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Cobinamide production of hydrogen in a homogeneous aqueous photochemical system, and assembly and photoreduction in a $(\beta\alpha)_8$ protein

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Abstract

Components of a protein-integrated, earth-abundant metal-macrocycle catalyst, purposed for hydrogen (H_2) production from aqueous protons under green conditions, are characterized. The cobalt (Co) -corrin complex, cobinamide, is demonstrated to produce H_2 ($4.4 \pm 1.8 \times 10^{-3}$ turnover number per hour) in a homogeneous, photosensitizer/sacrificial electron donor system, in pure water at neutral pH. Turnover is proposed to be limited by the relatively low population of the gateway Co(III) hydride species. A heterolytic mechanism for H_2 production from the Co(II) hydride is proposed. Two essential requirements for assembly of a functional protein catalyst complex are demonstrated, for interaction of cobinamide with the $(\beta\alpha)_8$, TIM-barrel protein, EutB, from the adenosylcobalamin-dependent ethanolamine ammonia-lyase from *Salmonella typhimurium*: (1) High affinity equilibrium binding of the cobinamide (dissociation constant, 2.1×10^{-7} M), and (2) in situ photoreduction of the cobinamide-protein complex to the Co(I) state. Molecular modeling of the cobinamide-EutB interaction shows that these features arise from specific hydrogen bond and apolar interactions of the protein with the alkylamide substituents and ring of the corrin, and accessibility of the binding site to solution. The results establish cobinamide-EutB as a platform for design and engineering of a robust H_2 production metallo-catalyst, that operates under green conditions, and utilizes advantages of protein as a tunable medium and material support.

Keywords

Green energy; Hydrogen; Cobinamide; Cobalt complex; Homogeneous catalysis

Introduction

The parallel trends of increasing global energy demand, and finite availability and adverse environmental impacts of fossil fuel energy sources, have accelerated the search for sustainable, “green” energy [1-4]. An attractive target is the production of hydrogen (H_2) from water protons [5, 6], according to the following reaction:

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Electronic Supplementary Material Table S1, extinction coefficients of Cbi redox states; Table S2, solid state structure parameters for $Co(dmgH)_2pyCl$; Table S3, extinction coefficients of cobaloxime redox states; Table S4, hydrogen production parameters for $Co(dmgH)_2pyCl$ in 1:1 v/v AcCN/water; Figure S1, absorption spectra of CN_2Cbi ; Figure S2, absorption spectra of Cbi redox states; Figure S3, solid state structure of $Co(III)(dmgH)_2pyCl$; Figure S4, absorption spectra of cobaloxime redox states; Figure S5, dependence of hydrogen production on irradiation time for $Co(dmgH)_2pyCl$ in water and 1:1 v/v AcCN/water; Figure S6, dependence of EY absorption on irradiation time in the hydrogen production system; Figure S7, representation of specific Cbi-EutB interactions; Figure S8, comparison of Cbi(I) absorption spectra in solution and in the presence of EutB.

The development of molecular catalysts for H₂ production has evolved to a focus on coordination complexes of earth-abundant metals, where Co, Ni, or Fe serve as the terminal sites of electron delivery and proton binding and combination [7-9]. The H₂ production performance features and mechanisms of different metallo-catalysts have been studied by using electrochemical (heterogeneous) and photochemical (homogeneous) reduction systems, with added acids, or the solvent, as proton source. Modifications of the ligand structure have been used to tune the metal redox and p*K*_a values, and to create proton binding and relay sites [10-13], to enhance catalyst performance and explore mechanism. A protein binding site offers an expanded repertoire for microscopic manipulation of catalytic performance [14]. Indeed, hydrogenase enzymes are prodigious H₂ production catalysts, albeit cost-limited and fragile for device applications [15, 16]. Recapitulation of the hydrogenase diiron site in a cytochrome *c* scaffold [17], and introduction of non-native Co complexes into the Photosystem I protein [18], represent recent photochemical approaches, that utilize protein machinery for H₂ production. Proteins can also oligomerize, to form novel material supports for the catalyst, and can integrate into higher-order biological structures [19-21]. Our long-term aim is to create protein-bound, site-specific Co macrocycle catalysts for fuels formation, that display photo- and chemical stability, and operate under biological conditions of pure water solution (at pH~7), and ambient temperature (*T*) and pressure (*p*). Here, we report H₂ production in water by the corrin-based macrocyclic Co complex, cobinamide (Cbi; Scheme 1), in a visible-light photosensitizer/sacrificial electron donor system [22, 23], and characterize the assembly and photoreduction of Cbi in a selected protein scaffold, the EutB protein of adenosylcobalamin-dependent ethanolamine ammonia-lyase [24, 25].

Co complexes have been studied as molecular H₂ production catalysts for over three decades [7-9, 26-30]. Sequential reduction of the Co(III) and Co(II) states leads to formation of Co(I) and formal Co(0), which are the active states in H₂ formation. The protonated Co(I) state, or Co(III) hydride [Co(III)–H], is the gateway species for H₂ production, either through Co(III)–H directly, or through the one-electron-reduced Co(II)–H species [7-9, 26-30]. Early photochemical studies used relatively saturated tetraaza equatorial ligands, such as the 1,4,5,8-tetrazacyclotetradecane (cyclam) complexes [electrochemical midpoint potentials, *E*_m[Co(II)/Co(I)] range, <–1.1 V [measured in acetonitrile (AcCN) solvent; vs standard hydrogen electrode, SHE] [31, 32], with relatively short-wavelength (ultraviolet, UV) absorbing photosensitizers, in organic solvents [28]. Cobalt dimethylglyoxime (dmgH), or cobaloxime, complexes, which exhibit raised *E*_m[Co(II)/Co(I)] values (typical range, –0.7 – –0.9 V vs SHE) and enhanced solubility in polar solvents, have been extensively studied as both electrocatalysts [27, 33] and photocatalysts [27, 29] for H₂ production. The recent green thrust in Co molecular catalyst development [7-9] has led to the use of water as both substrate and solvent, and to higher *E*_m[Co(II)/Co(I)] values, because higher *E*_m complexes generally have lower overpotentials for electrochemical reduction, and photoreduction can be accomplished by photosensitizers with longer wavelength (visible) absorption. Advances for cobaloxime catalysts include equatorial ligand annulation by difluoroboryl (BF₂) and diimine moieties, to stabilize the complex against decomposition, which also raises *E*_m[Co(II)/Co(I)] to –0.5 to –0.3 V (AcCN, vs SHE) [34-36]. A relatively high rate of H₂ production was attained with visible irradiation, by using Co(dmgh)₂pyCl [py, pyridine; Cl, chloride; *E*_m[Co(II)/Co(I)] = –0.89 V; AcCN vs SHE] [37], a photostable Eosin Y (EY) photosensitizer [38], and water-soluble triethanolamine (TEOA) as sacrificial electron donor in 1:1 v/v AcCN/water solution [22]. The EY/TEOA system was also used to drive H₂ production by Co(dmgh)₂(4-phospho-py)Cl in pure water at neutral pH [23].

Co(dm_g)₂(BF₂)₂ and Co(dp_g)₂(BF₂)₂ (p, phenyl) were optimized for photocatalysis in water by using a related fluorescein-derived photosensitizer, Rose Bengal, and triethylamine (TEA) [39]. In addition to the cobaloxime archetype, Co complexes with different types of polypyridine ligands are effective electrochemical [40–42] and photochemical [41, 43] H₂ production catalysts in aqueous solution. Quantitative comparisons of the various photoreduction systems are not possible, owing to different solution and irradiation conditions. However, a qualitative assessment is that the aqueous systems are characterized by turnover numbers (TON, total mol H₂ produced/mol Co complex) of approximately 30 – 300, over system competency periods of approximately 2 – 24 h.

The EutB subunit (453 residues, 49.4 kDa) of the coenzyme B₁₂ (adenosylcobalamin)-dependent enzyme, ethanolamine ammonia-lyase (EAL), from *Salmonella typhimurium* [44, 45], is a promising candidate for engineering of a Co catalytic center in a soluble protein. The high-resolution X-ray crystallographic structure of EAL from *Escherichia coli* has been reported [24], and a homology model and subunit preparation procedure for *S. typhimurium* EutB have been described [25]. The EutB subunit dimerizes [24], and the dimers form a hexameric, “trimer of dimers” oligomer [24, 25], which is depicted in Figure 1. Each EutB monomer contains one cobalamin binding site. The native function of EutB is to bind the adenosylcobalamin cofactor, which is the source of the primary carbon-based radical, 5'-deoxyadenosyl, that initiates radical-mediated catalysis in EAL. Thus, the protein is naturally engineered to control highly reactive species [46], as detailed spectroscopic and kinetic studies have shown [47–51]. EAL maintains function over a wide range of pH (5.8 – 9.2) [52, 53], which is relevant to formation and manipulation of the Co(III)–H species. The (β_α)₈-TIM barrel fold of EutB is comprised of a central cylindrical β-barrel, that is surrounded by eight α-helices [54]. This structural motif is robust [55], able to protect and stabilize reaction intermediates in the protein [56], and catalyzes a wide variety of natural reactions [52, 57–61]. Residues in the active site region of EutB can be mutated, without significantly destabilizing the protein structure [49].

Cbi was chosen as the H₂ production catalyst for development in the EY/TEOA photosensitizer system, for the following reasons: (a) Cbi has the same corrin macrocycle structure and pendant amide groups as cobalamin, but lacks the dimethylbenzimidazole nucleotide substituent. It is thus predicted to bind with high specificity at the cobalamin binding site in EutB. (b) Cbi is water-soluble and water-stable. (c) The Cbi Co(II)/Co(I) redox couple ($E_m[\text{Cbi(II)/Cbi(I)}] = -0.50 \text{ V; AcCN, vs SHE}$) [62, 63] is thermodynamically competent to drive the production of H₂ in water [$E_m(2\text{H}^+/\text{H}_2) = -0.42 \text{ V at pH 7.0}$]. (d) The E_m value for the Cbi(II)/Cbi(I) couple is accessible by using photo-reductants of moderate strength, under conditions that are not destructive to protein, in pure water. Previous photocatalytic H₂ production and CO₂ reduction studies of Cbi, cobalamin ($E_m[\text{Co(II)/Co(I)}] = -0.61 \text{ V; AcCN, vs SHE}$), and Co-tetraphenyl-porphyrin (Co-TPP; $E_m[\text{Co(II)/Co(I)}] = -0.62 \text{ V; AcCN, vs SHE}$), in 9:1 v/v AcCN/methanol solution with *p*-terphenyl/triethylamine (TEA) as photosensitizer/electron donor, showed that the Co corrins were more efficient catalysts than Co-TPP (approximately 10-fold higher initial rate of H₂ formation, and higher total yield of H₂) [64, 65]. The effect was explained by lower probability of proton attack on the corrin, which is more reduced (6 macrocycle double bonds) than the porphyrin (11 macrocycle double bonds). A similar conclusion was reached for electrocatalyzed haloalkane reduction rates [66]. A corrole (an aromatic form of the corrin, with 11 double bonds in the macrocycle, and phenyl substitution at the three methine positions), Co-*tris*-(5,10,15-pentafluorophenyl)-2,3,7,8,12,13,17,18-octafluorocorrole ($E_m[\text{Co(II)/Co(I)}] = -0.77 \text{ V; AcCN, vs SHE}$), was reported to electrochemically and photochemically catalyze H₂ production in aqueous 0.5 M sulfuric acid solution [67].

Here, we demonstrate H₂ production by Cbi, by using green light ($\lambda=520$ nm) and the established EY/TEOA photoreduction system [22] in pure water solvent, at neutral pH. High affinity binding of Cbi to the EutB protein and in situ formation of Cbi(I) are established. These are essential stages in the development of the protein-integrated, molecular H₂ production catalyst, which uses earth-abundant components and operates in the visible solar radiation range in pure water solution.

Materials and methods

Materials

TEOA, EY, AcCN, ethylenediaminetetraacetic acid (EDTA) and all other chemicals used for synthesis or sample preparation were obtained from commercial sources, and were used without further purification. Lysozyme (from hen egg white) and bovine serum albumin, were purchased from commercial sources and dialyzed in 10 mM potassium phosphate (pH 7.5), prior to use.

Methylcobinamide synthesis

The synthesis of methylcobinamide (MeCbi) was based on the method described by Zou et al [68]. A powder sample of 50 mg of MeCbl was dried inside a vacuum decanter, at 1.3×10^2 Pa, over phosphorus pentoxide (P₂O₅) for 12 h. The anhydrous MeCbi was then dissolved in 0.6 mL of anhydrous trifluoromethanesulfonic acid after being anaerobically transferred to glove box containing argon gas at 1.0×10^5 Pa. The mixture was stirred for 24 h. The solution was added to 100 mL of 1 M dibasic potassium phosphate (K₂HPO₄). An Amberlite XAD-2 column was used to remove the cleaved nucleotide, by washing with water and 10% v/v water/AcCN. The MeCbi was subsequently eluted by using 50% v/v aqueous AcCN. Solvent was removed by rotary evaporation to obtain the dry MeCbi. MeCbi was crystallized as the perchlorate salt. Note: Perchlorate salts may be explosive, and should be handled in small quantities with appropriate precautions.

Molar extinction coefficients for MeCbi in aqueous solution were obtained by conversion of a small amount of the compound to dicyanocobinamide (CN₂Cbi), for which a molar extinction coefficient at 368 nm of $30,400 \text{ M}^{-1} \text{ cm}^{-1}$ has been reported [69]. MeCbi was photolyzed aerobically in solution in the presence of 0.5 M potassium cyanide (KCN), which led to formation of CN₂Cbi. MeCbi was confirmed to have been quantitatively converted to CN₂Cbi by irradiation titration. Figure S1 and Figure S2 show absorption spectra, from which molar extinction coefficients for MeCbi, Cbi(II), and Cbi(I) in water were calculated, at 387 and 465 nm, by using Beer's Law. The values are presented in Table S1.

Co(III)(dmgH)₂pyCl synthesis

Synthesis of Co(III)(dmgH)₂pyCl was based on the previously reported method [70]. Dimethylglyoxime (12.86 grams) was dissolved in 300 ml of hot methanol. Separately, 11.9 g of Co(II) chloride hexahydrate was dissolved in 50 ml of water. The Co solution was added to the dimethylglyoxime methanol solution, to form dichlorocobaloxime. The cooled solution was stirred and bubbled with air for 6 h. The solution turned from red to brown. A saturated aqueous KCl solution (50 ml) was added, and the mixture was allowed to stand overnight. The crystals of chloroaquocobaloxime which formed were collected, and the identity confirmed by using nuclear magnetic resonance (NMR). Exchange of the water ligand with pyridine was accomplished by the addition of pyridine to a solution of chloroaquocobaloxime dissolved in chloroform. Excess pyridine was extracted from the solution by using water. The product was collected by crystallization, and the solid state structure was determined by using X-ray crystallography (Figure S3, Table S2). UV/Visible

extinction coefficients of the Co(III)(dmgH)₂pyCl and Co(II)(dmgH)₂py states were determined, as described above. The spectra are shown in Figure S4 and extinction coefficients are presented in Table S3.

Static absorption spectra

Static absorption spectra were collected for 250–700 nm by using a Shimadzu UV-1601 absorption spectrometer with 0.5 nm wavelength accuracy. Samples were contained in anaerobic quartz cuvettes (path lengths, 0.4 – 1.0 cm) with an airtight septum. UV/Visible absorption spectra of reaction solutions were taken before, during and after completion of the photo-initiated H₂ production reaction involving MeCbi and Co(dmgh)₂pyCl. All measurements were performed at 295 ± 1 K.

Light-driven hydrogen production and measurement by using gas chromatography

Preparation of samples—Solutions of MeCbi and Co(III)(dmgH)₂pyCl at varying concentrations (50 μM to 250 μM), and EY (50 μM), in water or water/AcCN solutions were made anaerobic by wet argon gas bubbling for 10–15 min at 295 K, in an anaerobic quartz cuvette fitted with an air-tight septum. An optimal organic dye photosensitizer concentration window of 40–100 μM was found in previous studies [71, 72]. A positive pressure of argon (1.2 × 10⁵ Pa) was maintained inside the cuvette during subsequent manipulations. Methane gas (100 μl, 99.98%) was injected, using a Hamilton gas-tight syringe, into the cuvette as the calibration standard for GC measurements.

Irradiation of samples—A continuous-wave irradiation apparatus, of home design and construction, was used for visible light-driven H₂ production. A 300 W xenon arc lamp was used as source. A copper sulfate cell (5.1 cm path length) was used to filter out near-infrared and infrared light. The beam was then collected with a series of lenses and directed through a 420 nm high pass filter (Newport, FSQ-GG420) before being directed through a 520 nm band pass filter (Newport, 10BPF10-520). The resulting output beam had a wavelength of 520 ± 2 nm. The output beam was split into 4 beams by using three 50% beam-splitters (Edmund Optics). Each beam line was equipped with a home designed and constructed cuvette holder with a built-in magnetic stirrer. The beam diameter was reduced to 0.3 cm before passing through the quartz sample cuvette. The power (*P*) of each beam was adjusted by using an iris, and measured with a power meter (Thorlabs, PM100D). In the reported H₂ production experiments, *P* = 500 μW. High-power irradiation of protein-containing samples was performed by using a green light-emitting diode (LED; *P* = 1 W, 520–530 nm), with attenuation to *P* = 75 mW, at the sample.

Gas chromatography—A Shimadzu GC-2014 gas chromatograph, fitted with a thermal conductivity detector, utilizing a 60/80 molecular sieve (5 Å) column and a 60/80 Carboxen 1000 column in series, was used to detect the presence of H₂ gas in the sample headspace. Argon was used as the carrier gas. Calibration of the gas chromatograph for moles of H₂, oxygen (O₂), methane (CH₄) and carbon monoxide (CO) gases inside the quartz cuvette was performed. The total volume of the cuvette, with an airtight rubber stopper in place, was measured to be 6.25 ml. Nanopure water (4 ml) was added to the cuvette and deaerated by bubbling with wet argon for 10 min. Multipoint calibration curves were constructed by injecting known volumes of H₂, O₂, CH₄ and CO gases into the headspace of the reaction vessel. The cuvette was agitated, to allow the gas to come into equilibrium with the solution. A deaerated 100 μl Hamilton gas-tight syringe was used to inject 100 μl of argon into the headspace and extract 100 μl of headspace gas, which was then injected into the gas chromatograph for detection. The area of the peak detected by the gas chromatograph thermal conductivity detector was plotted as a function of moles of calibration gas injected into the cuvette. A linear fit was used to determine the calibration constant. All fits resulted

in $R^2 > 0.95$ (R , Pearson's correlation coefficient). A baseline H_2 production rate was measured for each sample type, with all components present, except the Co complex. This baseline was subtracted from the H_2 production rate measured for the complete system, to obtain the reported value of H_2 production.

Protein preparation

EutB protein was purified from the *Escherichia coli* overexpression strain incorporating the cloned *S. typhimurium* EAL coding sequence [73] essentially as described [74], with the exception that the Shine-Dalgarno binding region and the start codon of the EutC subunit were deleted from the plasmid, resulting in the overexpression of only the EutB subunit. The enzyme was dialyzed against buffer containing 100 mM HEPES (pH 7.5), 10 mM potassium chloride, 5 mM dithiothreitol, and 10% glycerol [75], and neither Triton X-100 nor urea were used during the purification. The enzyme was dialyzed against 10 mM potassium phosphate buffer (pH 7.5). Dehydration of the protein in the dialysis tubing with Aquicide (CalBiochem) was used to concentrate the protein to 10-15 mg/ml. Protein concentration was determined by using a Bradford colorimetric protein assay and BSA standard. Fast protein liquid chromatography (FPLC) was used to confirm formation of the EutB oligomer [(EutB₂)₃, or EutB₆] under the sample conditions.

EutB endogenous tryptophan fluorescence quenching by MeCbi

The binding of MeCbi to EutB was measured by the quenching of the endogenous tryptophan fluorescence by the macrocycle [76]. Individual samples of 100 nM EutB (active site concentration) were prepared and MeCbi (1 nM to 100 μ M) was added to each sample. Fluorescence measurements were performed by using a spectrofluorometer (Fluoromax-4, Horiba Jobin Yvon). The excitation wavelength was set to overlap with the tryptophan excitation peak at 280 nm, and tryptophan fluorescence quenching was monitored at the peak of the tryptophan emission spectrum at 340 nm. Fluorescence counts for samples containing 100 nM EutB with no MeCbi were used as the baseline. The concentration of the MeCbi-EutB complex in solution was then calculated, as follows:

$$[\text{MeCbi-EutB}] = [\text{EutB}] \left[1 - \frac{F_S}{F_0} \right] \quad (2)$$

In Eq. 1, [EutB] is the total concentration of EutB active sites, and F_S and F_0 are the number of fluorescence counts collected from the sample and from EutB with no MeCbi present. The full quadratic form of the expression for equilibrium binding at a single site was used to fit the binding data. Inner filter effects [77] produced by the absorption of excitation and emission photons by MeCbi were evaluated by experiments performed with free tryptophan and MeCbi in solution. Inner filter effects were shown to contribute to a loss of fluorescence of <10% at [MeCbi] < 1 μ M.

Results

Cobinamide-catalyzed hydrogen production in water

Light-driven production of H_2 by Cbi in water is shown as a function of irradiation time in Figure 2. The reaction mixture included the photosensitizer/sacrificial electron donor combination, EY/TEOA, and a solution pH of 7.0. Green light ($\lambda = 520 \pm 2$ nm; $P = 500$ μ W) was used for the irradiation. A baseline H_2 production rate was measured, with all components present, except the Co complex. This baseline rate was subtracted from the H_2 production rate measured for the complete system. The rate of H_2 production is quantified by the turnover number (TON), which is defined as moles of H_2 produced divided by moles of catalyst in the sample solution. Figure 2 shows that the TON increases linearly at

irradiation times from 0-48 h, and then decreases after 48 h. Rates of H₂ production, in units of TON/h, were obtained from the slope of the initial, linear portion of TON versus irradiation time plots, and are presented for different Cbi concentrations in Table 1. The rate of H₂ production catalyzed by Cbi is optimal at lower catalyst concentrations, with a maximum of 4.4×10^{-3} TON/h at 50 μ M. Overall, the results demonstrate that Cbi catalyzes H₂ production in pure water at pH 7.0.

The proven [22] Co(dmgh)₂pyCl H₂ production catalyst was also studied, as a reference for comparison with Cbi in water, and for correlation of the homogeneous EY/TEOA system performance, under our conditions, with other studies of cobaloxime catalysts. The TON for light-driven production of H₂ by Co(dmgh)₂pyCl in water displayed a growth and plateau form as a function of irradiation time (Figure S5, Supplementary Material), as observed for Cbi, and previously in 1:1 v/v AcCN/water [22]. Values of H₂ production by Co(dmgh)₂pyCl in 1:1 v/v AcCN/water were also measured (Figure S5 and Table S3, Supplementary Material), and were comparable to those reported by Eisenberg and coworkers [22]. The H₂ production parameters for 125 μ M Co(dmgh)₂pyCl in water are presented in Table 1. Table 1 shows that the H₂ production rate and total H₂ production values Co(dmgh)₂pyCl are approximately 10²-fold larger, relative to the Cbi parameters.

Formation and stability of Cbi(I) under steady-state irradiation

The UV/visible spectrum in the region of Cbi(I) absorption ($\lambda_{\text{max}}=387$ nm) prior to, and during, photo-initiated H₂ production in 100% water solution is shown in Figure 3. The spectra indicate conversion of the initial MeCbi to Cbi(I) by steady-state irradiation. Figure 3 also shows that the concentration of Cbi(I) remains approximately constant throughout the 96 h irradiation window, shown in Figure 2. Thus, the Cbi is stable on the extended time scale of the H₂ evolution measurements. The results in Figure 2 also suggest that formation and the maintained presence of Cbi(I) are not rate limiting for H₂ production. The decline in H₂ production rate at >48 h in Figure 2 is attributed to photodecomposition of EY, as noted previously for the EY/TEOA system [22], and related [39] organic photosensitizers. Photosensitizer decomposition is consistent with the observed loss of the long wavelength EY absorption bands during irradiation (Figure S6, Supplementary Material). Demetallation, reduction/hydrogenation of the macrocycle, and ligand exchange [65, 78], are not significant, as shown by the enduring amplitude and line shape of the Cbi(I) absorption band at $\lambda_{\text{max}}=387$ nm in Figure 3.

Equilibrium binding of Cbi to the EutB protein

The binding affinity of the stable MeCbi for EutB was used to characterize catalytic complex assembly. The binding of MeCbi to EutB was monitored by using the quenching of the endogenous tryptophan fluorescence of EutB, that accompanies binding of the macrocycle. Figure 4 shows that the binding titration curve for MeCbi-EutB interaction follows the form of the Langmuir isotherm for binding at a single site. Therefore, binding at the six catalytic sites in the oligomer is independent. The deviation from the isotherm at site occupancies >80% (corresponding to approximately 1 μ M Cbi) arises from an inner filter effect., in which the free cofactor absorbance blocks the light necessary to excite tryptophan fluorescence. Fits of the data to the single-site binding model led to a dissociation constant, $K_D=210 \pm 40$ nM. The K_D value reported here for MeCbi binding to EutB is comparable to values obtained by tryptophan fluorescence quenching, for binding of adenosylcobalamin ($K_D=170$ nM) and adenosylcobinamide ($K_D=62$ nM) to the B₁₂-dependent enzyme, methylmalonyl-CoA mutase [76]. The K_D value of 210 nM indicates relatively high affinity binding of MeCbi to EutB at the cobalamin binding site.

Cbi(I) formation and protein stability under steady-state irradiation

Figure 5A shows the generation and stability of Cbi(I), and stability of the EutB protein, under the steady-state irradiation conditions typical of a high irradiation power ($P=75$ mW, $\lambda=520$ nm) H_2 production assay, on the minutes time scale. Figure 5A shows that Cbi(I) is rapidly formed and maintained in EutB. The absorbance at $\lambda=600$ nm is a signature of light scattering by denatured and aggregated protein. The EutB protein scattering remains at the baseline level throughout the irradiation period. The simultaneous satisfaction of the desirable features of rapid Cbi(I) formation and protein stability, for EutB, contrast with the behavior of two control proteins. Figure 5B shows that bovine serum albumin does not support the formation of Cbi(I), although it is stable to denaturation, under the irradiation conditions. Figure 5C shows partial conversion to Cbi(I) at early times in the presence of lysozyme, but that the level of Cbi(I) decays rapidly (<2 min) to a level of approximately 20% of total Cbi. The rapid decay of Cbi(I) corresponds to a rapid rise and continued increase of light scattering. The results indicate that EutB is distinguished by the ability to both support Cbi(I) formation, and to maintain native protein structure under high power irradiation, relative to the control proteins.

Discussion

Performance of Cbi as an H_2 production catalyst in water

Figure 2 shows that Cbi is a homogeneous catalyst for H_2 production in the light-driven, EY/TEOA photosensitizer/sacrificial electron donor system in water at neutral pH. This is the first report of photocatalytic H_2 production by Cbi in pure aqueous solution. The H_2 production parameter, TON/h, for Cbi is benchmarked against the proven H_2 production catalyst, $Co(dmgh)_2pyCl$ [22], by using values for this complex obtained in water. As shown in Table 1, under identical conditions and catalyst concentrations of $125 \mu M$, the initial rate of H_2 formation by $Co(dmgh)_2pyCl$ is larger than for Cbi, by 8.5×10^2 -fold. Production of H_2 by $50 \mu M$ Cbi in 9:1 v/v AcCN/methanol, by using the *p*-terphenyl/TEA photosensitizer/sacrificial electron donor pair [65], had an average rate of 34 TON in 24 h. This value is 3.2×10^2 -fold larger than the corresponding value presented in Table 1. Origins of the relatively low H_2 production rate of Cbi in pure water solution are addressed, below, by considering the general mechanisms proposed for H_2 generation by Co complexes.

Cbi-dependent hydrogen production: Mechanistic considerations

Scheme 2 shows possible pathways for H_2 formation by Co complexes [22, 28, 29, 33], following formation of the reduced precursor species, $Co(I)$, and its protonation to form $Co(III)-H$. For both Monometallic Pathway 1 (heterolytic mechanism, involving protonation of a single Co complex) and bimetallic (homolytic, involving combination of two Co complexes) pathways of H_2 production, the $Co(III)-H$ species is recognized as the critical intermediate [7-9, 22, 28-30]. The Monometallic Pathway 2 in Scheme 2, shows that $Co(III)-H$ can be reduced by an additional electron, to form the $Co(II)$ hydride, $Co(II)-H$. One or more of the pathways in Scheme 2 has been proposed for different Co complexes and solution conditions [23, 34-37, 39-43], including the presence of parallel hetero- and homolytic pathways. This is because of the sensitivity to both free energy barriers and equilibrium populations, and, in aqueous systems, multiple and pH-dependent proton sources [7-9]. The formation of H_2 through the $Co(II)-H$ intermediate has been proposed [28-30, 41, 42], including for the $Co(dmgh)_2pyCl$ /EY/TEOA (1:1 AcCN/water) [22] and Cbi/*p*-terphenyl/TEA (9:1 v/v AcCN/methanol) systems [65].

Figure 3 shows that Cbi(I) is formed in high yield from the initial $MeCbi(III)$ state. Therefore, we address the formation of the protonated Cbi(I) species, $Co(III)-H$, as a possible origin of the relatively low Cbi TON values. The pK_a value of the $Co(III)H/Co(I)$

conjugate acid/base pair determines the relative population of the Co(III)–H species at equilibrium, at a given solution pH value. Experimentally determined pK_a values of Co(III)–H species in different classes of complexes used in H₂ generation studies are relatively high, as follows: Co–HMD (HMD=5,7,7,12,14,14-hexamethyl-1,4,8,11-tetraazacyclotetradeca-4,11-diene), $pK_a=11.5$ [26, 79]; Co(dmgBF₂)₂ (dmgBF₂=difluoroboryl-dimethylglyoxime), $pK_a=13.3$ [80]; Co(dmgH₂(PnBu₃)) (PnBu₃=tributylphosphine), $pK_a=10.5$ [81, 82]. In contrast, the Cbi(III)–H/Cbi(I) pair in water has been reported to have $pK_a=1$ [83]. Therefore, at pH=7, the proportion of the total Cbi concentration, that is in the Cbi(III)–H form, is predicted to be approximately 10^{–6}. Proposed models for H₂ production imply that concentration terms in Co(III)–H contribute to the overall rate for H₂ production [22, 28–30]. Thus, a principal origin of the relatively low H₂ production activity of Cbi in our homogeneous system is the small proportion of Cbi(III)–H.

Insight into the H₂-producing Cbi species and mechanism is gained by estimation of the thermodynamic favorability of H₂ formation in the EY photosensitizer system, and overall consistency with experimental observations. The photoreduction mechanism in the Co(dmgH)₂pyCl/EY/TEOA system has been shown [22] to proceed by electron injection from the first excited triplet state of EY ($E_{00}[^3EY]=1.98$ V [38]). The free energy for this electron transfer reaction can be estimated from the following Weller equation [84]:

$$\Delta G_{3^*Co(z)/Co(z)} - 1 = \frac{RT}{nF} \{ E_m [EY^{\bullet+}/EY] - E_m [Co(z)/Co(z-1) - E_{00}[^3EY] \} \quad (3)$$

where R is the ideal gas constant, $n=1$ is the number of electrons transferred, F is the Faraday constant, $T=298$ K, and z is the formal charge on Co. Values for $E_m[EY^{\bullet+}/EY]=+1.02$ V (AcCN, vs SHE [8]; comparable to an earlier value of +1.1 V [38]) and $E_m[Co(II)/Co(I)]=-0.50$ V (AcCN, vs SHE [62, 63]) have been reported. Values for $E_m[Cbi(I)/Cbi(0)]$ and $E_m[Cbi(III)-H/Cbi(II)-H]$ have not been determined. However, density functional theory (DFT) calculations [85], performed for the dimethyl- and diphenyl-glyoxime ligated Co complexes, Co(dmgBF₂)₂ and Co(dpgBF₂)₂, predict that the differences, $E_m[Co(I)/Co(0)]$ minus $E_m[Co(II)/Co(I)]$, and $E_m[Co(III)-H/Co(II)-H]$ minus $E_m[Co(II)/Co(I)]$, have average values of -0.50 V and $+0.08$ V, respectively. Thus, by using these predicted shifts in E_m , with the known $E_m[Cbi(II)/Cbi(I)]$ value, the $\Delta G_{3^*Co(I)/Co(0)}$ and $\Delta G_{3^*Co(III)-H/Co(II)-H}$ values are estimated by using Eq. 3, to be +1 and -12 kcal/mol, respectively. Formation of the Cbi(II)-H species is thus predicted to proceed by photoreduction of the Cbi(III)-H species. The small proportion of Cbi in the Cbi(III)-H state, estimated as $\sim 10^{-6}$ above, would lead to a corresponding small proportion of Cbi(II)-H. The proposed mechanism of H₂ production through the small proportion of Cbi(II)-H is consistent with the approximately constant Cbi(I) concentration during photoreduction (Figure 3), and with pulsed-radiolysis studies, which show that Cbi(II)-H absorbs weakly in the visible region [65]. Thus, Cbi(II)-H would not be discernable, owing to the strong, overlapping and time-dependent absorption of EY and photodecomposition products.

We favor the heterolytic mechanism of protonation of Cbi(II)-H to form H₂ (Scheme 2), because of the low estimated concentration of Cbi(II)-H (on the order of 1 nM), and the steric hindrance to productive encounter from the non-planar corrin ring and alkyl-amide substituents (Scheme 1). The proposed mechanism suggests that the Cbi(II)-H species, itself, is an excellent H₂ production catalyst. Renormalizing the TON/h values in Table 1 to account for the low estimated active catalyst concentration leads to TON/h > 10²/h, which is comparable to values for other, higher- pK_a Co complexes in water [7–9].

Assembly of the Cbi-protein complex

MeCbi binds in a 1:1 stoichiometry with EutB, and displays relatively high binding affinity ($K_D=210$ nM), as shown in Figure 4. The equilibrium binding affinity is comparable to the Michaelis constant reported for the native adenosylcobalamin in *S. typhimurium* EAL ($K_D=500$ nM) [74], and in other adenosylcobalamin-dependent enzymes [76]. These results suggest that Cbi binds in the cobalamin binding site, in the native position. The low steric bulk of the axial methyl group in MeCbi suggests that the Cbi(III), Cbi(II), and Cbi(I) forms have comparable K_D values. Therefore, at EutB protein and stoichiometric Cbi concentrations in the 10-100 μ M range, the protein-catalyst complex is assembled to an extent of >95%. Site-specific, high-affinity binding of the Cbi is an essential prerequisite for implementing the protein engineering strategies that will optimize the reactivity of protein-bound Cbi for H₂ production. In addition, the Cbi K_D value, and the accessibility of the Cbi binding site, indicate that “healing” strategies for *in situ* maintenance of the Cbi-EutB H₂ production system, by exchange of turnover-damaged cofactor, are viable at modest, 10-100 μ M concentrations of replenishing Cbi.

Figure 6 depicts the structure of the EutB subunit from the *S. typhimurium* EutB structure model [25], with the Cbi positioned in the native cobalamin binding mode. Analysis of the Cbi-EutB interactions, by using LigPlot [86], shows that the amide substituents on the corrin ring make five specific hydrogen bonding interactions (inter-heteroatom distances, 2.67-3.21 Å) with EutB: C2-acetamide(N29) – Pro194(peptide-O); C7- acetamide(N40) – Leu225(peptide-O); C7- acetamide(O39) – Ser247(sidechain-O); C8-propionamide(N45) – Glu257(peptide-O); C18-acetamide(N62) – Met401(peptide-O). Hydrophobic contacts between the apolar regions of the Cbi macrocycle are formed by the following EutB residues: Asn193, Ala226, Phe 245, His 227, Phe 258, Ser295, Ile330, Phe329. These interactions are depicted, in detail, in Figure S7.

The multiple favorable interactions between protein and Cbi suggest a robust anchoring, for manipulation of the *in situ* Co chemistry, through site-directed mutagenesis of selected protein residues. A primary aim is to raise the effective concentration and accessibility of the Co center to protons. A pendant amine group in diphosphine complexes of iron, nickel, and Co facilitates intermolecular proton exchange with solution, and intramolecular proton exchange with the metal-hydride, which is essential for electrocatalytic H₂ production [10-13]. Engineered proximal and relay protonation sites in the protein could enhance such effects.

Photoreduction and stability of the Cbi-protein complex

During the irradiation times (>12 h), that were required for significant H₂ production from Cbi in solution, under the conditions of Figure 1, aggregation and precipitation of EutB were observed. Therefore, higher power, steady-state green light irradiation ($P=75$ mW; 520-530 nm) was used, in an effort to decrease the irradiation time interval. The baseline-level, light-scattering signal at 600 nm in Figure 5A shows that EutB was stable on at least the ~1 h time scale, under the high power irradiation conditions, in the presence of EY and TEOA. H₂ production by the Cbi-EutB complex was not detected under either the low or high power irradiation conditions. However, as also shown in Figure 5A, irradiation of the MeCbi-EutB complex in the EY/TEAO system led to prompt Cbi(I) formation. Under the conditions of Figure 5A ([EutB]=88 μ M; [Cbi]=50 μ M), the EutB-Cbi K_D of 210 nM indicates that the fraction of bound Cbi is 0.99. The Cbi(I) absorption maximum and line shape are not sensitive to binding to the EutB protein (Supplementary Material, Figure S8), as observed previously for the interaction of cobalamin cofactors with the binding site in EAL [51]. The formation of Cbi(I) is consistent with the relatively open binding site at the C-terminal end of the ($\beta\alpha$)₈-barrel, as shown in Figure 6, which would allow access of the photosensitizer

to the Cbi. The ability to rapidly form stable Cbi(I) in EutB by photoactivation is an essential prelude for the protein catalyst.

Figure 5B and C show that, unlike for EutB, significant Cbi(I) formation is not supported by the two control proteins, bovine serum albumin and lysozyme, under photocatalytic conditions. We propose that Cbi(I) reduces cystine disulfide bonds bovine serum albumin (34 cystines) and lysozyme (4 cystines). At neutral pH, subsequent protonation of the alkylsulfide groups is favored ($pK_a=8.3$ for cysteine [87]). The facile reaction of the Co(I) states of cobalamin and Cbi cofactors with soluble alkyl-disulfides to form Co(II) and the corresponding alkyl-sulfides has been reported [88, 89]. In contrast, as shown in Figure 5A, *S. typhimurium* EutB, which contains 8 cysteine residues, but no reported cystine [90], affords stable formation of Cbi(I). The absence of the quenching of Cbi(I) in EutB, relative to bovine serum albumin and lysozyme, arises from the absence of cystines in the EutB protein. Figure 5C also shows that lysozyme aggregates under irradiation.

Overall, the results in Figure 5 highlight the strict demands on a protein framework for stabilizing a low-redox potential metallocatalyst, that is generated by a photosensitizer system under high-intensity visible irradiation. The robust $(\beta\alpha)_8$ barrel structure [55] and amino acid side chain constitution of EutB supports both formation of Cbi(I) and maintenance of protein structure.

Conclusions

The production of H_2 by Cbi in pure water solution in the EY/TEOA photocatalytic system under mild, biological conditions of pH, T , and p is demonstrated. Rates of H_2 production in water are lower by $>10^2$ -fold, relative to cobaloxime and other Co coordination complexes [7-9]. It is proposed that this arises from the relatively low $pK_a=1$ for protonation of Cbi(I) to form the critical Cbi(III)-H intermediate in H_2 production. Thermodynamic considerations and the sustained Cbi(I) absorption support the further reduction of Cbi(III)-H by EY, to form the Cbi(II)-H species. Protonation of Cbi(II)-H is proposed as the principal mechanism of H_2 formation. Renormalization of H_2 TON values to the small (10^{-6}) proportion of Cbi(II)-H, suggests that this species is, itself, a viable catalyst, with performance comparable to recently characterized cobaloxime, polypyridinyl and other Co complexes in aqueous solution [23, 35-37, 39-43]. The water-soluble Cbi displays excellent stability ($<10\%$ loss in 96 h) in the Cbi(I) state under photoreduction conditions. Two essential requirements for catalyst-protein complex assembly and function are demonstrated for the Cbi-EutB protein system: (1) Relatively high affinity equilibrium binding of Cbi, such that $>95\%$ occupancy is achieved at free catalyst concentrations of 10^{-6} M, and (2) photoreduction to the catalytically competent Cbi(I) state. Molecular modeling of the Cbi-EutB interaction shows multiple hydrogen bond and apolar interactions, which support unique macrocycle positioning in the protein. This affords manipulation of the *in situ* Co reactivity, by using site directed mutagenesis to enhance local acidity, and implement proton delivery and relay groups. The results establish Cbi-EutB as a platform for design and engineering of a protein-based, H_2 production metallo-catalyst.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AcCN	Acetonitrile
Cbi	Cobinamide
CN₂Cbi	Dicyanocobinamide
dmgH	Dimethylglyoxime
EY	Eosin Y
MeCbi	Methylcobinamide
py	Pyridine
TEA	Triethylamine
TEOA	Triethanolamine
TPP	Tetraphenyl porphyrin

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Scheme 1.
Structures of cobinamide, Co(dmgh)₂pyCl, Eosin Y, and triethanolamine

Initial Hydride Formation **Monometallic Pathway 1: Co(III)H Precursor** **Monometallic Pathway 2: Co(II)H Precursor** **Bimetallic Pathway** **Scheme 2.**

Mechanisms proposed for H₂ production by cobalt complexes

Fig. 1. Oligomeric structure of the EutB protein, which shows the hexameric, or trimer of dimers $[(\text{EutB})_2]_3$ arrangement. The structure of EutB is presented in ribbon and transparent surface rendering, and the native cobalamin cofactor is represented by red sticks. There is one cobalamin binding site in each EutB, and six cobalamin binding sites in each oligomer. The tilt of the EutB monomers alternates above and below the equatorial plane. EutB monomers colored in green display the cobalamin binding site, above the plane.

Fig. 2.

Hydrogen (H₂) production by Cbi as a function of irradiation time in water. *Initial conditions:* 125 μ M MeCbi, 50 μ M EY, 5% TEOA, pH 7.0, 500 μ W irradiation ($\lambda = 520$ nm $\pm$ 2 nm), $T=295$ K. The amount of H₂ was determined by gas chromatography.

Fig. 3.

Time dependence of the UV/Visible spectrum of the Cbi H₂ production system in water in the region of Cbi(I) absorption. *Initial conditions:* 50 μ M MeCbi, 50 μ M EY, 5% TEOA, pH 7.0, 500 μ W irradiation ($\lambda = 520 \text{ nm} \pm 2 \text{ nm}$) and $T=295 \text{ K}$. Before irradiation (solid line). After 24 h of irradiation (dashed line). After 96 h of irradiation (dotted line).

Fig. 4.

Equilibrium binding titration curve for the MeCbi-EutB interaction. The binding was measured by using endogeneous tryptophan fluorescence quenching. The solid line represents the fit of the data to the full quadratic form of the single-site binding equation ($K_D=263$ nM). The average K_D value is 210 ± 40 nM ($N=2$ determinations). *Conditions:* EutB, equivalent to 100 nM binding sites in 10 mM potassium phosphate, pH 7.5, and $T=295$ K.

Fig. 5.

Time dependence of Cbi(I) formation and light scattering by solutions in the Cbi(I)-generating system under steady-state irradiation (75 mW, $\lambda = 520$ nm), for EutB and control proteins. (a) EutB, (b) BSA and (c) lysozyme. Cbi(I) formation (open symbols); light scattering (closed symbols). *Initial conditions:* 88 μ M EutB (6.5 mg/ml); 6.5 mg/ml BSA; 6.5 mg/ml Lysozyme, 50 μ M MeCbi, 50 μ M EY, 0.01% TEOA in potassium phosphate buffer at pH 7.5. The sample path length was 0.4 cm.

Fig. 6. Structure model, showing position of the cobalamin binding site at the C-terminal end of the $(\beta\alpha)_8$ barrel in EutB. The full EutB protein (top, middle) and the local region of the cobalamin binding site (bottom) are depicted. View is down the principal axis of the $\beta_8\alpha_8$ barrel in middle and bottom panels. Protein surface (transparent grey) and underlying secondary structure (dark grey) are represented. EutB oxygen (red) and nitrogen (blue) atoms that make direct hydrogen-bond contact with amide substituents on the corrin ring, and regions of hydrophobic interaction (black), are shown.

Table 1Hydrogen production parameters for cobinamide and Co(dmgh)₂pyCl complexes in water.^a

Concentration (μM)	H ₂ Production Initial Rate (TON/h) ^{b,c}	H ₂ Production Total TON ^b
Methylcobinamide		
50	$4.4 \pm 1.8 \times 10^{-3}$	0.45 ± 0.19^d
125	$2.0 \pm 0.8 \times 10^{-3}$	0.20 ± 0.09^d
250	$0.9 \pm 0.3 \times 10^{-3}$	0.09 ± 0.02^d
Co(dmgh) ₂ pyCl		
125	1.7 ± 0.2	60 ± 6

^aInitial conditions: 50 μM EY, 5% TEOA, pH 7.0, 500 μW irradiation ($\lambda = 520 \text{ nm} \pm 2 \text{ nm}$) in anaerobic solution.^bAverage of *N* 3 determinations, $\pm$ standard deviation.^cBaseline H₂ production rate was determined under identical conditions, in the absence of cobalt complex: $1.0 \pm 0.3 \times 10^{-3} \text{ mol H}_2/\text{mol EY/h}$ (TON/h, referenced to EY). Values in the table are corrected for the baseline H₂ production rate.^dReaction had not ceased by end of monitoring period (100 h). Values represent lower limits.