MeCbi^+ \cdot BF_4^-$.** The two previous preparations of $MeCbi^+$ in the literature[36,38] do not quantify the purity of the resultant $MeCbi^+$ compound by either $^1$H NMR of the aromatic region[43] or HPLC, making it impossible to determine if either synthesis gives sufficiently pure product for quantitative axial-base and other chemical precedent studies. Hence, it became necessary to evaluate both of these preparations as part of the present work and before commencing upon the desired studies.

Attempts to repeat the trifluoromethane sulfonic acid cleavage synthesis[38] (as detailed in the Experimental Section, and with ≥98% pure MeCbl by $^1$H NMR and HPLC) yielded only ~70% pure $MeCbi^+ \cdot OH^-$ by both $^1$H NMR and HPLC, at least in our hands by the procedure detailed in the Experimental Section and even after three attempts, and prior to any FPLC purification.[44] The most revealing indication of impurity is the existence of two peaks in the C10 aromatic region of the $^1$H NMR in a ~2.5:1 ratio indicating that two corrinoids are present; hence, we abandoned this synthesis.[45,46]

We returned, therefore, to the earlier $Ce(OH)_3$ preparation by Brown and Peck[36] (and even though the purity of the $MeCbi^+$ product was not stated therein), a synthesis based on the 1987 preparation of $AdoCbi^+ \cdot BF_4^-$ which has proven reliable over the intervening 13 years.[37] Repeating Brown and Peck's $MeCbi^+$ preparation *exactly* resulted in $MeCbi^+$ that was only ~80% pure by $^1$H NMR (Figure S1, Supporting Information; note that this synthesis includes the use of 0.1 M acetate buffer, pH = 5, as an eluent off an SP-Sephadex column). This synthesis was repeated four times in an attempt to improve the purity of $MeCbi^+$, including one attempt where the length of the Sephadex purification column was

(44) It should be noted that the ~70% purity we report was measured without the ion-exchange, FPLC step specified in the literature $MeCbi^+$ preparation.[38] However, in our analysis of the product using a C18 reversed phase analytical column (see Experimental Section), we could not get baseline separation between the peaks, indicating that HPLC cannot completely purify the cobinamide, at least in our hands. Of interest here is the report that an HPLC purification step was not necessary in the $CNCbi^+$ trifluoromethane sulfonic acid preparation (yielding $CNCbi^+$ 93% pure by HPLC) published earlier.[39] It would be of interest to check the purity of that product, too, by $^1$H NMR. Additionally, initial personal communications with the authors (ref 45) of this synthesis revealed that their synthesis also yielded a $MeCbi^+$ $^1$H NMR of the aromatic region with two peaks (attributed to a $CD_3$-$Cbi^+$ synthesis starting from impure $CD_3Cbl$). However, in a subsequent publication (ref 46), the authors state that their synthesis yields a $^1$H NMR of the aromatic region with one peak if pure MeCbl starting material is used. Note that the MeCbl we used with the trifluoromethane sulfonic acid preparation is of ≥98% purity by $^1$H NMR and HPLC.

(45) Personal communication, L. Marzilli, August 1999. We thank Prof. Marzilli for sharing this information.

(46) Trommel, J. S.; Warnchke, K., Marzilli, L. G. *J. Am. Chem. Soc.* **2001**, *123*, 3358−3366.

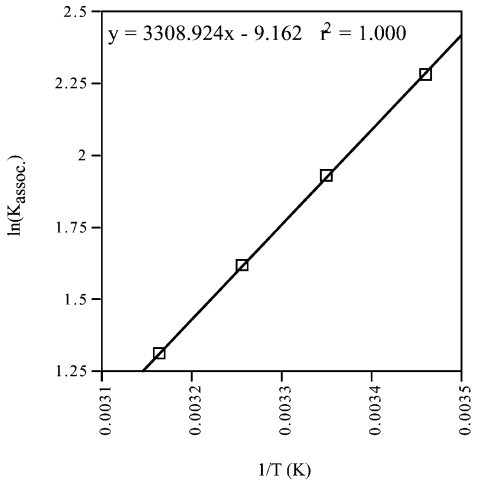
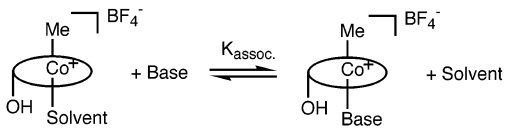



**Figure 3.** $\ln(K_{assoc})$ vs $1/T$ plot of $MeCbi^+$ plus neat *N*-MeIm in ethylene glycol. $\Delta H = -6.57(6)$ kcal/mol; $\Delta S = -18.2(2)$ eu.

doubled, but still using the literature[36] 0.1 M buffer as eluent. Each of these attempts gave $MeCbi^+$ of only ~80% purity. Eventually, we used a 10-fold lower strength buffer to more slowly elute $MeCbi^+$ off the Sephadex column (as was done in the 1987 $AdoCbi^+$ preparation[37]), resulting in $MeCbi^+$ of increased purity, ≥95% pure by both $^1$H NMR (Figure S2, Supporting Information) and HPLC (Figure S3, Supporting Information). Passing the purified cobinamide through an anion-exchange column established the counteranion to be $BF_4^-$, a weakly coordinating anion[47] suitable for our axial-base binding studies. Worth noting in the above is that our use of $^1$H NMR allowed us to quickly and quantitatively measure the purity of the cobinamide, a technique that is faster, direct, and therefore more definitive than either HPLC or UV−visible spectroscopy.[43]

**Methylcobinamide plus Exogenous Base Thermodynamic Parameters.** Titrating $MeCbi^+ \cdot BF_4^-$ with exogenous, coordinating axial-base results in a well-precedented solution color change from yellow to red, indicating conversion of the base-free ($\lambda_{max}$ = 462 nm) to the axial-base-on ($\lambda_{max}$ = 520 nm) state of the cobinamide (eq 4), Figure 2. Clean isosbestic points at 330, 380, and 500 nm are seen, consistent with only two absorbing species present, as desired. Analyzing this data at 520 nm by eq 5 derived previously[32] yields the $K_{assoc}$ values given in Table 2. The equilibrium measurements were then performed over a temperature range of 16−43 °C, and the resulting $\ln(K_{assoc})$ vs $1/T$ plot, Figure 3, yields the $\Delta H$ and $\Delta S$ values found in Table 2 (see also Figures S4−S42, Supporting Information).




(4)

$$\mathrm{Abs} = \frac{-(\mathrm{Abs} - \mathrm{Abs}_0)}{[\mathrm{base}]_0}\left(\frac{1}{K_{assoc}}\right) + \mathrm{Abs}_\infty \qquad (5)$$

The $K_{assoc}$, $\Delta H$, and $\Delta S$ results will first be discussed for each individual base in ethylene glycol solution, then in comparison between ethylene glycol and aqueous solutions, and finally in comparison to the thermodynamic studies done for $AdoCbi^+$ in ethylene glycol.[32,34,35] The $K_{assoc}$ values for *N*-MeIm binding to $MeCbi^+$ are almost equivalent to those for pyridine ($K_{assoc}$(*N*-MeIm) = 6.9(2) $M^{-1}$ vs $K_{assoc}$(py) = 6.2(4) $M^{-1}$); the thermodynamic parameters are within experimental error of each other as well (*N*-MeIm, $\Delta H = -6.57(6)$ kcal/mol, $\Delta S = -18.2(2)$ eu; py, $\Delta H = -6.2(6)$ kcal/mol, $\Delta S = -17(2)$ eu). This was an unpredicted result: for $AdoCbi^+ \cdot BF_4^-$, *N*-MeIm exhibits a lower $K_{assoc}$ value, but stronger $\Delta H$ value, when compared to pyridine.[34] Hence, it will be of special interest to see if imidazole bases react the same as, or differently from, pyridine bases when the reactions of $MeCbi^+$ with $RS^-$, for example, are studied, and in the presence of exogenous bases of different steric and electronic properties.[32−35] Of note here is that the range of p$K_a$ values available from pyridine bases provides a greater flexibility than is available with at least commercial imidazole bases.

The strongly electron-donating base 4-$Me_2$Npy (p$K_a$ = 9.7) yielded the expected greater $K_{assoc}$, a more favorable $\Delta H$, and a correspondingly more negative (unfavorable) $\Delta S$ than the other bases studied. This result is unexceptional, and is precedented both in studies of $MeCbi^+$ [42] in aqueous solution and in studies of $AdoCbi^+$ in ethylene glycol.[32] It should be noted that a linear correlation between base p$K_a$ and $K_{assoc}$ value is not observed in the present study, nor in the $AdoCbi^+$ study.[32]

As expected,[35] the sterically hindered bases 2-Mepy and 2,6-$Me_2$py show no increase in the UV−visible spectrum at 520 nm when added to the $MeCbi^+$ solution at concentrations up to [base] = 1.4 M, Figures S33 and S34 of the Supporting Information. As before, the steric bulk of the base prevents detectable coordination in the ground state.[35] Assuming that one could easily detect 10% binding by these bulky bases to $MeCbi^+$ (as can be detected for nonbulky bases such as *N*-MeIm and py), one can calculate an upper limit of $K_{assoc}$ = 0.07 $M^{-1}$ for these bases at 25 °C. Note that even these as-obtained commercial bulky bases, plus any impurities they may contain,[46] do not exhibit any detectable UV−visible detectable binding to $MeCbi^+$.

We also attempted to measure the $K_{assoc}$ for two bases expected to yield low $K_{assoc}$ values: the weakly basic 4-CNpy (p$K_a$ = 1.9) and the sterically bulky 1,2-$Me_2$Im, Figures S35−S37 of the Supporting Information. These bases exhibited a weaker interaction with $MeCbi^+$ in comparison to the other bases studied, with small, but repeatable, absorbance increases at 520 nm, but without clean isosbestic points. In addition, the data from the spectra do not result in a linear fit to eq 5, so that $K_{assoc}$ cannot be determined. In addition, the titrations with 1,2-$Me_2$Im were repeated with purified 1,2-$Me_2$Im[46] with equivalent qualitiative results. It should be noted that the *total* absorbance change for these at best weakly binding bases, $\Delta Abs_{max} < 0.05$ AU, is less than the *smallest* absorbance change detected for any of the other nitrogenous bases that were shown to bind to $MeCbi^+$,

(47) Strauss, S. *Chem. Rev.* **1993**, *93*, 927.

$\Delta Abs_{min} = 0.13$ (i.e., after the smallest aliquot of base is added). Hence, any axial-base binding that may be occurring with these bases is not quantifiable.[50]

**Comparison of $MeCbi^+$ Thermodynamic Parameters for Ethylene Glycol and Aqueous Solutions.** It is useful to compare the thermodynamic parameters for py in ethylene glycol with those for py in water, the only base studied herein with previously published $\Delta H$ and $\Delta S$ parameters.[40−42] The $K_{assoc}$ value determined herein for py binding to $MeCbi^+$ in ethylene glycol ($K_{assoc}$(ethylene glycol, py) = 6.2(4) $M^{-1}$) is within the range of values reported for aqueous solvent systems ($K_{assoc}$($H_2O$, py) = 6−9 $M^{-1}$, Table 1;[40−42] $K_{assoc}$(pH=7.2 buffer, py) = 6.9(1) $M^{-1}$, Table 2). The thermodynamic studies reveal a $\Delta H$(ethylene glycol) of −6.2(6) kcal/mol, 2−3 kcal/mol more favorable than the aqueous values of $\Delta H$($H_2O$) = −3.8(2) kcal/mol[42] and $\Delta H$(buffer) = −3.0(5) kcal/mol, and a compensatingly less favorable entropy measurement, $\Delta S$(ethylene glycol) = −17-(2) eu compared to $\Delta S$($H_2O$) = −8.8(5) eu[42] and $\Delta S$(buffer) = −7(2) eu. Since the Co−base bond energy should be be nearly invariant in ethylene glycol vs water, the $\Delta\Delta H$ = 2.4(6) kcal/mol may well be reflecting a weaker solvent−Co bond for ethylene glycol (estimated as ∼8 kcal/mol[34]) as compared to water, resulting in a greater $\Delta H$ of binding, eq 4. The entropic difference probably also contains a contribution from the Co−solvent bond, but its detailed interpretation is less obvious, since the individual entropic contributions from the $MeCbi^+$·solvent species vs the solvent itself are not known.

The thermodynamic parameters for *N*-MeIm follow a similar trend. The $K_{assoc}$ value for *N*-MeIm in both $H_2O$ and buffered solution (5 $M^{-1}$, Table 1,[40] and 4.9(2) $M^{-1}$, Table 2, respectively) are slightly lower than, but in the same general range as, the value measured in ethylene glycol (6.9(2) $M^{-1}$, Table 2). In addition, the $\Delta H$(ethylene glycol) = −6.9(2) kcal/mol value is somewhat more favorable than the values for a buffered aqueous solution ($\Delta H$(buffer) = −3.1(2) kcal/mol), and the $\Delta S$(ethylene glycol) = −18.2(2) eu is compensatingly less favorable than the value for the buffered aqueous solution, $\Delta S$(buffer) = −7.3(6) eu. In short, the same general trends are seen for *N*-MeIm as were seen for py. The thermodynamic values generated for py and *N*-MeIm in buffered aqueous solutions are of special interest since they are being used in conjunction with kinetic work with MetH(2−649), a fragment of the methionine synthase holoenzyme.[48]

**Comparison of Thermodynamic Parameters for Axial-Base Binding to $MeCbi^+$ vs $AdoCbi^+$.** The comparisons available in Table 3 reveal that the $K_{assoc}$ values for all the bases studied with $MeCbi^+$ are greater than those for the same bases plus $AdoCbi^+$.[32,34] This must be due to the change in $\beta$ ligand from methyl to the bulkier adenosyl, consistent with precedented steric trans influences of the $\beta$-alkyl ligand on the binding of α nitrogenous bases.[49] Comparing the enthalpy of binding for py and 4-$Me_2$Npy binding to $MeCbi^+$ vs $AdoCbi^+$, one finds that the $\Delta H$ values for both bases are more favorable for $MeCbi^+$ than $AdoCbi^+$, as one expects for a shorter, and stronger, Co−N(base) bond in the [MeCbi·base]$^+$ case, Table 3. These results are also consistent with the fact that the appended 5,6-dimethylbenzimidazole base binds more tightly to MeCbl ($pK_{base-off}$ of the conjugate acid of appended bzm = 2.89)[51] than AdoCbl ($pK_{base-off}$ = 3.67).[52]

**Table 3.** Comparison of Thermodynamic Parameters for $MeCbi^+$ and $AdoCbi^+$ plus the Axial Bases Which Yielded Detectable Binding (in Ethylene Glycol; 25 °C)

| base | $MeCbi^+$ [a] | $AdoCbi^+$ |
|---|---|---|
| | py ($pK_a$ = 5.29) | |
| $K_{assoc}$ | 6.2 ± 0.4 | 1.0 ± 0.2[b] |
| $\Delta H$ | −6.2 ± 0.6 | −3.3 ± 0.4[b] |
| $\Delta S$ | −17 ± 2 | −11 ± 1[b] |
| | *N*-MeIm ($pK_a$ = 7.33) | |
| $K_{assoc}$ | 6.9 ± 0.2 | 0.5 ± 0.1[c] |
| $\Delta H$ | −6.57 ± 0.1 | −7.8 ± 0.4[c] |
| $\Delta S$ | −18.2 ± 0.2 | −28 ± 1[c] |
| | 4-$Me_2$Npy (pKa = 9.7) | |
| $K_{assoc}$ | 18.0 ± 0.3 | 2.5 ± 0.1[b] |
| $\Delta H$ | −7.4 ± 0.5 | −6.5 ± 1.0[b] |
| $\Delta S$ | −30 ± 2 | −20 ± 3[b] |

[a] This work. [b] Reference 32. [c] Reference 34.

*However*, comparing the enthalpies of binding *for N-MeIm*, Table 3, one finds that $\Delta H$ for $MeCbi^+$ is actually 1.2(4) kcal/mol *less* favorable for the electron-donating Me moiety than for the electron-withdrawing Ado moiety in $AdoCbi^+$: specifically, $\Delta H$ = −6.57(10) kcal/mol for $MeCbi^+$ vs −7.8(4) kcal/mol for $AdoCbi^+$;[35] the entropy is a compensatingly less negative −18.2(2) eu for $MeCbi^+$ than the −28-(1) eu for $AdoCbi^+$, Table 3. *N*-MeIm has previously been noted to exhibit an unusually strong $\Delta H$ value when compared to the pyridine ligands studied; the earlier $AdoCbi^+$ results[34,35] were explained by invoking $\sigma$ and $\pi$ orbital effects that are known to differ significantly between imidazole and pyridine ligands.[53−56] It appears that the electronic difference between a Me vs Ado trans ligand may be manifesting itself in different orbital interactions (less favorable *N*-MeIm $\sigma$ or

(48) Dorweiler, J. S.; Finke, R. G.; Matthews, R. G. Manuscript in progress; to be submitted to *Biochemistry*.

(49) Pratt, J. M. In *Chemistry and Biochemistry of $B_{12}$*; Banerjee, R., Ed.; John Wiley & Sons: New York, 1999; Chapter 5.

(50) We considered the possibility that the sterically bulky 1,2-$Me_2$Im base could in principle be operating by deprotonating ethylene glycol; the resulting alkoxide could then coordinate to $MeCbi^+$. One can calculate from the $pK_a$ values of 1,2-$Me_2$Im ($pK_a$ = 7.85) and ethylene glycol ($pK_a$ = 14.8) that 3.2 × $10^{-4}$ M to 1.2 × $10^{-3}$ M alkoxide is produced from 0.05−0.7 M 1,2-$Me_2$Im (the concentrations used in the $MeCbi^+$ titrations). This alkoxide binding hypothesis for the observed spectral changes was tested experimentally by adding 4.8 × $10^{-3}$ M Proton Sponge to a solution of 1 × $10^{-4}$ M $MeCbi^+$ in ethylene glycol at 25 °C (this amount of Proton Sponge generates 4.8 × $10^{-3}$ M alkoxide, 4 times the maximum amount generated in the 1,2-$Me_2$Im titration). No change from the original base-free $MeCbi^+$ UV−visible spectrum was observed after adding the Proton Sponge; hence, this alkoxide binding possibility can be ruled out.

(51) Brown, K. L.; Hakimi, J. M.; Nuss, D. M.; Montejano, Y. D.; Jacobsen, D. W. *Inorg. Chem.* **1984**, *23*, 1463−1471.

(52) Brown, K. L.; Hakimi, J. M.; Jacobsen, D. W. *J. Am. Chem. Soc.* **1984**, *106*, 7894−7899.

(53) Rohmer, M.-M.; Strich, A.; Veillard, A. *Theor. Chim. Acta* **1984**, *65*, 219−231.

(54) Al-Jaff, G.; Silver, J.; Wilson, M. T. *Inorg. Chim. Acta* **1990**, *176*, 307−316.

(55) Scheidt, W. R.; Chipman, D. M. *J. Am. Chem. Soc.* **1986**, *108*, 1163−1167.

(56) Asperger, S.; Cetina-Cizmek, B. *Croat. Chem. Acta* **1996**, *69*, 1305−1328.

$\pi$ effects in MeCbi·*N*-MeIm$^+$, or possibly an increase in destabilizing d$\pi$−p$\pi$, filled−filled orbital interactions[53]), leading to the unusual thermodynamic parameters; at least this is one consistent, precedented[34,35,53−56] explanation. Detailed MO calculations will be required to try to understand these [MeCbi·*N*-MeIm]$^+$ vs [AdoCbi·*N*-MeIm]$^+$ results in greater detail, although these appear to be a bit beyond the current capabilities of methods such as density functional theory (DFT). Whatever the exact explanation proves to be, the *interesting and noteworthy result is that the base that Nature has chosen (histidine and its model congener, N-MeIm) are somewhat unusual*. An entatic active site,[57] that is, an unusual metalloprotein active site poised for catalysis, is one descriptor that comes to mind.

## Conclusions

A synthesis and improved purification leading to 95% pure MeCbi$^+$·$BF_4^-$ is now available. A $K_{assoc}$, $\Delta H$, and $\Delta S$ study for a series of exogenous, nitrogenous bases binding to MeCbi$^+$ has also been completed, the necessary prerequisite to further chemical precedent studies of MeCbi$^+$·base in the methionine synthase elementary reactions shown in eqs 1−3. The results show the following: First, they show that the bases studied have larger $K_{assoc}$ values (where measurable) when binding to MeCbi$^+$ than when binding to AdoCbi$^+$ under analogous conditions. Second, comparison of the MeCbi$^+$ binding parameters of py to those for *N*-MeIm reveal that these two bases bind similarly, within experimental error, to MeCbi$^+$, contrary to what was seen earlier with AdoCbi$^+$.[32] Third, the *pyridine* bases follow the expected trends with the base with the highest p$K_a$ of those studied, 4-$Me_2$Npy, exhibiting the highest $K_{assoc}$ value and base with the lowest p$K_a$, py, exhibiting the lowest detectable $K_{assoc}$ value. Fourth, there is no detectable binding ($K_{assoc} = 0.07$ $M^{-1}$) for the sterically bulky bases 2-Mepy or 2,6-$Me_2$py with MeCbi$^+$. Finally, *N*-MeIm, a base that is a close congener to the imidazole base provided by the biological His759 residue in methionine synthase, exhibits unusual $\Delta H$ and $\Delta S$ values for the formation of MeCbi$^+$·*N*-MeIm, results interpreted as consistent with competing $\sigma$ and $\pi$ effects for imidazole[34,35] bases binding to alkylcobinamides.

(57) Williams, R. J. P. *Eur. J. Biochem.* **1993**, *234*, 363−381 and references therein.

**Acknowledgment.** Support provided to J.S.D. by an NIH postdoctoral fellowship, Grant HL10173, is gratefully acknowledged. We thank K. Doll for helpful discussions, especially regarding the MeCbl plus trifluoromethane sulfonic acid synthesis.

**Note Added in Proof:** In a final attempt to understand why the MeCbl plus trifluoromethane sulfonic acid synthesis failed in our hands, two of us (R.G.F., with assistance from K. Doll) noticed that the choice of *overnight* reaction times chosen by another of us (J.S.D.) contrasts, significantly, with the literature treatment of MeCbl for "7−10 days"[38] with trifluoromethane sulfonic acid. HPLC studies (Alltech C18 Versapack column, isocratic, 1 mL/min, 30% $CH_3CN$/70% pH 4.5 acetate buffer) of our reaction after 5 days did show that two major and three minor product peaks are present, along with a bit of remaining starting material. This leaves little doubt that the FPLC purification step (and probably longer reaction times) are crucial to the success of the literature MeCbl plus trifluoromethane sulfonic acid synthesis,[38] just as column chromatography is crucial to the present MeCbl plus $Ce(OH)_3$ synthesis, vide infra.

Of interest here is that Brown and co-workers used a much shorter reaction time, "24 hrs at room temperature", for NCCbl plus trifluoromethane sulfonic acid.[39] The unexpected, approaching 10-fold differences between the times required for these two syntheses is surprising and not understood, especially since MeCbl and NCCbl should both be primarily in the protonated, base-off form. Understanding these differences (e.g., possibly $Co^{III}CblH^+$ or HCN catalysis?) may yield insights crucial to shortening the time for a more convenient MeCbl plus trifluoromethane sulfonic acid synthesis.

**Supporting Information Available:** Complete synthetic details of the two preparations of MeCbi$^+$; $^1$H NMR spectrum and HPLC trace of MeCbi$^+$ following a purification using 0.1 M acetate buffer and 0.01 M phosphate buffer; UV−visible spectra of MeCbi$^+$ plus exogenous bases; and the data analysis used to obtain the $K_{assoc}$, $\Delta H$, and $\Delta S$ values. This material is available free of charge via the Internet at http://pubs.acs.org.

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