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A Blue Corrinoid from Partial Degradation of Vitamin B_{12} in Aqueous Bicarbonate: Spectra, Structure, and Interaction with Proteins of B_{12} Transport

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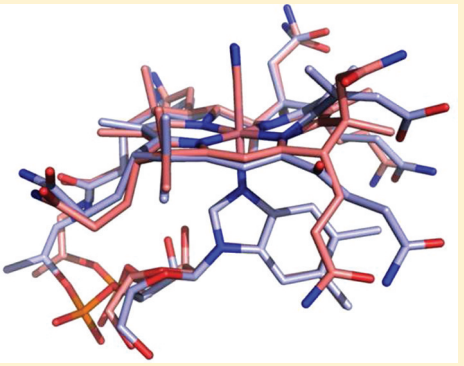
Supporting Information

ABSTRACT: Cobalamin (Cbl) is a complex cofactor produced only by bacteria but used by all animals and humans. Cyanocobalamin (vitamin B_{12}, CNCbl) is one commonly isolated form of cobalamin. B_{12} belongs to a large group of corrinoids, which are characterized by a distinct red color conferred by the system of conjugated double bonds of the corrin ring retaining a Co(III) ion. A unique blue Cbl derivative was produced by hydrolysis of CNCbl in a weakly alkaline aqueous solution of bicarbonate. This corrinoid was purified and isolated as dark blue crystals. Its spectroscopic analysis and X-ray crystallography revealed B-ring opening with formation of 7,8-*seco*-cyanocobalamin (7,8-*s*CNCbl). The unprecedented structural change was caused by cleavage of the peripheral C−C bond between saturated carbons 7 and 8 of the corrin macrocycle accompanied by formation of a C═C bond at C7 and a carbonyl group at C8. Additionally, the C-amide was hydrolyzed to a carboxylic acid. The extended conjugation of the π-system caused a considerable red shift of the absorbance spectrum. Formation and degradation of 7,8-*s*CNCbl were analyzed qualitatively. Its interaction with the proteins of mammalian Cbl transport revealed both a slow binding kinetics and a low overall affinity. The binding data were compared to those of other monocarboxylic derivatives and agreed with the earlier proposed scheme for two-step ligand recognition. The obtained results are consistent with the structural models of 7,8-*s*CNCbl and the transport proteins intrinsic factor and transcobalamin. Potential applications of the novel derivative for drug conjugation are discussed.



Vitamin B_{12} (cyanocobalamin, CNCbl) is a unique cobalt complex[1,2] (Figure 1A) that consists of two major structural elements: (1) the highly substituted corrin ring with the central Co(III) ion and (2) the nucleotide appendage with a 5,6-dimethylbenzimidazole (DMB) base.[3,4] The compounds with similar construction are called "complete" corrinoids with reference to the presence of the nucleotide appendage.[5] DMB can be displaced from its normal "base-on" position at the central Co(III) ion by another ligand, e.g., a cyanide ion.[4] The transition to a "base-off" form is also facilitated by low pH values, due to protonation of DMB. Substitution of DMB with adenine, as seen in pseudocoenzyme B_{12}, also shifts the equilibrium to the base-off form.[6] In some enzymes, the base-off form of Cbl cofactors is required for catalysis,[7,8] whereas the proteins of Cbl transport preferentially bind the chemically more stable base-on forms.[9,10]

Co(III) corrinoids display a typical red color conferred by the system of conjugated double bonds of the corrin ring. The Co(III) form is stabilized by high-affinity axial ligands, like cyanide and alkyl groups.[4] Otherwise, corrinoids are prone to reduction and produce a brown-yellow Co(II) or green Co(I) form, depending on the strength of the reducing agents,[11] yet these forms are readily oxidized by atmospheric oxygen with restoration of the "normal" red color.[4,12,13]

Three acetamide and four propionamide side chains (indexed as *a*, *b*, *c*, *d*, *e*, *f*, and *g*) project to either the "upper" or the "lower" face of the corrin ring (see Figure 1A).[3] They provide specific hydrogen bonding interactions between the corrinoid and an enzyme or a Cbl-transporting protein (see below for further discussion).[7,14−17] Selective hydrolysis of the *b*-, *e*-, and *d*-propionamide groups of CNCbl produces B_{12} carboxylic acids,[18−22] which have lower affinities for the transporting proteins.[19]

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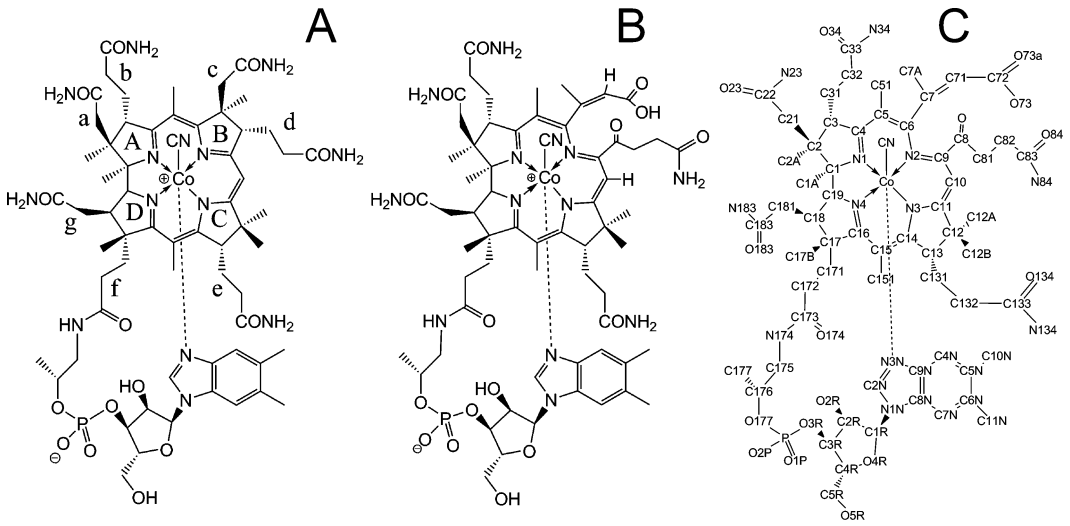

Figure 1. (A) Structural formula of vitamin B_{12} (CNCbl), with indices used for rings and side chains of the corrin ligand. (B) Structural formula of the blue corrinoid, 7,8-*seco*-cyanocobalamin (7,8-*s*CNCbl), with the altered ring B segment highlighted and (C) atom numbering used.

Cbls are produced only by bacteria, and elucidation of the complex pathway of this biosynthesis has become a milestone in the B_{12} field.[23,24] All mammals need Cbls and obtain them via an intricate mechanism of intestinal uptake and blood transportation.[9,15] The proteins of Cbl transport, intrinsic factor (IF), transcobalamin (TC), and haptocorrin (HC), have different ligand specificities, which results in a selective accumulation of Cbl in the tissues and clearance of its modified derivatives from the body.[9,15] The sources of the vitamin for mammals are either intestinal bacteria (herbivores) or food of an animal origin (carnivores and humans). Cbl deficiency causes pernicious anemia and/or various nervous disorders, both syndromes being lethal if left untreated.[9,25]

In contrast to the completely characterized pathway of Cbl biosynthesis,[23,24] very little is known about its natural degradation. In fact, vitamin B_{12} (CNCbl) is very stable if stored in aerated aqueous solutions at pH 4.[26,27] Selective chemical modifications of Cbl have been used for the preparation of vitamin B_{12} derivatives.[18,28,29] Thus, brief treatment with either strong acid or alkali causes cleavage of the nucleotide from the corrin ring.[18,26] On the other hand, incubation under relatively mild acidic or alkaline conditions leads to a stepwise hydrolysis of the *b*, *d*, and *e* amide groups.[18,21] The C3, C8, and C13 atoms of the corrin ring may become deprotonated under alkaline conditions that differ in strength. The accompanying increase in the electron density of the deprotonated corrin ligand causes the so-called "self-reduction"[4,18] of some Cbls with formation of Co(II) corrins (often followed by a cascade of other reactions). The typical sequence of such reactions leads to formation of a lactam ring, where C7 and C8 at ring B become connected via the *c* acetamide side chain. Lactone formation at the same position is a competing process, which is efficient in reactions with halogens or under treatment with dilute alkali.[4,18]

None of the mentioned transformations cause a pronounced change in the color of the product, as long as the cobalt ion is maintained in its normal Co(III) oxidation state. Indeed, the changing color of Co(III) corrins indicates a modification in the structure of the chromophore. Thus, in "stable yellow corrinoids" (or "xanthocorrinoids"), the chromophore of the corrin ligand is interrupted (saturated) at C5 and C6.[30,31] Cleavage of the corrin macrocycle at the same positions by ozonolysis[32] or with singlet oxygen[33] also yields yellow corrinoids. In contrast, a "blue corrinoid" was described as a minor component isolated from products of B_{12} biosynthesis, where C18 and C19 are unsaturated and thus extend the corrin chromophore.[34] Green Co(III) corrins were obtained under pyrrolysis, which caused loss of the *c* side chain and complete unsaturation of the pyrrole ring B.[35]

In this work, we describe an unexpected and highly unusual modification of CNCbl, which occurs in weakly alkaline aqueous solution and in the presence of bicarbonate ion. A blue corrinoid named 7,8-*seco*-cyanocobalamin (7,8-*s*CNCbl) is formed under these conditions, as the most abundant polar product together with a mixture of red corrinoids. We have characterized the spectral and structural features of 7,8-*s*CNCbl, as well as its patterns of interaction with the relevant proteins of Cbl transport in mammals. As delineated below, the unusual color is caused by the opening of ring B accompanied by introduction of additional unsaturated groups adjacent to the conjugated system. The observed changes in this part of the Cbl structure significantly affected the velocity of their primary attachment to the specific proteins of Cbl transport, as well as their overall affinity.

EXPERIMENTAL PROCEDURES

Materials. All salts and solutions used for synthesis and isolation were purchased from Sigma-Aldrich. TLC plates Polygram Sil G and Alugram Sil G (both 20 cm × 20 cm, 0.2 mm gel) were from Macherey-Nagel. RP18 F254s TLC plates were from Merck. CNCbl, AdoCbl, and H_2OCbl were from Sigma-Aldrich. Monocarboxylic *b*-, *c*-, *d*-, and *e*-acids were prepared as described previously.[18,19,21] Partially purified monocarboxylic derivative c-COOH(C8OH) was produced by R. Alberto (University of Zürich, Zürich, Switzerland) as a byproduct of alkaline hydrolysis of B_{12} lactone when reacted with amines (unpublished results). This derivative was purified by preparative TLC as described below.

Methods. *Synthesis of the Blue Corrinoid.* CNCbl (350 mg, 250 μmol) was dissolved in 35 mL of 0.2 M $NaHCO_3$ buffer (pH 9.0) (final CNCbl concentration of 7 mM), and the solution was incubated at 50 °C for 1−3 weeks (yields of 3−6%). Then, concentrated HCl was added dropwise to a final

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concentration of 0.25 M (pH ≈3). Precautions against intensive bobbling under neutralization of bicarbonate were taken. The mixture of corrinoids was desalted by phenol/chloroform extraction (see below). The desalted aqueous solution (25 mL, 250 μmol of corrinoids) was applied to a QAE-Sephadex column (25 mL). Ion-exchange chromatography was performed as described below. A mixture of monocarboxy derivatives of Cbl (73 μmol, 12 mL) was eluted with 0.05 M P_i buffer (pH 7.5) and subjected to phenol/chloroform extraction. This preparation was purified by preparative TLC in the ammonium medium. Detailed TLC procedures are described below. Ten Polygram Sil G plates (20 cm × 20 cm) were used (7 μmol = 10 mg/plate). A blue band separated from a number of red bands, whereupon it was extracted, and the extract was desalted. The major red bands were also extracted and used for characterization of the major contaminants. The sample with the blue corrinoid as the major component (9 μmol, 0.6 mL) was subjected to preparative TLC in the acetate medium on two Alugram Sil G plates (20 cm × 20 cm) (Polygram Sil G is not recommended for this procedure). The blue band was extracted, and the extract was desalted. The obtained aqueous solution with 7 μmol of the blue corrinoid (7,8-*s*CNCbl) was lyophilized and the residue redissolved in water. An analogous procedure was used for the final purification of *b*-, *d*-, and *e*-monocarboxylic analogues.

Phenol/Chloroform Extraction. An aqueous solution of corrinoids in a buffer (pH ≈3) was mixed with phenol and chloroform (1:1) in the proportion of 1 mL of the organic phase per 5 μmol of corrinoids. The sample was vigorously shaken and briefly centrifuged (20 s). The lower organic phase contained the corrinoids. If the extraction was incomplete, the extraction was repeated with fresh organic solvent. The organic phase was pooled and vigorously shaken with an equal volume of 10 mM HCl, and the aqueous layer was discarded after a brief centrifugation. Then, 0.5 volume of chloroform, 0.5 volume of acetone, and 0.5 volume of water were added to 1 volume of the phenol/chloroform phase. The mixture was vigorously shaken and briefly centrifuged, and the upper aqueous phase with corrinoids was collected. The obtained solution was shaken with 2 volumes of diethyl ether to remove remains of phenol, and the upper organic phase was discarded. The sample was either lyophilized or used directly depending on the situation.

Ion-Exchange Chromatography on Sephadex QAE. A desalted aqueous mixture, containing carboxylic derivatives after hydrolysis of Cbl, was loaded on Sephadex QAE [prewashed with 2−3 bed volumes of 0.2 M P_i buffer (pH 7.5) and 2−3 volumes of water]. A column of 25 mL was used to load and separate 250 μmol of corrinoids containing approximately 50% of the negatively charged derivatives. All uncharged molecules (e.g., CNCbl, dicyanocobinamide, etc.) were eluted with water. The negatively charged molecules remained bound at the top of the column. Washing with water was continued until the main part of the column became colorless. The corrinoids with one negative charge (including the blue corrinoid and the monocarboxylic acid derivatives) were eluted with the front of 50 mM P_i buffer (pH 7.5). The dicarboxylic acid derivatives, with two negative charges, were eluted with the front of 0.2 M P_i buffer (pH 7.5). The final regeneration of the matrix was performed with 1 M HCl and water.

Preparative and Analytical TLC. A desalted aqueous solution of corrinoids was applied to a TLC plate (20 cm × 20 cm, 0.2 mm silica gel). For preparative TLC, 5−7 μmol of corrinoids (7−10 mg) was placed in 10 μL portions on the plate to make a 20 cm streak. Polygram Sil G plates with a plastic support were used during development in the ammonium medium (3:1 2-butanol/water mixture, 2 mM NH_4OH, and 0.2 mM KCN), whereas Alugram Sil G plates with the aluminum support were employed for development in the acetate medium (2.5:1 2-butanol/water mixture and 0.1 M acetate). The aluminum plates demonstrated a better separation of monocarboxylic derivatives under acetate development. Chromatography was continued until a clear separation of the bands was achieved (between 4 h and an overnight run, depending on the situation). Analytical TLC was conducted similarly except for a smaller loading of the sample (1−4 μL portions in separate spots).

Extraction of Corrinoids from a TLC Plate. A streak with a particular corrinoid was cut out of the TLC plate (20 cm × 20 cm), and the gel was scraped out. The material from one plate was placed into a 2 mL tube, where it was made into a powder with a plastic pestle and subjected to extraction with 0.7 mL of 0.1 NH_4OH and 20 mM KCN (2−3 min). The suspension was centrifuged and the liquid collected. The pellet was subjected to another round of extraction, whereupon the two supernatants were pooled and neutralized by HCl (final concentration of 0.12 M). Then, the corrinoids were desalted by phenol/chloroform extraction.

Isolation and Crystallization. A sample of 3.8 mg of the blue corrinoid was dissolved in 1 mL of H_2O_{dest} and lyophilized. The residue was dissolved in 100 μL of $H_2O_{dest.}$ and precipitated from acetone. The mother liquor was removed, and the precipitate was redissolved in water (100 μL). The blue corrinoid 7,8-*s*CNCbl crystallized at 4 °C after addition of 2 mL of acetone. The mother liquor was removed, and the crystals were dried for gravimetric (3.1 mg) and spectroscopic analysis.

Spectroscopy. UV−vis spectra were recorded on a Hitachi-U3000 (Hitachi, Tokyo, Japan) or Varian Cary 50 spectrophotometer [λ_{max} (log ε) values in nanometers]. CD spectra were recorded on a Jasco J715 instrument [λ_{max} or λ_{min} (log ε) in nanometers]. Fluorescence spectroscopy was performed using a DX.17 MV stopped-flow spectrofluorometer (Applied Biophysics), with excitation at 525 nm (1.5 mm slit), a ≥550 nm emission cutoff, and a voltage of 685 V, or a SPEX FluoroLog instrument (HORIBA Jobin Yvon), with excitation at 525 nm (2 nm) and emission at 550 nm (5 nm). For ^1H and ^{13}C NMR,[36] we employed 500 MHz Varian Unity and 600 MHz Bruker UltraShield Inova spectrometers. For ^{31}P NMR, we used a Bruker AM 300 instrument (121.5 MHz). HSQC: 2K × 512 complex data points, SW2 11 ppm centered around the residual water signal, SW1 0−200 ppm, 128 scans per increment. HMBC: 2K × 512 data points (real), SW2 11 ppm centered around residual water signal, SW1 0−230 ppm, 256 scans per increment. ROESY: 2K × 512 data points (real), SW1 and SW2 11 ppm centered around residual water signal, 32 scans per increment, 1.5 s predelay, 250 ms mixing time for H_2O suppression with WATERGATE echo. All experiments were conducted with 2 mg of the blue corrinoid dissolved in 600 μL of deuterated solvent at 25 °C. The spectra were referenced to the residual solvent signal (4.77 ppm for D_2O, 3.31 ppm for CD_3OD and CD_3OH). For mass spectrometry, fast-atom-bombardment (FAB) mass spectra[37] were recorded on a Finnigan MAT95S instrument (positive ion mode, matrix of glycerine, ionization with Cs gun at 20 kV). Electrospray ionization (ESI) mass spectra were recorded on a Bruker apex

Table 1. Rate Constants for the Interaction between Different Corrinoids and B_{12}-Binding Proteins[a]

	IF		TC		HC	
	k_{+1}, k_{-1}	k_{+2}, k_{-2}	k_{+1}, k_{-1}	k_{+2}, k_{-2}	k_{+1}, k_{-1}	k_{+2}, k_{-2}
CBC[b]	6.7×10^7	complex	6.1×10^7	none	1.28×10^8	none
	complex	complex	4×10^{-7}	none	4×10^{-7}	none
$K_{1/2}$ (CBC)	1×10^{-13} M		7×10^{-15} M		6×10^{-15} M[b]	
CNCbl[b]	7.4×10^7	none	6.8×10^7	none	9.0×10^7	none
	4×10^{-7}	none	3×10^{-7}	none	$\leq 5 \times 10^{-7}$	none
$K_{1/2}$ (Cbl)	5×10^{-15} M		4×10^{-15} M		6×10^{-15} M	
b-acid	5.0×10^6	0.002	8×10^6	7.4×10^{-4}	2.8×10^7	none
	3.7×10^{-3}	0.0011	6.3×10^{-4}	3.6×10^{-4}	$\leq 1 \times 10^{-6}$	none
$K_{1/2}$ (*b*-acid)	2.6×10^{-10} M		2.6×10^{-11} M		3.6×10^{-14} M	
c-acid	2.2×10^6	4.0×10^{-3}	5.7×10^6	1.5×10^{-3}	4.3×10^6	8.6×10^{-4}
	0.041	4.8×10^{-3}	1.0×10^{-3}	4.2×10^{-4}	7.1×10^{-3}	2.1×10^{-4}
$K_{1/2}$ (*c*-acid)	1×10^{-8} M		3.9×10^{-11} M		3.3×10^{-11} M	
d-acid	4.6×10^7	4×10^{-3}	3.4×10^7	none	7.6×10^7	none
	5×10^{-4}	4×10^{-4}	$\leq 9 \times 10^{-6}$	none	$\leq 1 \times 10^{-6}$	none
$K_{1/2}$ (*d*-acid)	1×10^{-12} M		2.6×10^{-13} M		1.3×10^{-14} M	
e-acid	3.4×10^7	3.2×10^{-3}	3.7×10^7	2.3×10^{-3}	5.4×10^7	none
	7.5×10^{-3}	1.3×10^{-3}	2.8×10^{-3}	1×10^{-3}	$\leq 1 \times 10^{-6}$	none
$K_{1/2}$ (*e*-acid)	6.4×10^{-11} M		2.3×10^{-11} M		1.8×10^{-14} M	
7,8-*s*CNCbl	2.5×10^6	0.15	8.9×10^6	2.0×10^{-3}	5.2×10^6	none
	1.9	0.12	4.1×10^{-3}	1.6×10^{-3}	1.7×10^{-5}	none
$K_{1/2}$ (7,8-sCNCbl)	3.4×10^{-7} M		2.0×10^{-10} M		3.3×10^{-12} M	

[a]Dimensions of the rate constants are M^{-1} s^{-1} (k_{+1}) and s^{-1} (all others). None means that the corresponding step of the binding is either absent or invisible (its rate constant became part of k_{-1}). Complex means that the ligand binding mechanism is more complex than the two-step scheme in Figure 7C. The coefficient of half-saturation ($K_{1/2}$) is equal to $K_1/(1 + 1/K_2)$ where K_1 and K_2 are the k_{-1}/k_{+1} and k_{-2}/k_{+2} ratios, respectively. [b]Data from ref 10.

ULTRA FTMS (7T) instrument [positive ion mode, spray voltage of 4.5 kV, 1:1 (v/v) MeOH/H_2O mixture as the solvent, in source CID, mass selective quadrupole, collision cell CID].[38]

Binding of the Blue Corrinoid to B_{12}-Binding Proteins Monitored by Fluorescence Spectroscopy. Interaction between different corrinoids and the B_{12}-binding proteins (IF, TC, and HC) was monitored indirectly using a fluorescent analogue of Cbl (called CBC[39]) as a reference ligand. The details of the method are explained elsewhere.[10,39] The relevant rate constants for the association were calculated by computer fitting, as described elsewhere.[10,39] The dissociation reaction was analyzed when mixing the tested protein−ligand complex (0.5 or 0.1 μM) with an equal concentration of exogenous CBC. The reaction starts with dissociation of the tested ligand from the protein, which is followed by the binding of CBC. The latter process is accompanied by amplification of the fluorescence. The dissociation was measured over 20, 800, or 3600 s, depending on the ligand. The relevant rate constants were calculated by computer simulations (see Table 1).[10,39]

The concentrations of the B_{12} "red acids" used in these experiments were calculated from the optical densities of the corresponding dicyano forms [obtained after 5 min at 95 °C with 10 mM KCN in 0.1 M sodium phosphate buffer (pH 12)] registered on the Varian Cary 50 spectrophotometer. The following coefficients of molar absorbance of dicyanocorrins were used: ε_{367} = 30700 for the γ-peak,[4] and ε_{540} = 9000 and ε_{578} = 9900 for β,α-peaks. Another procedure implied measurements of monocyano forms (neutral pH without cyanide added): ε_{361} = 28060 for the γ-peak,[4] and ε_{520} = 7900 and ε_{578} = 8800 for β,α-peaks. Spectral features of the blue corrinoid are described in Results.

X-ray Analysis. Crystals of the blue corrinoid (7,8-*s*CNCbl) were grown from a water/acetone mixture. A small crystal specimen was immersed in hydrocarbon oil, picked up with a rayon loop, and quickly cooled to cryotemperature by immersion in liquid nitrogen. Diffraction experiments were conducted on beamline PXIII at the Swiss-Light-Source/Paul Scherrer Institute (Villigen, Switzerland), which was equipped with a CCD detector and a gas-stream low-temperature [103(2) K] device. Data pertaining to diffraction data collection and structure refinement are summarized in Table S1 of the Supporting Information.

Indexing of diffraction images, intensity integration, and data scaling were performed with Automar (MarResearch). The crystal was monoclinic (space group $P2_1$) with the following unit cell constants: a = 13.015(3) Å, b = 22.401(5) Å, c = 14.248(3) Å, and β = 111.33(3)°. The structure was determined by direct methods as implemented in SHELXS-97[40] and refined against F^2 values using SHELXL-97.[40] Full matrix least-squares anisotropic refinement converged at an R_1 of 0.0783 for all data. No absorption correction was applied to the data. Scattering factors, including real and imaginary dispersion corrections, were taken from the International Tables of Crystallography. The solvent region was modeled using one acetone and seven water molecules with anisotropic

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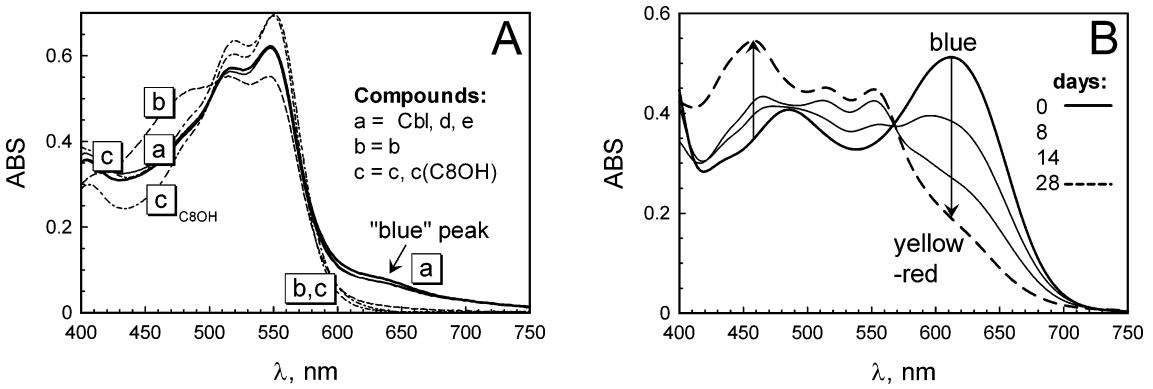

Figure 2. Synthesis and degradation of the blue corrinoid registered by changes in the absorbance spectra. (A) Conversion of different derivatives of Cbl (all monocyano forms) to the blue corrinoid after 1 week in 0.2 M $NaHCO_3$ at pH 9 and 50 °C. The members of group a (CNCbl, *d*-, and *e*-monocarboxylic acids) are shown as overlapping solid lines. The spectrum of the *b*-monocarboxylic form is shown as a dashed line. The spectra of two *c*-monocarboxylic forms (with or without C8OH) are indicated by dashed−dotted lines labeled c_8 and c, respectively. (B) Degradation of the purified blue corrinoid in 0.2 M $NaHCO_3$ at pH 9 and 37 °C. Similar spectroscopic records were observed over 5 min via reduction with $NaBH_4$.

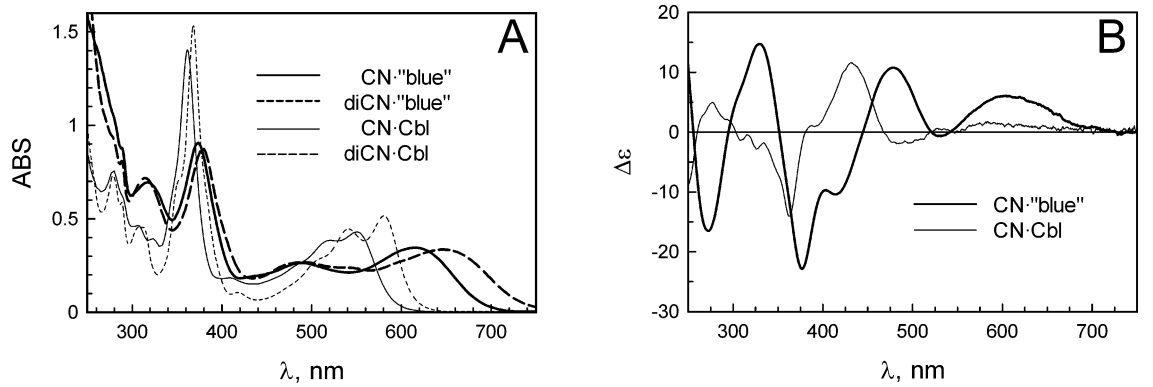

Figure 3. Spectral properties. (A) Absorbance spectra of the blue corrinoid (monocyano and dicyano forms) and CNCbl and diCNCbl. All CN forms were dissolved in H_2O and diCN forms in 10 mM KCN (pH 12). All ligand concentrations were 50 μM. (B) CD spectra of blue corrinoid 7,8-*s*CNCbl and of vitamin B_{12} (CNCbl) in H_2O.

atomic displacement parameters (adp). Restraints for adp's (DELU and SIMU, as well as ISOR for solvent atoms) were applied. Hydrogen atom positions were calculated and refined as "riding" on their respective non-hydrogen atom. For hydroxyl groups, the torsion angle around the C−O bond was also refined. The isotropic adp for each H atom was set to 1.5 times (for methyl and hydroxyl groups), and the equivalent isotropic adp of the adjacent non-H atom was 1.2 times (for all other hydrogen atoms).

RESULTS

Synthesis of the Blue Corrinoid (7,8-*s*CNCbl) and Variants of the Procedure. The formation of the blue corrinoid (7,8-*s*CNCbl) involved hydrolysis of CNCbl at pH 9.0 in the presence of bicarbonate. Incubation of CNCbl in 0.2 M $NaHCO_3$ at pH 9.0 and 50 °C for 3 weeks under atmospheric air provided 7,8-*s*CNCbl in a yield of ∼6%. Several variants of this procedure in which the yields were slightly inferior were tested: 1 and 2 weeks at 50 °C gave the blue corrinoid in yields of 3 and 5%, respectively, 3 weeks at 37 °C a yield of 2%, 4 days in 0.1 M $NaHCO_3$ at pH 10 and 50 °C a yield of 3%, 1 week in 0.5 M $NaHCO_3$ at pH 9.0 and 50 °C a yield of 4%, etc. The isolation procedure (as schematically depicted in Figure S1 of the the Supporting Information; see Experimental Procedures for the details) included the following major steps: (1) separation of corrinoids by ion-exchange chromatography, (2) preparative TLC in the ammonium medium, and (3) preparative TLC in the acetate medium. This method allowed separation and characterization of the major products of hydrolysis. From a reaction of CNCbl for 3 weeks in 0.2 M $NaHCO_3$ at pH 9.0 and 50 °C, the following material balance was obtained: 45% of the original CNCbl was recovered; 5% consisted of different neutral corrinoids ($z = 0$); 30% accounted for the derivatives with one negative charge ($z = -1$), where 7,8-*s*CNCbl represented ∼6% and the monocarboxylic forms *b*, *d*, *e*, and *c*(C8OH) corresponded to 5, 2, 1, and 3%, respectively; 15% consisted of various dicarboxylic acid forms ($z = -2$); and the remaining 5% included numerous derivatives with higher negative charges. The detection of the specified corrinoids was conducted by comparison to the pure standards on TLC followed by extraction of the spots and analysis on the mass spectrometer.

Reaction of CNCbl at pH 9 in 0.2 M Tris buffer in the absence of bicarbonate revealed only traces of a "blue form" (<1% vs 5% in a parallel experiment in bicarbonate buffer). The presence of 20 mM KCN in the medium precluded formation of the blue derivative but caused gradual accumulation of the incomplete forms (cobinamide and its carboxylic acids). Treatment of AdoCbl and H_2OCbl with 0.2 M $NaHCO_3$ at pH 9.0 was not accompanied by the appearance of a blue form.

Incubation of CNCbl in 0.2 M $NaHCO_3$ at pH 9.0 and 50 °C for 1 week was compared to the same procedure, where the

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monocarboxylic acids *b*, *c*, *c*(C8OH), *d*, and *e* were used as starting materials. The absorbance spectra of these samples after incubation for 1 week are shown in Figure 2A. As the blue corrinoid has a specific absorption at longer wavelengths (see below and Figure 3A), its presence could be easily discovered even in a crude mixture, as shown for for CNCbl, *d*, and *e* forms (group a in Figure 2A). The derivative *c*(C8OH) did not undergo a change in its original spectrum, whereas the "ordinary" *c* acid showed an increase in absorbance at 400–500 nm, which probably indicates a partial self-reduction though without formation of the blue form. The *b*-carboxylic derivative generated a considerable amount of a yellow corrinoid (with an absorbance peak at ≈470 nm) but no blue forms. Synthesis of the blue corrinoid was apparently unaffected by a decreased level of oxygen, which was removed by vacuum and nitrogen bobbling (hydrolysis under nitrogen).

Exploratory studies of the conversion of the blue corrinoid to red derivatives were conducted. The blue corrinoid (7,8-*s*CNCbl) itself was not stable under the conditions of the reaction and underwent a gradual conversion to red corrinoids. In fact, treatment of the purified blue corrinoid at pH 9 (50 °C) or pH 12 (37 °C) caused a change in color from blue to yellow-red with the approximate half-reaction times ($t_{1/2}$) of 10 days and 0.8 day, respectively. This reaction was seemingly accompanied by further transformations (probably self-reduction), because a pronounced absorbance peak was found at 460 nm (Figure 2B).

Addition of $NaBH_4$ powder in trace amounts to a solution of 7,8-*s*CNCbl also caused a change in color in 4–5 min, compatible with a successful reduction. The spectral transition resembled the blue → red transformation at alkaline pH, discussed above (Figure 2B). The "red corrinoid" isolated by TLC in acetate medium (see Experimental Procedures) had a stable color, which did not change after its incubation in bicarbonate buffer. This red corrinoid appears to be a monocarboxylic derivative of B_{12}. Its structure is currently under investigation.

Spectroscopic Characteristics. Absorbance and CD Spectra of the Blue Corrinoid. The absorbance spectrum of the blue corrinoid (7,8-*s*CNCbl,) strongly deviated from those of all known corrinoids, e.g., CNCbl:[4,26] its α-band displayed a maximum at 615 nm. UV–vis ($c = 5 \times 10^{-5}$ M, H_2O): λ_{max} (log ε) 615 (3.84), 489 (3.72), 373 (4.26), 317 nm (4.14) (see Figure 3A, thick solid line). The shift to a longer wavelength increased even further (e.g., of the α-band to ∼650 nm) after conversion of the blue monocyano derivative to its dicyano form (Figure 3A, thick dashed line).

Similar shifts to longer wavelengths were observed in the corresponding CD spectra (Jasco J715; $c = 5.35 \times 10^{-5}$ M, H_2O): λ_{max}, λ_{min}, 600.5 (12.0), 530.5 (−1.1), 414 (−19.8), 377 (−43.3), 329.5 (27.6), 271.5 (−31.4), 231 nm (54.6) (mol^{-1}, cm^3, cm^{-1}); λ_0, 538.5, 522, 444, 351, 295, 255 nm (see Figure 3B). The observed pronounced red shift of all the major peaks (+65 nm for the α-band) indicated expansion of the existing conjugated system.

Mass Spectrometric Characteristics. ESI mass spectra indicated that 7,8-*s*CNCbl is a complete corrinoid with an M_w of 1370.5. High-resolution ESI mass analysis of the $[M + H]^+$ ion of 7,8-*s*CNCbl provided an *m*/*z* value of 1370.5384, corresponding to $C_{63}H_{86}O_{16}N_{13}CoP$ (calcd *m*/*z* 1370.5385, giving a molecular formula of $C_{63}H_{85}O_{16}N_{13}CoP$). Therefore, 7,8-*s*CNCbl, compared to vitamin B_{12}, contains one fewer nitrogen and one fewer hydrogen atom per molecule, but two additional oxygen atoms. In the FAB mass spectrum of 7,8-*s*CNCbl, the most dominant peak is at *m*/*z* 1344.49, arising from a fragment ion due to loss of the cyanide ligand. A weak peak at *m*/*z* 1370.42 corresponds to the pseudomolecular ion $[M + H]^+$ (in the ESI mass spectrum, this peak is the base peak).

NMR Spectroscopic Characteristics and Solution Structure. Full assignments of signals of ^1H and ^{13}C spectra of the blue corrinoid (7,8-*s*CNCbl) were obtained from ^1H–^{13}C heteronuclear (HSQC and HMBC) and ^1H–^1H homonuclear correlations (COSY and ROESY).[36] The spectra were recorded in D_2O, or for the assignment of exchange labile protons, in a H_2O/D_2O mixture (see Figure 4). Because of

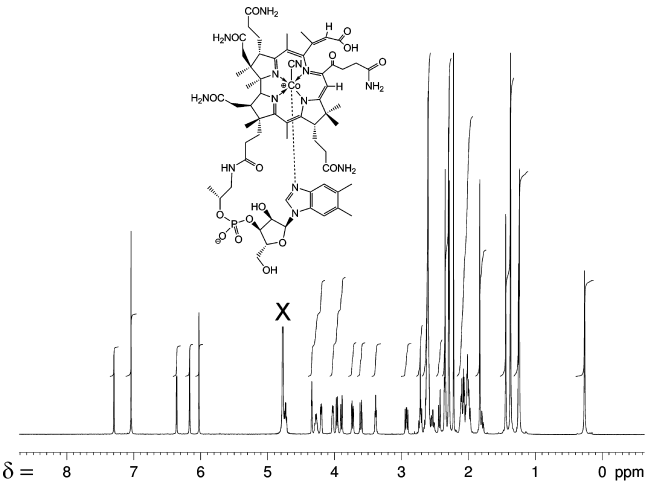

Figure 4. ^1H NMR spectrum (500 MHz) of the blue corrinoid (7,8-*s*CNCbl) in D_2O (25 °C; X marks the residual signals due to solvent).

signal overlap in the high-field region in aqueous solution, additional ^1H NMR spectra of 7,8-*s*CNCbl were recorded in CD_3OD and CD_3OH (see Figure S2 of the Supporting Information).[43] The ^1H NMR spectrum of 7,8-*s*CNCbl in D_2O revealed diagnostic differences, when compared to the spectrum of vitamin B_{12} (CNCbl).[41,42] Specifically, an additional signal at 6.16 ppm was observed in the spectrum of 7,8-*s*CNCbl. Its directly bound carbon had a chemical shift of 130.4 ppm. Such a pair of chemical shift values would be typical for a (new) vinyl-CH group. In the high-field region of the ^1H NMR spectrum of 7,8-*s*CNCbl, several characteristic signals of the *d*-side chain of the CNCbl spectrum were missing, such as H8, or were shifted to lower field [H81 and H82 (see Figure 1 for the deduced structure and for the atom numbering of 7,8-*s*CNCbl)].

With the help of two-dimensional, homo- and heteronuclear NMR analyses of spectra of 7,8-*s*CNCbl, 84 protons and 62 carbons were assigned (see Table S2 of the Supporting Information for the data for measurements in D_2O). In this way, all but the cyanide carbon and a proton of the *c*-acid function were identified. The data indicated that only the region of ring B appeared to be modified significantly, requiring more specific NMR investigations (as described in the Supporting Information and Figure S3). In short, the molecular constitution of the blue corrinoid (7,8-*s*CNCbl) was completely (and nearly unambiguously) established, and a detailed picture of the solution structure was obtained from the NMR spectra. Only, the proposed *Z* configuration of the unsaturated *c*-side

chain remained tentative. In contrast, the structure and the orientation of the *d*-side chain toward the "lower" face of the corrin ligand were deduced beyond any doubt. As the *d*-side chain showed no NOE's to methyl group 7A, while NOE's between the *c*- and *d*-side chains were observed, both of them were shown to be oriented to the same (α) face of the corrin ligand. This implied a different orientation of the *c*-side chain, compared to that in vitamin B_{12}. The blue corrinoid was thus shown to represent a ring-modified 7,8-*seco*-CNCbl in a typical base-on form (as depicted in Figure 1B). Indeed, its NMR-derived three-dimensional structure, as well as its gross conformational properties, turned out to be completely compatible with those derived (subsequently) by X-ray crystallography.

Crystal Structure. The crystal structure of 7,8-*s*CNCbl was determined using synchrotron radiation X-ray diffraction data extending to a resolution of 1.0 Å. The asymmetric unit of the monoclinic crystal contained one B_{12} molecule, one acetone, and seven partially disordered water molecules (crystallographic residuals at the close of the refinement are given in Table S1 of the Supporting Information).

The crystal structure provided the three-dimensional molecular structure of 7,8-*s*CNCbl (see Figure 5 and Figure S4 of the Supporting Information) and revealed relevant differences, when compared to that of CNCbl[3] (see Figure 6), based on the geometry and the refined adp's (atomic displacement parameters). Ring B of 7,8-*s*CNCbl was confirmed to be broken up between atoms C7 and C8. At C8, a planar carbonyl group was present at C8, with a carbon–oxygen distance of 1.23(1) Å. The former *c*-side chain now exhibited a double bond between C7 and C71 [1.34(1) Å] in a *Z* configuration. The carboxamide of this side chain was converted to a carboxylic acid group. Atom type assignment (N vs O) was based on atomic displacement parameters indicating the presence of two oxygen atoms. One of these terminal oxygen atoms was treated as protonated, based on the respective distances to C72 [1.29(1) Å for O73A vs 1.23(1) Å for O73B], as well as the nonbonding interaction of O73A with the phosphate group of a neighboring, symmetry-equivalent B_{12} molecule (with a distance between O73A and O5P of 2.5 Å). The X-ray crystal analysis thus confirmed the NMR-deduced structure and provided detailed insights into the bonding properties in the blue corrinoid, 7,8-*s*CNCbl (a stereoview is displayed in Figure S5 of the Supporting Information).

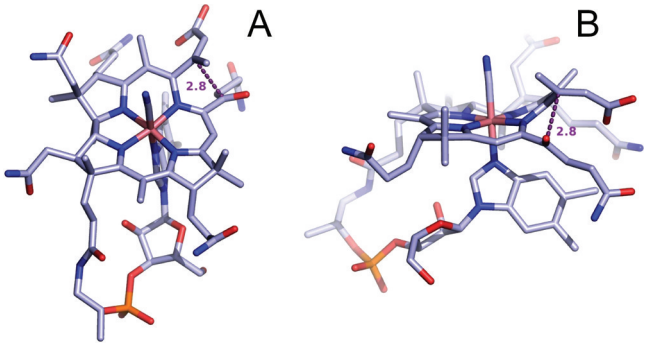

Figure 5. Crystal structure of 7,8-*s*CNCbl shown as a sticks in two different views (A and B). C, N, O, and P atoms are colored light blue, blue, red, and orange, respectively. H atoms have been omitted for the sake of clarity. The distance between atoms C7 and C8 is given in angstroms. This figure was prepared using PyMOL (http://www.pymol.org/).

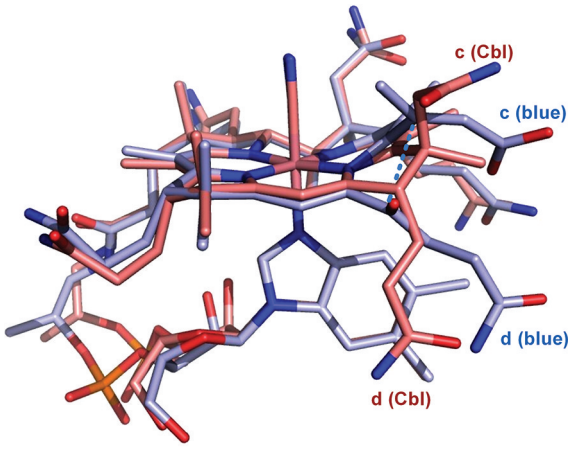

Figure 6. Overlay of crystal structures of CNCbl (vitamin B_{12}, pink) and of 7,8-*s*CNCbl (light blue). The structures were superimposed using the four corrin nitrogen atoms. Pyrrole rings B and C are placed in front of the image. The largest deviations are evident in the area of pyrrole ring B, where side chains *c* and *d* are indicated. This figure was prepared using PyMOL (http://www.pymol.org/).

The geometry of the inner coordination sphere of the Co atom was found to be similar to that in CNCbl,[3,44] except for a slightly elongated bond to the N2 atom of the corrin ring (see Figure 6). Bond distances in the equatorial plane are 1.886(9) Å (Co–N1), 1.965(8) Å (Co–N2), 1.897(8) Å (Co–N3), and 1.914(8) Å (Co–N4). The distances to the axial ligands are 1.887(11) Å (Co–Cβ) and 2.012(8) Å (Co–Nα). The Co atom is slightly out of the plane of the four corrin nitrogen atoms and is shifted by 0.021(4) Å toward the α-dimethylbenzimidazole (DMB) ligand. The conformation of the nucleotide loop is comparable to the one observed in the structure of CNCbl.[3] Slight changes are observed in the conformation around the phosphate group, which are most likely caused by differences in crystal packing (see Figure 6).

A number of polar interactions were observed between symmetry-equivalent B_{12} molecules in the crystal structure (see Figure S5 of the Supporting Information). The *c*-carboxylic acid group is hydrogen bonded to the phosphate group as well as to the *a*-acetamide group of symmetry-equivalent molecules. The newly introduced oxo group at C8 does not form any polar interactions. The cyanide ligand also accepts a hydrogen bond from the ribose hydroxymethyl group of a neighboring molecule. Of the seven water molecules in the final structure, only two are very well-defined with atomic displacement parameters comparable to those of the B_{12} molecule. These water molecules are interacting with the *e*-propionamide as well as with the amide and the phosphate group of the nucleotide loop. One of the waters forms an additional hydrogen bond to the *b*-propionamide group of a neighboring molecule. An acetone molecule is sequestered among the DMB moiety, the C15 methyl group, and the *d*-side chain (including the C8 oxo group) of different B_{12} molecules. In addition, its carbonyl group is hydrogen bonded to the *a*-acetamide group of another neighboring B_{12} molecule.

Interaction with the Specific Transporting Proteins. The blue corrinoid (7,8-*s*CNCbl) and the Cbl *b*-, *c*-, *d*-, and *e*-monocarboxylic acids were used to test the binding sites of the three specific transporting proteins, IF, TC, and HC, where the

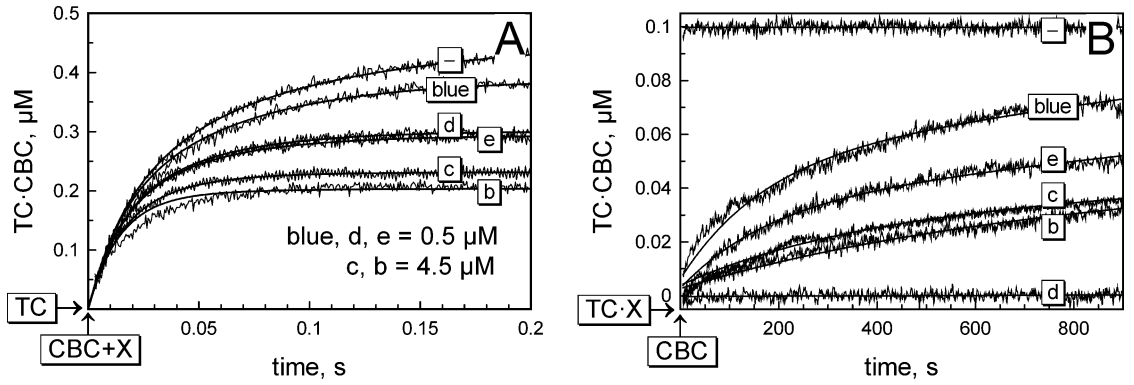

Figure 7. Binding to and dissociation from TC. (A) Binding reactions. The specific protein TC (0.5 μM) was mixed with the fluorescent ligand CBC (0.5 μM) and the tested monocarboxylic acid derivative [none, 4.5 μM for *b* or *c*, 0.5 μM for *d* or *e* (blue)], whereupon the change in fluorescence was recorded (0.2 M P_i buffer at pH 7.5 and 22 °C). The signal was recalculated to the concentration of the TC·CBC complex using the amplitude of the TC + CBC reaction. The fitting simulations are shown as solid lines. The calculated rate coefficients are listed in Table 1. (B) Dissociation reaction. TC (0.1 μM) was mixed with the tested ligand (none or 0.1 μM, 2 min), whereupon CBC (0.1 μM) was injected and the change in fluorescence was recorded. The signal was recalculated to the current TC·CBC complex concentration using the maximal amplitude of the TC + CBC reaction. The fitting simulations are shown as solid lines. The calculated rate coefficients are listed in Table 1.

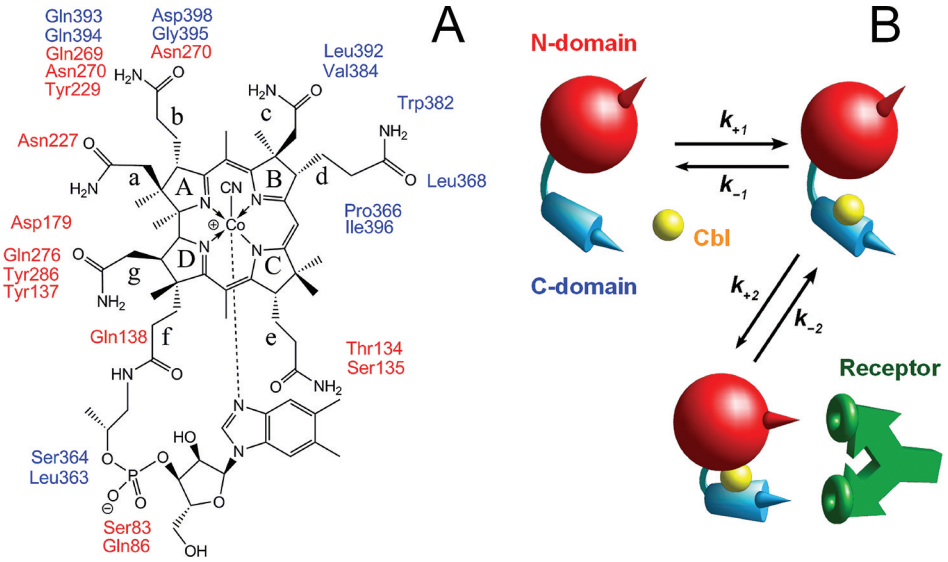

Figure 8. Major interactions of CNCbl with the specific transporting proteins. (A) Formula of vitamin B_{12} (CNCbl) and major interactions with amino acid residues of TC:[14] red for the N-terminal domain of TC and blue for the C-terminal domain of TC. The residues are numbered according to mature bovine TC, the structure of which was determined first.[14] (B) General scheme of interaction between a specific transporting protein and a corrinoid. The ligand binds to the high-affinity C-terminal domain, and the N-terminal domain stabilizes the sandwichlike complex. The two assembled domains form a receptor-compatible interface in the case of IF[48] and, possibly, TC.

specificity for Cbl decreases in the following order: IF > TC > HC.[9,10,15] The binding and dissociation reactions were followed indirectly by monitoring the response from a fluorescent derivative CBC (CNCbl with a conjugated and fluorescent rhodamine appendage[10,39]).

The binding of different corrinoids to a B_{12}-binding protein was tested in a competitive assay, where CBC and the examined ligand X were simultaneously injected into the protein (all at final concentrations of 0.5 μM) (see an example in Figure 7A). Binding of the reporting ligand CBC to the protein caused an increase in fluorescence, which was affected by the presence of a competing ligand. The observed binding kinetics obeyed the scheme of an effectively irreversible process under conditions of the experiment and within the time scale of 0.2 s:

$$E{\cdot}X \leftarrow X + E + CBC \rightarrow E{\cdot}CBC$$

where E is the binding protein, CBC is the fluorescent probe, and X is the tested ligand. Examples of the recorded binding curves for TC are presented in Figure 7A. The rate constants were calculated by computer fitting, and the results are listed in Table 1.

Dissociation from the specific protein was registered during the course of displacement of the tested ligand by CBC, where the preformed E·X complex (preincubation for 2 min) was mixed with added CBC. Dissociation of X and binding of CBC were accompanied by an increase in fluorescence. The examples of displacement curves over time are shown for TC in Figure 7B. The protein ligand interactions followed in most cases the scheme with at least two sequential steps discussed previously[45] (see Figure 8B). It should, however, be mentioned that the high-affinity ligands (e.g., Cbl; $K_d \approx 10^{-15}$ M) seemingly bind to and dissociate from the proteins in one step, probably because of a very fast second transition. The full reaction scheme (see below) included three steps and five rate

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constants:

$$E_2{\cdot}X \leftrightarrow E_1{\cdot}X \leftrightarrow X + E + CBC \rightarrow E{\cdot}CBC$$

The interaction between CBC and the protein (E) has been described as an irreversible single-step process, being a fair assumption under the conditions used in the experiment. The rate constants of primary binding k_{+1X} and k_{+CBC} were determined during the binding experiment (Figure 7A). The three remaining constants were evaluated by repeated iterative computer fitting, in which the reaction with trial rate constants was simulated first as E + X binding (2 min) and then as dissociation of X and binding of CBC. The values of rate constants were adjusted at each cycle of the simulations until the proper approximation of curves was reached (see examples in Figure 7B). The results are listed in Table 1, together with the constant of half-saturation ($K_{1/2}$) that is equal to $K_1/(1 + 1/K_2)$, where K_1 and K_2 are the k_{-1}/k_{+1} and k_{-2}/k_{+2} ratios, respectively.

DISCUSSION

Vitamin B_{12} (CNCbl) is rather stable under neutral and slightly acidic aqueous conditions,[4,27] whereas it readily degrades to various derivatives in an alkaline medium.[4,18] Introduction of bicarbonate into a slightly alkaline medium produced an unexpected outcome. Under these conditions, vitamin B_{12} was partially degraded and an unusual polar Cbl derivative was detected, because of its intensive blue color.

Most essential aspects of the structure of the unprecedented blue corrinoid (7,8-*s*CNCbl) were unambiguously deduced by NMR spectroscopic investigations, as delineated in Results. A disconnected B-ring with a double bond (C7═C71) and a ketone group (C8) at the points of disconnection was revealed. Extension of the original conjugated system in 7,8-*s*CNCbl by the two novel elements caused an unanticipated 60−65 nm red shift of the long-wave peaks (Figure 3B).

The X-ray structure of 7,8-*s*CNCbl crystallized from aqueous acetone is now available at 1 Å resolution, and it was compared to that of CNCbl.[3,44] The new unsaturated vinyl and carbonyl functionalities at C7 and C8 are both twisted out of the plane of the conjugated π-system of the corrin ring, with torsion angles of −64.1° (C5−C6−C7−C71) and −47.3° (C10−C9−C8−O8A), respectively. The unsaturated centers at C7 and C8 have remarkably short contacts (2.8 Å). This pushes C7 far above the calculated plane of the remaining corrin ring and locks the conformation of the unsaturated *c*-side chain. Mostly because of these changes in ring B, the fold angle[3] of the corrin ring in 7,8-*s*CNCbl is increased to a record value of 37.0(3)°. Indeed, compared to the available Cbl structures, the *c*-side chain and methyl group C7A have swapped their orientations. In 7,8-*s*CNCbl, this methyl group now points upward, toward the β-cyano ligand, whereas the carboxylic acid moiety is oriented toward the DMB base (see Figure 5 and Figure S4 of the Supporting Information).

The actual chemical steps involved in the formation of the blue corrinoid (7,8-*s*CNCbl) are far from being clear, yet on the basis of our experimental observations, several key steps may be pointed out. First of all, dissociation of the proton from C8 is required. (Self-)reduction of Cbl is suggested as another important step, because coordination of external CN^- and formation of the dicyano-Cbl anion preclude generation of the blue derivative (presumably by hindering cobalt reduction). AdoCbl was also a poor source of the blue ligand (probably because of a higher electron density at the cobalt ion provided by the adenosyl group). However, at pH 10−12, this resistance was overcome, and a still uncharacterized blue corrinoid was derived from AdoCbl.

The further formal steps require cleavage of a stable (C−C) bond between saturated carbon centers at β-positions C7 and C8 of CNCbl, as well as introduction of two novel double bonds (C7═C71 and C8═O). Any precedence for such a reaction in the B_{12} field is lacking. However, such types of transformations may be achieved in radicaloid or in carbocationic species, examples being provided by, for example, "Baeyer−Villiger"-type reactions (with percarboxylic acids) or "Grob"-type fragmentations.[46] Indeed, in the observed partial degradation process of CNCbl, the "activated" C8 atom may be a radical (after self-reduction of Cbl) or an electrophilic center (e.g., via self-reduction and reduction of another Cbl).

Different variants of the reaction were examined in an exploratory way, to gain insights into its mechanism. Surprisingly, a decrease in the level of oxygen in the medium had a marginal effect on the obtained yield of the blue corrinoid, suggesting that molecular oxygen played a lesser role in this transformation. On the other hand, bicarbonate appeared to assist in this unusual transformation. The absence of bicarbonate in the medium switches the whole pathway to the "normal" reaction sequence of alkaline hydrolysis,[4,18] where traces of the blue form could still be found, probably because of a small amount of dissolved atmospheric CO_2.

Comparison of the reaction progress for Cbl and its monocarboxylic derivatives showed that *d*- and *e*-acids were good precursors of the blue corrinoid (Figure 3A). Quite surprisingly, *b*-monocarboxylic acid produced no blue derivative at all. At the same time, a significant amount of a yellow corrinoid did accumulate, in contrast to all other reactions tested. It seems that *b*-COOH accelerates synthesis of other products from the reduced Cbl. The reaction sequence for the formation of "yellow corrinoids" leads to interruption of the original conjugation pattern between C5 and C6.[30,31] Typically, this transformation involves the *c*-chain, which makes it unavailable for reactions at position C8. The *c*-acid derivatives [*c* and *c*(C8OH)] were also insensitive to bicarbonate treatment (Figure 3A). This can be rationalized by the presence of a carboxylic group in the proximity of C8 (possibly interfering with bicarbonate action), where *c*(C8OH) cannot even undergo self-reduction.

Qualitatively, the kinetics of formation and decomposition of the blue corrinoid (7,8-*s*CNCbl) could be described as CNCbl → 7,8-*s*CNCbl → red (at pH 9 and 50 °C, k_{+1} = 0.01 day^{-1} and k_{+2} = 0.12 day^{-1}) based on accumulation of the blue derivative over time (3, 5, and 6% after 7, 14, and 21 days, respectively) and its decay measured in separate experiments (e.g., the one shown in Figure 2B). The formation of 7,8-*s*CNCbl was only slightly dependent on the bicarbonate concentration if we were working in the interval of 0.1−0.5 M. The decomposition was pH-dependent. Thus, the blue corrinoid was stable at neutral and acidic pH, but it underwent additional transformations in the alkaline medium leading to regeneration of the red color. The obtained product was a red monocarboxylic acid. Conversion of the color could also be achieved in a few minutes by reduction with $NaBH_4$, presumably by reduction of at least the keto group at C8. The discussed reductive conversions and the structural assay of the red product are the subjects of ongoing investigations.

The ease of formation of the unusual blue corrinoid (7,8-*s*CNCbl) under mild reaction conditions raises the question about its ability to bind to proteins of Cbl transport. Interactions between corrinoids and the specific transporting proteins (IF, TC, and HC) involve multiple contacts of the corrin amide side chains with the protein (Figure 8A shows an example of such contacts for the TC·Cbl complex[14]). The two-domain structure (Figure 8B) was hypothesized for all proteins of Cbl transport based on the binding studies conducted for two large IF fragments.[45,47] This suggestion was corroborated by the structural analysis of the high-resolution crystals produced first for TC[14] and afterward for IF.[17] According to the suggested binding scheme,[45] the corrinoid ligand interacts at the first step with the high-affinity C-terminal domain, whereupon the two domains are assembled into a sandwichlike structure with Cbl placed in the center. The assembly of two domains by a ligand is critical for recognition by the receptor of the saturated IF[47,48] and possibly TC.

In our previous binding assay, the base-on, base-off, and baseless corrinoids were tested.[10] We found that the primary attachment is insensitive to the correct positioning of the nucleotide base (or even its total absence). The presence and proper orientation of the base become, however, important at the second step in which final adjustments at the domain−domain interface take place.[10]

In this work, several monocarboxylic acids (*b*, *c*, *d*, *e*, and 7,8-*s*CNCbl) were used to probe the binding sites of IF, TC, and HC. In contrast to the corrinoids with a modified nucleotide base, introduction of a carboxylic group at one of the side chains was accompanied by noticeable changes in both k_{+1} and $K_{1/2}$ (Table 1). The observed kinetic patterns helped to verify the scheme in Figure 8, where the *b*-, *c*-, and *d*-chains bind to the C-domain (step 1) and the *a*-, *b*-, *e*-, *f*-, and *g*-chains interact with the N-domain (step 2). It was found that the *d*-COOH substitution produced just a minor effect on k_{+1} and $K_{1/2}$ because only one contact with the C-domain was affected. The *e*-COOH derivative bound with a high k_{+1} to all proteins as no interaction between the *e*-chain and the C-domain takes place in step 1, yet the overall affinity for IF and TC ($K_{1/2}$) was low because the binding of the N-domain to the *e*-chain was disturbed (step 2). Modification of the *b*-amide affected both binding steps (Table 1) because multiple contacts with both domains were disrupted. Introduction of *c*-COOH instead of *c*-amide also led to a general deterioration of affinity caused by repulsion from negative charges on the C-domain in the corresponding binding region of all proteins. The blue corrinoid (7,8-*s*CNCbl) with a broken B-ring and misplaced *c*-acid and *d*-amide bound very poorly to IF and TC. At the same time, its affinity for HC was actually better than that of the normal *c*-acid, probably because the carboxylic group was moved aside from its proximity to the negatively charged Glu-380 of HC.

A structural model of the interaction of the blue corrinoid (7,8-*s*CNCbl) with the most specific protein intrinsic factor is shown in Figure 9. It is based on the crystallographic analysis of IF−Cbl complexes,[17,48] where the "western" half of the corrin ring mainly interacts with the side chains of the α-domain (pink) and the "eastern" half contacts the β-domain (light blue). The structure of blue B_{12} was superimposed onto the cobalamin bound to human intrinsic factor (purple in Figure 9) using the four nitrogen atoms of the corrin ring. The conformations of amide side chains *a*, *b*, and *d*−*f* are slightly different in blue B_{12}, most likely because of interactions within

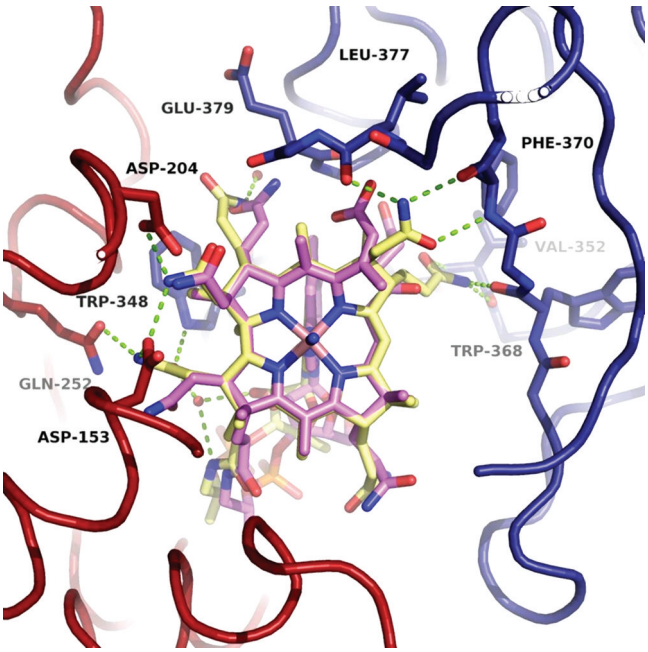

Figure 9. Structural model of major interactions of CNCbl and 7,8-*s*CNCbl with the mammalian transporting protein intrinsic factor (IF). Schematic representation of the polar interactions between human intrinsic factor and a bound cobalamin (Protein Data Bank entry 2pmv[17]). The N-terminal α-domain is colored pink and the C-terminal β-domain light blue. Amino acid residues interacting with the amide side chains of the bound cobalamin (yellow) are shown as sticks. Hydrogen bonds are shown as green dashed lines. The structure of blue B_{12} superimposed onto the bound cobalamin is colored purple. This figure was prepared using PyMOL (http://www.pymol.org/).

the crystal, yet this minor change will not impair the binding of the blue derivative. The carboxylic acid group in the *c*-side chain, however, can obviously not form the same polar interactions as the carboxamide group of the unmodified cofactor. In addition, the flexibility of the side chain is restricted because of the double bond and the tight interaction between C7 and C8. In the conformation observed in the crystal structure, the carboxylate group would severely clash with main chain atoms of Leu-377 and Glu-379 of the C-terminal domain of intrinsic factor hindering successful ligand binding.

The mentioned distortions in 7,8-*s*CNCbl lead to a great loss of the affinity for all proteins, including HC (the protein with the lowest specificity). The evaluated constants of half-saturation (Table 1) indicate that 7,8-*s*CNCbl will bind under physiological conditions to only HC. Weak binding to TC is possible, but the presence of HC in blood will cause fast abstraction of the blue corrinoid from TC.

One can speculate about whether B_{12} derivative 7,8-*s*CNCbl might be useful for drug conjugation, because cancer cells accumulate large amounts of Cbl to satisfy their demands in synthetic processes.[19,49,50] The mechanism of corrinoid accumulation by tumors is presently under review,[49] and HC (or a protein with properties resembling those of HC) was recently selected as the binding target in cancer cells.[49] The blue corrinoid will interact with HC only under physiological conditions, and it might provide a promising starting material for conjugation via a carboxylic group. A relatively weak interaction with HC might be an additional advantage, because it will facilitate exchange of the ligand between different pools

of HC (e.g., blood pool and tumor pool) preventing it from being trapped by HC in blood.

CONCLUSIONS

A novel corrinoid with an atypical blue color was produced during the course of mild alkaline hydrolysis in the presence of bicarbonate. The blue corrinoid was the one of the major products among a great variety of other usual derivatives of Cbl hydrolysis. The physicochemical and spectral characteristics of the novel compound were described. The major structural changes included hydrolysis of the *c*-amide and an unprecedented opening of the pyrrole B-ring. This is accompanied by the introduction of two double bonds that contribute to the existing conjugation pattern, causing the atypical color of the blue derivative. The patterns of interaction of this new corrinoid with the proteins of Cbl transport were used to verify the two-step model of protein−corrin interactions. The potential applicability of the novel corrinoids as drug carriers is indicated.

ASSOCIATED CONTENT

Supporting Information

A more detailed description of NMR data as well as the scheme of purification of 7,8-*seco*-cyanocobalamin. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

CCDC 818044 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from http://www.ccdc.cam.ac.uk/data_request/cif, by contacting data_request@ccdc.cam.ac.uk or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, U.K.

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ABBREVIATIONS

AdoCbl, adenosylcobalamin; adp, atomic displacement parameter; CBC, fluorescent conjugate of Cbl; Cbl, cobalamin; CNCbl, cyanocobalamin; Cbi, cobinamide; Cba, cobamide; DMB, 5,6-dimethylbenzimidazole; HC, haptocorrin; IF, intrinsic factor; P_i buffer, sodium phosphate buffer; 7,8-*s*Cbl, 7,8-*seco*-cobalamin; 7,8-*s*CNCbl, 7,8-*seco*-cyanocobalamin; TC, transcobalamin.

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