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The Electrochemistry of 5-Hydroxybenzimidazolylcobamide

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Methanogenic archaea typically contain 5-hydroxybenzimidazolylcobamide (cba-HBI) as the prosthetic group of a number of methyltransferases involved in their central metabolic pathways. In this paper the (acidic) dissociation constants and standard oxidation–reduction potentials of the Co^{3+}/Co^{2+} and Co^{2+}/Co^{1+} couples of isolated aquo-cba-HBI were measured. Comparison of the data to those established for 5,6-dimethylbenzimidazolylcobamide (cobalamin) showed that the 5-hydroxybenzimidazolyl (HBI) nucleotidic base hardly affected the redox potentials. HBI, however, proved to be the weaker ligand, thus favoring "base-off" formation. The implications for the functioning of cba-HBI in biochemical methyl group transfer reactions are discussed. © 1995 Academic Press, Inc.

***Key Words:* 5-hydroxybenzimidazolylcobamide; cobalamin; corrinoid; cyclic voltammetry; redox potential; ligand interaction.**

Corrinoids, cobalt-containing macrocycles (Fig. 1), act as catalysts in distinct types of biochemical reactions like C-C rearrangements (1) and methyl group transfers (2, 3). The reactions take advantage of the particular chemical properties of the corrinoids that are directly related with the redox state of the central cobalt atom (2–4). The cobalt atom may occur in three oxidation states, Co(III), Co(II), and Co(I). In cob(III)amide the cobalt is six-coordinated by four equatorial nitrogens derived from the tetrapyrrole (corrin) macrocycle. An axially bound nucleotide serves as the lower (α-)ligand and the upper (β-)position may be occupied by ligands like water, cyanide, a methyl, or a 5′-deoxyadenosyl group. Pentacoordinated cob(II)amide is an excellent radical trap and in the Co^{2+} redox state the corrinoid is involved in C-C rearrangements (5). The square-planar, four-coordinate cob(I)amide, one of nature's most powerful nucleophiles, acts as a catalyst in methyl group transfers (2–4).

The *N*-heterocyclic base of the nucleotide moiety is usually 5,6-dimethylbenzimidazole (DMBI)[2] (5, 6). Corrinoids present in methanogenic archaea typically contain 5-hydroxybenzimidazole (HBI) as the nitrogenous base (7). In these organisms 5-hydroxybenzimidazolylcobamide (cba-HBI; "Factor III") is the prosthetic group of a number of methyltransferases involved in the central methanogenic pathways from hydrogen and CO_2 (8, 9), methanol (10), or acetate (11, 12). Substitution of DMBI for HBI will likely affect the electrochemical properties of the corrinoid and as the consequence its catalytic action. These properties are well documented for 5,6-dimethylbenzimidazolylcobamide (cobalamin, cba-DMBI) (13–17). Since no such information was available for cba-HBI, we studied its electrochemistry. The results are discussed in view of its role in biochemical methyl group transfer.

EXPERIMENTAL PROCEDURES

Materials. Cyclohexylaminoethanesulfonic acid, 2-[tris(hydroxymethyl)-methylamino]ethanesulfonic acid, 3-(cyclohexylamino)-1-propanesulfonic acid, and aquo-cobalamin were purchased from Sigma Chemical Co. (St. Louis, MO). DEAE–Sepharose-Cl-6B was from Pharmacia LKB Biotechnology A. B. (Uppsala, Sweden). Octadecyl C_{18} was purchased from J. T. Baker (Phillipsburg, NJ) and Fractogel EMD SO_3-650 (M) was obtained from Merck–Schuchardt A. G. (Darmstadt, Germany). Sep-Pak C_{18} cartridges were from Waters Associates (Milford, MA). Tetrabutylammonium *p*-toluenesulfonate was prepared by neutralization of a 1.2 M aqueous solution of tetrabutylammonium hydroxide (Fluka Chemie AG, Buchs, Switzerland) and by *p*-toluenesulfonic acid (BDH Chemicals Ltd., Poole, England). Gasses were supplied by Hoek–Loos (Schiedam, The Netherlands). Buffers (100 mM concentrations) were prepared by adjusting solutions of the following compounds with NaOH to the indicated pH ranges: phosphoric acid (pH 2–2.5 and pH 11.5–12.5), citric acid (pH 3–6.5), 2-[tris(hydroxymethyl)-methylamino]ethanesulfonic acid (pH 7–7.5), Tris (pH 8–9), cyclohexylaminoethanesulfonic acid

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[2] Abbreviations used: DMBI, 5,6-dimethylbenzimidazole; HBI, 5-hydroxybenzimidazole; cba-DMBI, cobalamin; cba-HBI, 5-hydroxybenzimidazolylcobamide; SHE, standard hydrogen electrode.

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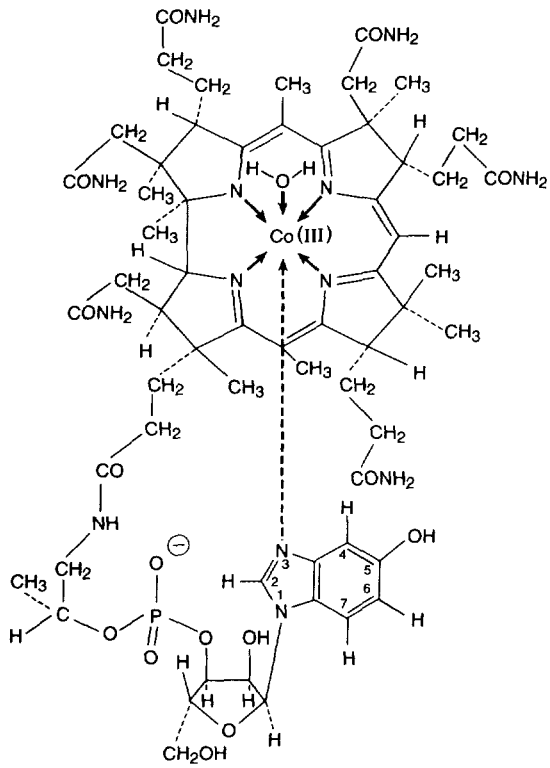


FIG. 1. Structure of Coα-[α-(5-hydroxybenzimidazolyl)]-Coβ-aquocobamide (cba-HBI).

(pH 9.5), and 3-(cyclohexylamino)-1-propanesulfonic acid (pH 10–11). A mixture of HCl and KCl was used as a buffer at pH 1–1.5. Solutions of perchloric acid, acetic acid, and sulfuric acid were used to acquire pH 0.5 and below.

Corrinoid isolation. Cba-HBI was purified from cell-free extract of *Methanosarcina barkeri* strain MS (DSM 800) as the cyano-form. Organisms were grown and extracts prepared as described before (18). Extraction in hot methanol containing 0.01% KCN and subsequent purification employing DEAE–Sepharose Cl-6B, octadecyl C_{18}, and Fractogel EMD SO_3-650 (M) column chromatography proceeded essentially according to Pol *et al.* (19). After each ion chromatography step CN-cba-HBI-containing fractions were combined, concentrated, and desalted with Sep-Pak C_{18} cartridges. For the preparation of the cob(II)amide and of aquo-cob(III)amide, pure CN-cba-HBI was reduced by addition of 25 μl of hydrogenase from *M. barkeri* (0.12 μmol benzylviologen reduced min^{-1} ml^{-1}) (18), followed by incubation under a hydrogen atmosphere for 48 h at 37°C. Thereafter, the cob(II)amide was applied under strict anaerobic conditions to a Sep-Pak C_{18} cartridge. Following thorough washing with distilled water, the corrinoid was eluted from the cartridge with 80% methanol. Aquo-cba-HBI was obtained in 92% yield by exposure of cob(II)amide to air and subsequent purification on a Fractogel EMD SO_3-650 (M) column. The nucleoside of cba-HBI, 5-hydroxy-1-α-ribofuranosylbenzimidazole, was obtained by hydrolysis of CN-cba-HBI with cerous hydroxide and purified as described by Pol (20).

Analytical procedures. Ultraviolet–visible absorption spectra in the above-mentioned buffers were recorded at 22°C using an Hitachi U-3200 spectrophotometer. Direct electrochemistry (cyclic voltammetry) of 20-μl amounts of corrinoid solution (0.1 or 0.3 mM) took place at 22°C under an argon atmosphere using a nitric acid-treated glassy carbon, a platinum, and a saturated calomel three-electrode system. Methodology and microapparatus were as described by Hagen (21). Redox potentials as a function of the pH were determined by diluting (1:1) the corrinoid stock solution with buffer of the desired pH. Reactant adsorption was prevented by the presence of 50 mM tetrabutylammonium *p*-toluenesulfonate (15).

RESULTS

Dissociation Constants of 5-Hydroxybenzimidazolylcobamide in the Different Oxidation States

The pK_a values were derived from the pH-dependent changes in the uv–visible light spectra of aquo-cob(III)amide-HBI and of the cob(II)amide (Table I). The former compound showed three pK_a values. The $pK_a = 7.8 \pm 0.2$ and $pK_a = 9.7 \pm 0.2$ closely agreed the values reported by Pol *et al.* (22) and are assigned to the aquo- and hydroxo-cba-HBI equilibrium and to the deprotonation of the 5-OH group in HBI, respectively. At pH < -1 an additional $pK_a = -1.3 \pm 0.2$ was found. This pK_a value reflects the association/dissociation of the nucleoside with the central cobalt, the so-called "base-on"/"base-off" equilibrium. The pH-dependent uv–visible light spectra of cob(II)amide are shown in Fig. 2. Here we measured two pK_a values (Table I). As above, the high $pK_a = 9.8 \pm 0.4$ was attributed to the ionization of the HBI phenolic group. The lower $pK_a = 3.6 \pm 0.1$ was assigned to the base-on/base-off equilibrium. The spectrophotometric determination of the ionization constants of Co(I)-cba-HBI was hampered by its extreme oxygen sensitivity. Since in this oxidation state the corrinoid is exclusively present in the uncoordinated, base-off state (23), the pK_a values of isolated 5-hydroxy-1-α-ribofuranosylbenzimidazole were considered a good approximation of the pK_a values of the base bound to the cob(I)amide. Two pH-dependent shifts in the uv–visible spectrum of the isolated nucleoside were found showing a $pK_a = 10.1 \pm 0.1$ and a $pK_a = 5.25 \pm 0.1$ which were assigned to the (de)protonation of the 5-OH group and of N(3) of HBI, respectively (6, 20).

Reduction of the Cob(III)amide to Cob(II)amide

Cyclic voltammetry was used for determination of the standard potential of reduction for the Co(III)/Co(II) redox couples of cba-HBI as a function of pH. Reduction of the Co(III) corrinoid was a slow reaction but at a scan rate of 4 mV/s the $n = 1$ electron transfer was fully reversible, as judged from the reproducible cathodic-to-anodic peak potential separation of 64 ± 3 mV (Fig. 3A). A minor distortion around 375 mV is observed in the voltammogram presented in the figure, but this was also found in the absence of corrinoid and therefore ignored. The response was optimal in the presence of 50 mM tetrabutylammonium *p*-toluenesulfonate. The redox potentials were measured over a pH range varying between 2 and 12. Between pH 3.5 and

TABLE I

Ultraviolet–Visible Light Spectral Data of 5-Hydroxybenzimidazolyl-cob(III)amide and -cob(II)amide

Compound	pH	Extinction maxima and coefficients nm ($\epsilon \times mM^{-1} cm^{-1}$)	pK_a values
Cob(III)amide			
H_2O-cba-HBI	−1.6	540(12.0), 512(sh)[a](10.6), 416(5.0), 358(26.0), 276(sh)(24.3)	−1.3 ± 0.2
	6.0	522(8.9), 497(8.4), 410(3.7), 352(27.0), 290(sh)(16.8), 274(18.5), 258(sh)(19.3)	7.8 ± 0.2
HO-cba-HBI	9.0	532(9.7), 510(sh)(9.1), 418(4.2), 357(21.4), 300(sh)(14.8), 280(16.4)	9.7 ± 0.2
	12.0[b]	535(9.9), 510(sh)(9.2), 418(4.4), 356(22.0), 325(18.3), 280(sh)(12.9)	
Cob(II)amide			
Cba-HBI	2.0	470(9.6), 411(sh)(6.6), 316(22.8), 290(21.2), 267(23.1)	3.6 ± 0.1
	7.0	474(9.4), 407(7.9), 312(27.7)	9.8 ± 0.4
	11.5	587(sh)(2.5), 526(sh)(5.2), 476(8.5), 405(6.9), 362(sh)(10.8), 313(25.7)	

[a] sh, shoulder.
[b] These values were taken from Ref. 19 and used to calculate the other ϵ values.

7 a constant $E^0 = 226 \pm 9$ mV vs the standard hydrogen electrode (SHE) was observed. Below pH 3 and between pH 8 and 9 E^0 varied as a function of pH, whereas above pH 10 the redox potential remained constant at $E^0 = 130 \pm 11$ mV vs SHE (Fig. 4).

Reduction of the Cob(II)amide to Cob(I)amide

Measurements of the Co^{2+}/Co^{1+} redox couple used the aquo-cob(III)amide as the starting material. Prior to the voltammetric run, the compound was quantitatively reduced to the Co(II) level at the initial potential of the scan (−150 mV vs SHE) (15). At a scan rate of 50 mV/s the one-electron transfer reaction was fully reversible over the pH range tested, as judged from the cathodic/anodic peak potential separation of 59 ± 2 mV (Fig. 3B). This fast scan rate allowed the determination of the redox potential of the base-on cob(II)amide/cob(I)amide couple without interference of the base-on/base-off reaction, which can precede the actual reduction of Co^{2+} at low scan rates (13, 14). The electrode response was independent of 50 mM tetrabutylammonium *p*-toluenesulfonate. The experiments were conducted at pH values ranging between 2 and 12. The E^0 values thus determined are shown in the lower part of Fig. 4. E^0-stable regions occurred around pH 2–3 ($E^0 = -500 \pm 3$ mV) and between pH 5.5 and 10 ($E^0 = -592 \pm 2$ mV). Above pH 10 a third region may be present with a slightly, but not significantly, lower $E^0 = -598 \pm 11$ mV vs SHE. Below pH 2.5 the voltammogram showed a catalytic character and simultaneously gas bubbles appeared in the sample. This was due to the formation of the cobalt hydride by protonation of cob(I)amide-HBI and its decomposition into the cob(II)amide and molecular hydrogen (13, 24). Because this reaction interfered in the determination of the redox potentials of the Co^{2+}/Co^{1+} couple, no data could be collected at pH values below 2.5.

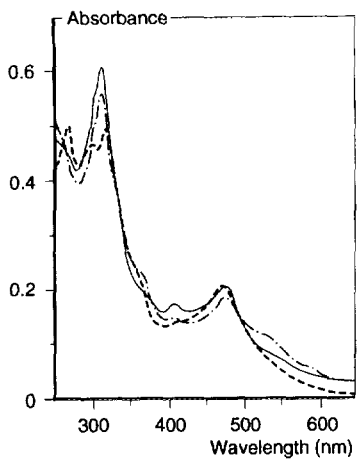


FIG. 2. Ultraviolet–visible light spectra of 0.022 mM 5-hydroxybenzimidazolylcob(II)amide recorded at pH 2.0 (---), pH 7.0 (——), and pH 11.5 (– · –).

DISCUSSION

In this study we measured the acidic dissociation constants of cba-HBI as well as the standard redox potentials as a function of pH of the Co^{3+}/Co^{2+} and the Co^{2+}/Co^{1+} couples. The results are summarized in the E^0–pH diagram shown in Fig. 4. In the figure the voltammetrically determined redox potentials could be fitted into pH-invariant and pH-dependent sections bounded by the spectrophotometrically established pK_a values (13, 14). The diagram provides the information for thermodynamic calculations of all possible reactions. It allows an easy comparison of the electrochemical properties of cba-HBI and those of the ubiquitously occurring cobalamin, or between free and enzyme-bound cba-HBI.

The presence of HBI affects the electrochemistry of cba-HBI in relation to cobalamin in a number of aspects (Table II). The 5-OH group introduces an additional

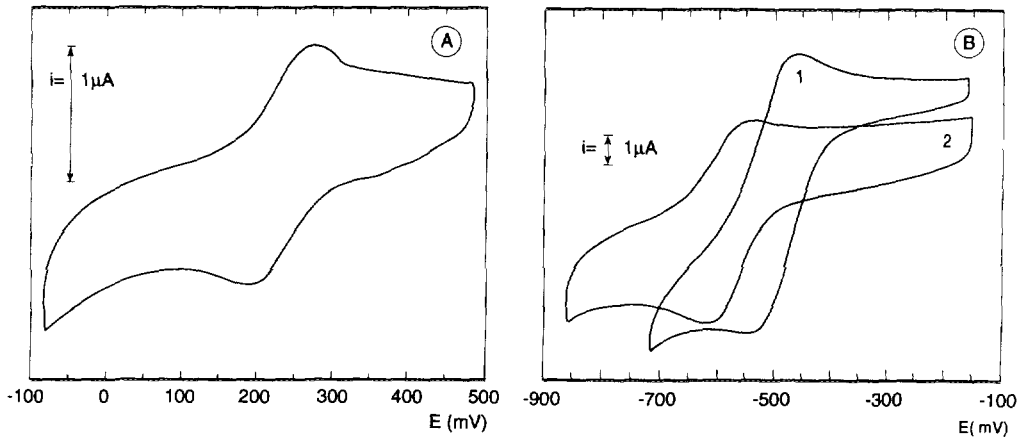


FIG. 3. Cyclic voltammograms of (A) the Co^{3+}/Co^{2+} and (B) the Co^{2+}/Co^{1+} couples of cba-HBI. Experimental conditions: (A) H_2O–cba-HBI (20 μl, 0.1 mM) in 50 mM sodium citrate buffer (pH 6.0) containing 50 mM tetrabutylammonium *p*-toluenesulfonate; potential scan rate, 4 mV/s; temperature, 22°C. (B) Aquo-cba-HBI (20 μl, 0.3 mM) in 50 mM Tris/Cl^- buffer, pH 8.0 (1), or 50 mM sodium citrate buffer, pH 3.0 (2); potential scan rate, 50 mV/s; temperature, 22°C. In Fig. 3B voltammograms 1 and 2 show the response of the "base-on" Co^{2+}/Co^{1+} and "base-off" Co^{2+}/Co^{2+} couple, respectively. The potential axis is defined versus the standard hydrogen electrode.

ionizable function. The pK_a(5-OH) was about constant among the different redox states of the central cobalt atom, indicating that coordination to the corrin nucleus only slightly influences this ionization. However, since the 5-hydroxy function in HBI is a better electron donor than the 5-methyl group in DMBI the protonation constant of the N(3) of the base, as well as the base-on/base-off equilibrium constants, was significantly higher (Table II). Remarkably, the HBI base had only little effect on the redox potentials of the cob(III)amide/cob(II)amide and cob(II)amide/cob(I)amide couples. Reduction of the base-on cba-HBI species took place at values 10–12 mV higher than those of the respective cobalamins. HBI, being the weaker base, thus, somewhat facilitates the reduction of the cobalt ion. As expected, reduction of the base-off species occurred at nearly identical E^0 values, since cba-HBI and cobalamin contain structurally the same corrin nucleus.

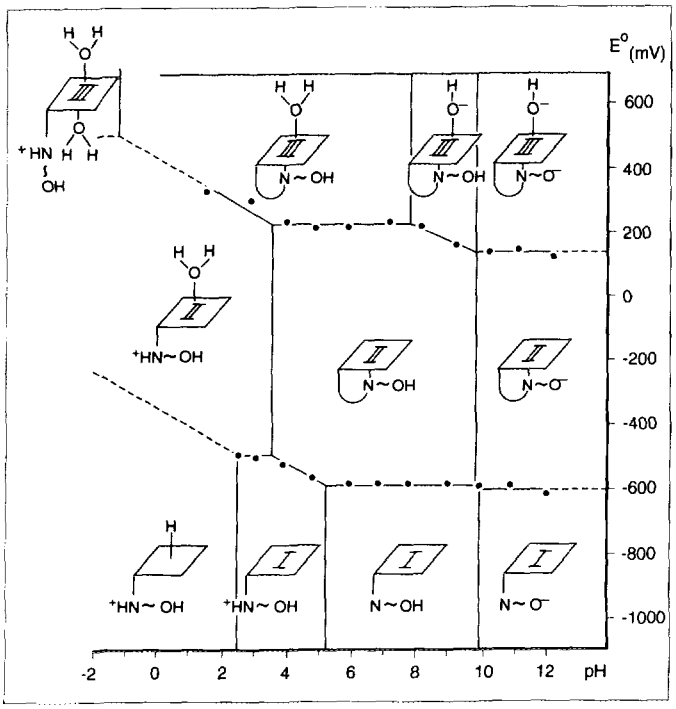


FIG. 4. Standard redox potential–pH diagram of the Co^{3+}/Co^{2+} and Co^{2+}/Co^{1+} couples of cba-HBI at 22°C. The corrin moiety of cba-HBI is schematically represented by the square plane with the formal redox state of the cobalt indicated by roman numbers; the N-(3) and 5-hydroxy group of the HBI-base are illustrated by N~OH. Dashed lines represent areas of theoretical redox potentials assuming a $\Delta E^0/\Delta pH = -59$ mV/pH.

In view of the relatively subtle differences in the chemical properties with respect to cobalamin, one now might ask which advantage methanogenic bacteria could derive from the specific presence of cba-HBI in their metabolism. Indeed, when growth medium of *Methanobacterium thermoautotrophicum* was supplemented with DMBI, the compound was incorporated as cobalamin, without a notable effect on the growth rates and methanogenic capacities (26). Methanogenic bacteria, thus, could simply lack the possibilities of DMBI biosynthesis. From a chemical point of view, a more positive answer cannot be ruled out. In methanogens and other anaerobic microorganisms, cba-HBI is the prosthetic group of a number of methyltransferases involved in the central metabolism (8–12, 27). Superreduced cob(I)amide is the catalytically active species and methyl-cob(III)amide is an intermediate in the subsequent methylation and demethylation steps (2–4, 10, 18, 28). In the chemical and biochemical transmethyla-

TABLE II

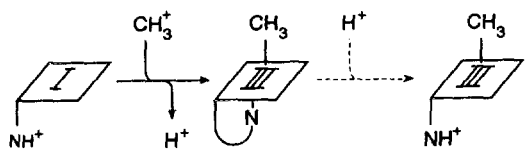
Comparison of the Electrochemical Properties between 5-Hydroxybenzimidazolylcobamides and Cobalamins

Compound	pK_a values		E^0 values (mV)			Refs.
	pK_a(N-3)/ "Base-on/off"	pK_a(5-OH)	"Base-off"	"Base-on"	Hydroxo	
Co(III) (→ Co(II))						
Cba-HBI	−1.3 ± 0.2	9.7 ± 0.2	516[a]	226 ± 9	130 ± 11	This paper
Cobalamin	−2.4		515	208; 216 ± 8[b]	208 - 59/pH	11, 13, 14 16, 25
Co(II) (→ Co(I))						
Cba-HBI	3.6 ± 0.1	9.8 ± 0.4	−500 ± 3	−592 ± 2	−600 ± 4	This paper
Cobalamin	2.7		−494	−604	−604	13, 14
Co(I)[c]						
Cba-HBI	5.25 ± 0.1	10.1 ± 0.1				This paper
Cobalamin	4.7					11

[a] The value was calculated by extrapolation of the data shown in Fig. 4.
[b] Measured by cyclic voltammetry (P. Daas, unpublished results).
[c] The pK_a values are those measured for the isolated nucleosides.

tions, base-on/base-off interconversions are of key importance (Scheme 1). Compared to cobalamin, cba-HBI apparently favors base dissociation, *without* necessarily negatively effecting base coordination. Methylation of (base-off) cob(I)amide yielding (base-on) methyl-cob(III)amide takes advantage of nucleotide coordination, which stabilizes the Co–C bond formed (4, 29). This so-called thermodynamic *trans*-effect is determined by the difference in pK_a values between the N(3) of the uncoordinated base and the base-on/base-off equilibrium constant of the methylated cobamide. Despite the individual differences in the constants (22, 30), nearly identical ΔpK_a values are found for cba-HBI and cobalamin methylation (Scheme 1). Conversely, the *trans*-effect will stabilize the Co–C bond against heterolytic cleavage during the methyl group displacement, and the preceding formation of the base-off methyl derivative will facilitate Co–C bond cleavage (4, 29). Due to the higher pK_a, base dissociation in methyl-cba-HBI is clearly favored with respect to methylcobalamin.

From the above discussion it could be concluded that cba-HBI seems to be better adapted to a specific role in transmethylation reactions. However, it should be stressed that the considerations apply to aqueous solutions of the corrinoids. Electrochemical and base-off/base-on properties may be altered within a protein environment. For few enzymes that contain HBI or the structurally related 5-methoxybenzimidazole as the nitrogenous base, oxidation–reduction potentials were actually measured (11, 12, 31). In the enzymes, cob(II)amide was specifically present either in the base-on ($E^0(Co^{2+}/Co^{1+}) = -630$ mV) (31) or thermodynamically unfavorable base-off state ($E^0 = -486$ and -504 mV) (11, 31), thus showing standard redox potentials that were close to those measured here for the respective free cba-HBI derivatives (Table II). However, a cba-HBI-containing enzyme isolated from *M. barkeri* involved in acetyl-CoA cleavage and having an as yet undefined base configuration had a significantly higher $E^0(Co^{2+}/Co^{1+}) = -426$ mV vs SHE (12). Our data pre-



	pK_a (N-3)	pK_a (on/off)	ΔpK_a	(ΔG_{stabil} kJ/mol)
cba-HBI	5.25	3.3	1.95	(-11.1)
cobalamin	4.7	2.7	2.0	(-11.4)

SCHEME 1

dict that this may be achieved by keeping the corrinoid in the base-off state. Substitution of water by a less nucleophilic fifth ligand or a weakening of the aquo–cobalt bond by hydrogen bridges then would contribute to a further increase of the redox potential.

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