Decyanation of vitamin B₁₂ by a trafficking chaperone

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The mystery of how the cyanide group in vitamin B₁₂ or cyanocobalamin, discovered 60 years ago, is removed, has been solved by the demonstration that the trafficking chaperone, MMACHC, catalyzes a reductive decyanation reaction. Electrons transferred from NADPH via cytosolic flavoprotein oxidoreductases are used to cleave the cobalt–carbon bond with reductive elimination of the cyanide ligand. The product, cob(II)alamin, is a known substrate for assimilation into the active cofactor forms, methylcobalamin and 5'-deoxyadenosylcobalamin, and is bound in the "base-off" state that is needed by the two B₁₂-dependent target enzymes, methionine synthase and methylmalonyl-CoA mutase. Defects in MMACHC represent the most common cause of inborn errors of B₁₂ metabolism, and our results explain the observation that fibroblasts from these patients are poorly responsive to vitamin B₁₂ but show some metabolic correction with aquocobalamin, a cofactor form lacking the cyanide ligand, which is mirrored by patients showing poorer clinical responsiveness to cyano- versus aquocobalamin.

cobalamin | flavin oxidoreductase | methylmalonic aciduria | homocystinuria | cyanide

Vitamin B₁₂ (or cyanocobalamin, CNCbl) was discovered exactly 60 years ago in the laboratories of Folkers and Smith as the anti-pernicious anemia factor (1, 2), and its structure was revealed a few years later by the crystallographic studies in the Hodgkin laboratory (3). B₁₂ is one of the most complex cofactors in nature and belongs to the family of tetrapyrrolic-derived macrocyclic compounds. Five of the six ligands to the central cobalt atom in free B₁₂ are endogenous, being provided by the corin ring itself (Fig. 1A). In CNCbl, the sixth ligand, which occupies the upper axial position, is a cyano group. Although the biological origin of the cyano group in vitamin B₁₂ is unknown, it is the cofactor form commonly used in multivitamin formulations and one that individuals on vitamin supplementation are regularly exposed to. Decyanation of CNCbl is a prerequisite for its conversion to the active cofactor forms used by mammals in which the cyano ligand is replaced by a methyl group (in methylcobalamin) or a 5'-deoxyadenosyl group (in coenzyme B₁₂ or 5'-deoxyadenosylcobalamin) (Fig. 1B).

Defects in B₁₂ metabolism are inherited as autosomal recessive disorders and are classified as belonging to one of eight genetic complementation groups, *cblA*–*G* and *mut* (4). Two loci, *cblG* and *mut*, encode B₁₂-dependent enzymes: methionine synthase, which is cytoplasmic, and methylmalonyl-CoA mutase, which is mitochondrial. The remaining loci encode functions needed for intercompartmental trafficking and assimilation of the cofactor into its active forms. B₁₂ is present at relatively low concentrations in human tissues (5) and is reactive in all three of its biologically relevant oxidation states, factors that pose challenges for its unescorted delivery to target enzymes (6). The problem is further exacerbated by the use of the "base-off/His-on" conformation of the cofactor by both mammalian enzymes, in which the lower axial dimethylbenzimidazole ligand is not coordinated to the cobalt and is replaced instead by a histidine residue donated by the respective proteins (7, 8) (Fig. 1B). The "base-off" species is rare at physiological pH, its concentration being governed by the pK_a for protonation of the lower axial base [–2.13, 0.1, 2.9, and 3.7 for aquocobalamin,[†] CNCbl, methyl-, and 5'-deoxyadenosylcobalamin, respectively (9)]. We have pro-

posed that to circumvent problems of poor cofactor availability in the needed conformation, mammalian B₁₂-binding chaperones and processing enzymes stabilize the base-off state and deliver the cofactor to target enzymes (6, 10). This model is consistent with the observation that very little B₁₂ in mammalian tissues is free; in fact, >95% of B₁₂ is estimated to be protein-bound (11).

Patients with inborn errors of cobalamin metabolism belonging to the *cblC* group exhibit impairments in both B₁₂-dependent enzymatic functions found in mammals, methionine synthase, and methylmalonyl-CoA mutase, which is consistent with the gene product MMACHC (for methylmalonic aciduria *cblC* type with homocystinuria) being involved early in the assimilation pathway (11). The gene corresponding to the *cblC* locus was identified recently, and the encoded protein does not bear any obvious sequence similarity to other proteins with the exception of the very C-terminal domain, which bears resemblance to the bacterial TonB protein (12). The latter serves in the two-component transport systems in Gram-negative bacteria and functions to open the hatch on the periplasmic side of the outer membrane transporter thereby allowing vectorial passage of consignments into the cell (13). By analogy, MMACHC is postulated to accept the cobalamin cargo as it exits the lysosomal compartment via an as yet uncharacterized transporter (6).

In this work, we report the discovery that human MMACHC is a CNCbl decyanase, which catalyzes the reductive decyanation of CNCbl by using reducing equivalents furnished by cytosolic diflavin oxidoreductases, namely, methionine synthase reductase and novel reductase 1 (NR1). Furthermore, we demonstrate that the product of the decyanation reaction, cob(II)alamin is bound to MMACHC in the base-off conformation, which is consistent with the proposed model for targeted intracellular delivery of B₁₂ from chaperones to cobalamin-dependent enzymes.

Results and Discussion

MMACHC Binds Cobalamin. We have purified recombinant human MMACHC to >95% homogeneity, and based on analytical ultracentrifugation studies, we found that it exists as a monomer with a native molecular mass of ≈29 kDa. As isolated, the protein is devoid of color and a visible absorption spectrum and does not have bound metal as revealed by metal analysis (data not shown). Binding of CNCbl to MMACHC elicits very minor absorption changes in the visible spectrum (Fig. 2A), suggesting that the cofactor remains in the "base-on" state upon binding. Isothermal titration calorimetry demonstrates that MMACHC binds CNCbl stoichiometrically ($n = 1.2 \pm 0.1$) with a K_d of $15.5 \pm 1.1 \mu\text{M}$ (Fig. 2B). Apparently, the available energy derived from cofactor binding is insufficient for converting

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[†]Aquo- and hydroxocobalamin differ by the protonation state of the upper axial ligand, i.e., water versus hydroxide ion. The pK_a for this transition is 7.8 (9). Hence, a mixture of species exists at physiological pH with aquocobalamin being the major one.

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Fig. 1. Structures of B₁₂ derivatives. (A) Structure of free base-on cobalamin showing that five of the six ligands are provided by the corrin ring. The lower axial ligand is the endogenous base, dimethylbenzimidazole. The upper axial ligand is variable, as indicated. MeCbl, AdoCbl, and H₂OCbl refer to methylcobalamin, deoxyadenosylcobalamin, and aquacobalamin respectively. (B) Conversion of vitamin B₁₂ to the active cofactor forms of B₁₂ requires a decyanation step. The product, cob(II)alamin, can be used for the synthesis of MeCbl or AdoCbl, which are bound in the base-off/His-on state in methionine synthase and methylmalonyl-CoA mutase, respectively. The differences in the UV-visible absorption spectra for these cobalamins lead to orange or red compounds as denoted.

base-on CNCbl to the base-off form, a transition that occurs with a pK_a that is ≈ 7 units below physiological pH (9). In contrast, the pK_a for protonation of base-on 5'-deoxyadenosyl- and methylcobalamin are ≈ 3 and 4 units, respectively, below physiological pH (9), and binding of these cofactor derivatives to MMACHC is accompanied by a large blue shift in their α/β bands from ≈ 525 to 460 nm, which is diagnostic of base-off alkylcobalamins (Fig. 3). The apparent nonselectivity of MMACHC for the upper axial ligand of cobalamin is consistent with its postulated role as a porter that accepts B₁₂ derivatives delivered to the lysosomal compartment. We note that alkylcobalamins are very photolabile and therefore easily degraded during processing of foods. Intracellularly, MMACHC is unlikely to be exposed to 5'-deoxyadenosylcobalamin, a cofactor derivative that is synthesized in the mitochondrial compartment. Upon cofactor binding, MMACHC is able to expend the binding energy to achieve the base-off conformation in some, but not all, cobalamin derivatives.

MMACHC Catalyzes the Decyanation of CNCbl. Addition of NADPH and either human methionine synthase reductase (14, 15) or

NR1 (16), members of the dual flavoprotein oxidoreductase family, results in conversion of MMACHC-bound CNCbl to cob(II)alamin, as evidenced by the increase in absorption at 473 nm ($k_{\text{obsd}} = 0.10 \pm 0.004 \text{ min}^{-1}$ and $0.068 \pm 0.007 \text{ min}^{-1}$ for methionine synthase reductase and NR1, respectively) (Fig. 4A). In contrast, reduction of free CNCbl by methionine synthase reductase or NR1 in the absence of MMACHC is not detectable (Fig. 4A *Inset*). Although glutathione was reported to be required for decyanation of CNCbl catalyzed by cell extracts (17), we find that its presence (at a concentration of 1 mM) is without effect on either the rate of decyanation or the product that is formed (data not shown). Unlike titanium citrate, a low potential reductant (18), glutathione is unable to replace the NADPH/diflavin oxidoreductase system as a source of reducing equivalents for decyanation of CNCbl by MMACHC (data not shown). In contrast to CNCbl, neither methyl- nor 5'-deoxyadenosylcobalamin bound to MMACHC undergoes reductive elimination in the presence of NADPH and methionine synthase reductase (data not shown). Very negative potentials ($E \approx$ approximately -1 V versus the standard hydrogen electrode) are needed for

Fig. 2. Binding of CNCbl to MMACHC. (A) UV-visible absorption spectra of 10 μM free CNCbl (dashed line) or bound to 20 μM MMACHC (solid line) in 100 mM Hepes (pH 7.0), 150 mM KCl, and 5% glycerol. (*Inset*) Comparison between base-on (dashed line) and base-off (solid line) CNCbl in water and 6% HClO_4 , respectively. (B) Binding of CNCbl to MMACHC was monitored by isothermal titration calorimetry of 25 μM MMACHC with CNCbl (1–500 μM) as described in *Materials and Methods*. Analysis of the titration data reveals stoichiometric binding and yields a K_d of $15.5 \pm 1.1 \mu\text{M}$.

Fig. 3. Binding of methyl- or 5'-deoxyadenosylcobalamin to MMACHC. UV-visible absorption spectra of 10 μ M free methyl- or 5'-deoxyadenosylcobalamin (dashed line) or bound to 20 μ M MMACHC (solid line) in 100 mM Hepes (pH 7.0), 150 mM KCl, and 5% glycerol are shown.

reducing methyl- and 5'-deoxyadenosylcobalamin (19, 20), which are considered to be outside the range of biological reductants. Based on its enzymatic activity, we propose to rename the MMACHC protein cyanocobalamin decyanase.

Although methionine synthase reductase functions in reductive activation of the cytoplasmic cobalamin-dependent enzyme, methionine synthase, the function of NR1, a paralog, is not known. Mutations in methionine synthase reductase are associated with the *cbI*E class of cobalamin disorders and result in an isolated functional deficiency of methionine synthase, i.e., the mitochondrial cobalamin function is not impaired. The ability of either flavoprotein to reduce cyanocobalamin decyanase-bound CNCbl precludes the need for a dedicated cytoplasmic oxidoreductase for this reaction. In solution, reduction of CNCbl to cob(II)alamin is complex, and the redox potential exhibits a steep dependence on the concentration of cyanide (21).

Base-Off Cob(II)alamin Is the Product of MMACHC-Catalyzed Decyanation. The EPR spectrum of the decyanation product bound to cyanocobalamin decyanase is of base-off cob(II)alamin rather

than the base-on species found in solution (Fig. 4B). A classic point of distinction in the EPR spectra of the two species is in the high-field region where the eight-line hyperfine splitting pattern appears as singlets in the base-off state versus triplets in the base-on state of the free cofactor. This difference in the morphology of the hyperfine lines results from superhyperfine coupling of the unpaired electron on cobalt and a nitrogen atom (with an $I = 1$ nuclear spin) in the base-on species. Furthermore, the spacing between the hyperfine lines increases from 110 G in free base-on cob(II)alamin to 140 G in the CNCbl decyanase-bound base-off state.

The pK_a for protonation of the lower axial base in cob(I-I)alamin, the product of the decyanase reaction, is estimated to be 3.1 (22), which is substantially higher than that for the substrate, CNCbl. The energetically less demanding conversion of base-on to base-off cob(II)alamin appears to be within reach of the binding energy stored in the holo-CNCbl decyanase and yields a product that is in the correct conformation needed by the downstream cobalamin-dependent enzymes, methionine synthase and methylmalonyl-CoA mutase (Fig. 1B).

Conclusions

Our demonstration that human MMACHC is a CNCbl decyanase resolves a question that has endured since the discovery of vitamin B₁₂, i.e., what is the mechanism of biological processing of its cyanide ligand. Although the origin of the cyano ligand in CNCbl is not known, some bacteria have the capacity for cyanide biogenesis, and cyanide is used both as a metal ligand as in hydrogenases and as a secondary metabolite (23). Our results explain the observation that fibroblasts from the *cbI*C class of patients exhibit some metabolic correction when supplemented with aquocobalamin but not with CNCbl, and they exhibit normal internalization of cobalamin, but enhanced efflux of CNCbl compared with aquocobalamin (24). These observations with cultured primary cell lines are mirrored by the poorer clinical and biochemical responses in *cbI*C patients receiving intramuscular CNCbl versus aquocobalamin injections (25, 26). Together with the studies on patients and their fibroblasts (24–26), our results support the proposed model for cobalamin trafficking in which MMACHC accepts the cofactor from the lysosomal compartment and in partnership with a cytoplasmic reductase, catalyzes the reductive elimination of CNCbl to base-off cob(II)alamin, which is a metabolic prerequisite for the assimilation of this cobalamin precursor to its active cofactor forms (Fig. 5). The proposal that CNCbl is converted via the

Fig. 4. Decyanation of CNCbl by MMACHC. (A) Spectrum of MMACHC reconstituted with CNCbl (blue) and its conversion to cob(II)alamin (red) in the presence of methionine synthase reductase and NADPH. (Inset) Rate of cob(II)alamin formation from 20 μ M MMACHC/CNCbl in the presence of 4 μ M methionine synthase reductase (●), 4 μ M NR1 (▲), or in the absence of MMACHC (open symbols). (B) EPR spectra of cob(II)alamin (100 μ M) generated in solution or formed by decyanation of 100 μ M MMACHC/CNCbl as described in *Materials and Methods*.

Fig. 5. Model for the decyanase activity of MMACHC. The dark blue domain in MMACHC represents the C-terminal TonB-like domain, which is proposed to open the “hatch” on the lysosomal transporter, in analogy with the role of TonB in bacterial two-component transport systems (6, 12).

action of a β -ligand transferase to glutathionyl-CoA, which is subsequently converted via a reductase to a highly reactive cob(I)alamin species (17), is not supported by this model. During the synthesis of methyl- and 5'-deoxyadenosylcobalamin, cob(I)alamin is transiently generated from cob(II)alamin bound to methionine synthase and to adenosyltransferase, respectively, thus averting the problem of delivering an exogenously generated and supernucleophilic cob(I)alamin species to these enzymes.

Materials and Methods

Cloning and Expression of MMACHC. The cDNA encoding MMACHC was kindly provided by D. Rosenblatt (McGill University, Montreal, ON, Canada). The gene was cloned into the pET-28b(+) vector (Novagen) by using the NcoI and XhoI restriction enzyme sites. *Escherichia coli* BL21(DE3) transformed with the plasmid construct was grown in Luria-Bertani (LB) medium containing kanamycin (50 μ g/ml) at 37°C, induced with 0.5 mM IPTG (at $A_{600\text{ nm}} = 0.3\text{--}0.7$), and then grown overnight at 20°C.

Protein Purification. Human MSR and NR1 were prepared as described (27, 28). Recombinant MMACHC with a C-terminal His tag was purified from cells (≈ 15 g wet weight) suspended in 100 ml of 100 mM potassium phosphate buffer (pH 7.0) and 5% glycerol (buffer A) containing 0.15 mg/ml lysozyme and one tablet of complete EDTA-free protease inhibitor (Roche). After sonication, the cell lysate was centrifuged and loaded onto a DEAE-Sephacose column, the flow-through was collected, and KCl (150 mM) and imidazole (10 mM) were added to achieve the specified final concentrations and loaded onto a Ni-nitrilotriacetic acid column (Qiagen) equilibrated with buffer A, 150 mM KCl, and 10 mM imidazole. The column was washed extensively, and proteins were eluted with a 10–300 mM imidazole gradient. MMACHC fractions eluted between 100 and 200 mM imidazole and were pooled and dialyzed against 100 mM Hepes (pH 7.0), 150 mM KCl, and 5% glycerol (buffer B).

Decyanation of CNCbl. The anaerobic assay mixture (in 200 μ l of buffer B) contained 200 μ M NADPH and 4 μ M MSR or NR1, and the absorbance baseline was adjusted after a 5-min incubation. The reaction was initiated with 20 μ M premixed CNCbl/MMACHC (1/1 molar ratio). Spectra were recorded at ambient temperature by using a spectrophotometer housed in an anaerobic chamber (Coy Laboratory). The decyanation rate was monitored at 474 or 550 nm and determined by fitting the data to a single exponential function.

Isothermal Titration Calorimetry. MMACHC (30–35 μ M) was titrated at 4°C with $59 \times 5\text{-}\mu$ l injections of CNCbl (1–550 μ M) in buffer B. The K_d was determined by using a single-site binding model and is the average of three independent experiments (29).

EPR Spectroscopy. The EPR sample for decyanation contained MMACHC-CNCbl (100 μ M each), 200 μ M NADPH, and 20 μ M methionine synthase reductase or NR1 in anaerobic buffer B. The mixture was incubated under anaerobic and dark conditions for 30 min at ambient temperature before freezing in liquid nitrogen. Cob(II)alamin reference samples were prepared by photolysis of 100 μ M 5'-adenosylcobalamin in buffer B (base-on) or 1% H_3PO_4 (base-off) as described in ref. 30. Spectroscopic parameters were: temperature, 100 K; microwave power, 20 mW; microwave frequency, 9.38 GHz; receiver gain, 1×10^5 ; modulation amplitude, 10.0 G; modulation frequency, 100 kHz.

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