Synthesis and Properties of Formylcobalamin and Propionylcobalamin, Novel Acylcobalamins

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Summary Formylcobalamin (formyl-Cbl), a C1-unit carrying corrinoid, and propionylcobalamin (propionyl-Cbl) were synthesized for the first time, and their properties were compared with those of acetylcobalamin (acetyl-Cbl). Formyl-Cbl, acetyl-Cbl, and propionyl-Cbl were decomposed by a NH2OH treatment, forming formo-, aceto-, and propionohydroxamic acids, respectively, which offers a proof for the presence of “activated” acyl groups and for their structures of Co-acyl-Cbls. These results, together with chromatographic, electrophoretic, and spectroscopic properties, indicate that the acyl-Cbls synthesized are actually formyl-Cbl, acetyl-Cbl, and propionyl-Cbl. Spectroscopic and electrophoretic properties were consistent with the σ-donor strength or trans-effect increasing in the order: formyl<acetyl<propionyl. All acyl-Cbls underwent the Co-C bond cleavage upon photolysis with a tungsten light bulb as well as upon treatment with alkali, forming aquacobalamin (aqCbl) and hydroxocobalamin (OH-Cbl), respectively, under air. Formyl-Cbl was thermally unstable and decomposed to aqCbl, and the thermal stability in neutral solution was much lower than in diluted HCl. In contrast, acetyl-Cbl and propionyl-Cbl were heat-stable under these conditions. Formyl-Cbl was essentially unsusceptible to unbufferized KCN solution, although acetyl-Cbl and propionyl-Cbl were rapidly converted to dicyanocobalamin under the same conditions. Such abnormal behaviors of formyl-Cbl suggest that it mainly exists in a hydrated form as formaldehyde.

Key Words formylcobalamin, acetylcobalamin, propionylcobalamin, acylcobalamin, vitamin B12

Cobamide coenzymes including adenosylcobalamin (AdoCbl) are naturally occurring organometallic compounds that contain a unique cobalt-carbon (Co-C) σ-bond (Fig. 1) (1–4). Methylcobalamin (MeCbl) was first synthesized as an analog of AdoCbl (5), and was found later to be active in the enzymatic synthesis of methionine by Escherichia coli extracts (6) and to be present in human blood plasma and other natural source materials (7). The metabolic roles and the mechanisms of action of these two forms of vitamin B12 are quite different: AdoCbl participates as a coenzyme in intramolecular group-transfer reactions and ribonucleotide reduction, whereas MeCbl participates in intermolecular methyl transfer reactions (3, 4). To catalyze chemically challenging reactions, the former utilizes the high reactivity of a super-active primary carbon radical which is formed by the homolytic cleavage of its Co-C bond, while the latter a cob(I)alamin (CblI) species whose Co(I) is a super-nucleophile (3, 4).

No organocobalamins, other than MeCbl, carrying a C1 unit have been known to date. In this paper, we wish to report the synthesis of formylcobalamin (formyl-Cbl) for the first time (Fig. 1). Some properties, especially the stability of the Co-C bond, of this novel acylcobalamin (acyl-Cbl) are also described here, compared with those of propionylcobalamin (propionyl-Cbl), another novel acyl-Cbl, and acetylcobalamin (acetyl-Cbl). The synthesis (8–10) and some properties (8, 10, 11) of acetyl-Cbl were reported earlier by other investigators.

MATERIALS AND METHODS

Chemicals. Crystalline cyanocobalamin (CN-Cbl) was obtained from Glaxo Laboratories Ltd. (Greenford, U.K.). All other chemicals were reagent grade commercial products and were used without further purification.

Analytical procedures. The concentrations of acyl-Cbls were determined spectrophotometrically after converting them to dicyanocobalamin [(CN)2-Cbl] by photo-irradiation at 0˚C for 5 min with a 200-W tungsten light bulb as well as upon treatment with alkali, forming aquacobalamin (aqCbl) and hydroxocobalamin (OH-Cbl), respectively, under air. Formyl-Cbl was thermally unstable and decomposed to aqCbl, and the thermal stability in neutral solution was much lower than in diluted HCl. In contrast, acetyl-Cbl and propionyl-Cbl were heat-stable under these conditions. Formyl-Cbl was essentially unsusceptible to unbufferized KCN solution, although acetyl-Cbl and propionyl-Cbl were rapidly converted to dicyanocobalamin under the same conditions. Such abnormal behaviors of formyl-Cbl suggest that it mainly exists in a hydrated form as formaldehyde.

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Filter paper No. 50. The solvent systems used were (C) water-saturated 2-butanol and (D) water-saturated 2-butanol containing 1% acetic acid. Paper electrophoresis was conducted in 0.03 M sodium citrate buffer (pH 3.5) at a voltage gradient of 22 V/cm for 4 h, and the mobility of CN-Cbl was taken as 0.

Synthesis of acetyl-Cbl and propionyl-Cbl. Acetyl-Cbl was prepared as described by Müller and Müller (8) using 0.4 mL of acetic anhydride and Cbl I obtained by the reduction of 20 mg of aquacobalamin (aqCbl) with 0.4 g of zinc powder in 10 mL of 10% NH4Cl under N2. Propionyl-Cbl was also prepared by the same procedure, except that 0.4 mL of propionic anhydride was used instead of acetic anhydride. Cbls were desalted by phenol extraction and then passed through a phospho-cellulose (pH 6.0) column. The slowly moving band contained the desired product acyl-Cbl, whereas neutral by-products moved faster. Unreacted aqCbl was adsorbed on the top of the column. The yields of acetyl-Cbl and propionyl-Cbl were 87% and 67%, respectively.

Synthesis of formyl-Cbl. Formyl-Cbl was synthesized similarly, except that 30 mg of aqCbl was reduced in the suspension of 1 g of NH4Cl and 0.4 g of zinc powder in 10 mL of N,N-dimethylformamide, and that the mixed acid anhydride from 6 mL of formic acid and 2 mL of acetic anhydride was used. After 5 min of the reaction of CblI with the mixed anhydride, Cbls were desalted by phenol extraction and then passed through a phospho-cellulose (pH 6.0) column. The slowly moving band contained both formyl-Cbl and acetyl-Cbl. Formyl-Cbl was separated from acetyl-Cbl by silica gel G column chromatography using solvent A. Formyl-Cbl moved more slowly than acetyl-Cbl, whereas aqCbl formed by decomposition during the procedure was adsorbed on the top of the column. The yield of formyl-Cbl was 18% on the basis of aqCbl used.

Identification of acyl-Cbls. NH2OH·HCl (1 g) was dissolved in 20 mL of water and passed through a Dowex 1-X8 (OH− form) column to obtain 0.18 M NH2OH. Acyl-Cbls (5–10 mg) were allowed to react with 0.14 M NH2OH at room temperature for a week. The degradation products from their upper axial ligands were then analyzed by paper chromatography. The solvent systems used were (E) 1-butanol/acetic acid/water (4 : 1 : 5 by vol) and (F) amyl alcohol/formic acid/water (75 : 25 : 75 by vol). Hydroxamic acids were located by spraying 1.25% FeCl3 in 1 N HCl (13).

RESULTS AND DISCUSSION

Identification and chromatographic, electrophoretic, and spectroscopic properties of acyl-Cbls

Acetyl-Cbl undergoes the heterolytic cleavage of its Co-C bond by treatment with nucleophilic reagents, such as OH−, NH3, and NH2OH, in the dark (10). Upon the reaction of acetyl-Cbl with aqueous NH3 in the presence of methyl iodide, MeCbl and acetate are formed, suggesting the intermediate formation of CblI (10) (Eqs. 1 and 2).

Acetate formation in the KCN-decomposition of ace-
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Table 2. Chromatographic and electrophoretic behaviors of acyl-Cbls.

<table>
<thead>
<tr>
<th>Cbl</th>
<th>$R_f$ values</th>
<th>Mobility on paper electrophoresis at pH 3.5 (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TLC</td>
<td>Paper chromatography</td>
</tr>
<tr>
<td></td>
<td>Solvent A</td>
<td>Solvent B</td>
</tr>
<tr>
<td>Formyl-Cbl</td>
<td>0.21</td>
<td>0.11</td>
</tr>
<tr>
<td>Acetyl-Cbl</td>
<td>0.38</td>
<td>0.18</td>
</tr>
<tr>
<td>Propionyl-Cbl</td>
<td>0.47</td>
<td>0.23</td>
</tr>
<tr>
<td>CN-Cbl</td>
<td>0.29</td>
<td>0.13</td>
</tr>
<tr>
<td>MeCbl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AdoCbl</td>
<td>0.24</td>
<td>0.11</td>
</tr>
<tr>
<td>aqCbl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Absorption spectra of acyl-Cbls in water.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\lambda_{max}$ in nm ($\epsilon \times 10^{-3}$ in M$^{-1}$cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formyl-Cbl</td>
<td>334 (16.4), ~361s, 520 (8.3)</td>
</tr>
<tr>
<td>Acetyl-Cbl</td>
<td>321 (14.8), 362 (13.3), 510 (8.7)</td>
</tr>
<tr>
<td>Propionyl-Cbl</td>
<td>321 (16.0), 360 (13.7), 511 (8.9)</td>
</tr>
</tbody>
</table>

1 s, shoulder.

Acyt-Cbl as well as spectroscopic evidence for Cbl$^1$ formation in alkaline decomposition was also demonstrated (11). In the reaction of acetyl-Cbl with NH$_2$OH, acetohydroxamic acid is formed, which offers a proof for the presence of an “activated” acetyl group and for the structure of Co-acetyl-Cbl (10). Hence, we attempted to identify the new acyl-Cbls using this type of reaction. Acetyl-Cbl and propionyl-Cbl were decomposed by NH$_2$OH completely within 3 d, whereas formyl-Cbl partly remained unreacted at this point but was completely decomposed within a week, suggesting that formyl-Cbl is less susceptible to this nucleophile than acetyl-Cbl and propionyl-Cbl. As shown in Table 1, formyl-Cbl, acetyl-Cbl, and propionyl-Cbl provided degradation products with essentially the same $R_f$ values as authentic formo-, aceto-, and propionohydroxamic acids, respectively, upon paper chromatography in two solvent systems. These results, together with chromatographic, electrophoretic, and spectroscopic properties described below, indicate that the acyl-Cbls synthesized are actually formyl-Cbl, acetyl-Cbl, and propionyl-Cbl.

Table 2 summarizes the behaviors of acyl-Cbls in TLC and paper chromatography. It seems reasonable that $R_f$ values increased with the length (hydrophobicity) of an acyl group in all the solvent systems used. The red color of acyl-Cbls turned yellow upon acidification with diluted HCl, and then the yellow color turned pink upon photo-irradiation. Table 3 shows absorption maxima ($\lambda_{max}$) together with molar extinction coefficients ($\epsilon$) in the optical spectra of acyl-Cbls in water. Like cobalamin coenzymes, all acyl-Cbls were converted to aqCbl by photolysis in the absence of KCN and to (CN)$_2$-Cbl in the presence of KCN (data not shown). Slight bathochromic shifts (9–10 nm) in the $\alpha/\beta$ band were observed with formyl-Cbl, as compared with acetyl-Cbl and propionyl-Cbl, which suggests that the -CHO group in formyl-Cbl mainly exists in a hydrated form.

Table 2 summarizes the electrophoretic properties of acyl-Cbls at pH 3.5 as well. When the electrophoretic mobility of CN-Cbl (net charge 0) is taken as 0, mobility reflects the net charges of Cbls at pH 3.5. The net charges of alkyl- and acyl-Cbls reflect their base-/base-off equilibria (Eq. 3) at this pH, suggesting that the so-called trans-effect (14) or the $\sigma$-donor strength of an upper axial ligand increases in the order: formyl$<$acetyl$<$propionyl. Although the methyl group is a stronger $\sigma$-donor than acyl groups, methyl-Cbl showed much smaller mobility (Table 2). Such a discrepancy might be due to the bulkiness of the acyl groups or their hydrated forms.

Determination of $pK_a$ values in the base-on $\leftrightarrow$ base-off equilibrium

Figure 2A shows the spectral changes of acyl-Cbls upon acidification. Like acetyl-Cbl (10), formyl-Cbl and propionyl-Cbl underwent a color change from red to yellow in 0.1 N HCl—that is, all acyl-Cbls existed in the base-off form at pH 1 by protonation of its lower axial ligand, i.e., the 5,6-dimethylbenzimidazole (DBI) moiety (Eq. 3). Again, a slight bathochromic shift (8 nm) in the spectrum of the base-off form was observed with formyl-Cbl, as compared with acetyl-Cbl and propionyl-Cbl. The apparent $pK_a$ values of formyl-Cbl, acetyl-Cbl, and propionyl-Cbl for the base-on $\leftrightarrow$ base-off equilibrium were determined from the pH-absorbance profiles at two wavelengths to be 3.7, 3.9, and 4.0, respectively (Fig. 2B). As to Eq. 3, a six-coordinate aqua complex
may also be involved in a formal sense as an intermediate in the equilibrium of DBI displacement upon acidification of cobalamins, but Firth et al. reported that the proportion of a six-coordinate aqua complex in neutral aqueous solution is negligible with AdoCbl and ethyl-Cbl (15). They demonstrated as well with organo-Cbls that a strong \( \pi \)-donor ligand increases an equilibrium constant (\( K \))—that is, it favors the formation of a five-coordinate complex (14). Therefore, the above-mentioned order of apparent pK\(_a\) values of acyl-Cbls seems to be reasonable by assuming the equilibrium shown in Eq. 3. Although the pK\(_a\) value of the DBI moiety in the base-off form would be close to that of free DBI (4.7), namely, not affected by an upper axial ligand, the fraction of the five-coordinated complex would increase with the trans-effect (\( \pi \)-donor strength) of an acyl group in the order: formyl<acetyl<propionyl. This conclusion is consistent with the result of paper electrophoresis.

**Photo-sensitivity**

Like acetyl-Cbl (8, 10) and other organocobalamins, formyl-Cbl and propionyl-Cbl underwent rapid Co-C bond homolysis by photo-irradiation with a tungsten light bulb, forming aqCbl under aerobic conditions. When monitored by the increase of A\( _{350} \), photolysis proceeded linearly with the time of photo-irradiation. The first-order rate constants (\( k \)) were 0.55, 0.79, and 0.59 min\(^{-1}\) for formyl-Cbl, acetyl-Cbl, and propionyl-Cbl, respectively (Fig. 3).

**Thermal stability in neutral solution and in acidic solution**

Formyl-Cbl in 0.1 M potassium phosphate buffer (pH 7.0) was thermally very unstable, and its spectrum was rapidly converted to that of aqCbl upon the heat treatment at 100°C. When thermal decomposition was monitored by the increase of A\( _{350} \), i.e., the formation of aqCbl, more than 50% of formyl-Cbl underwent

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**Fig. 2.** Spectral changes of acyl-Cbls upon acidification (A) and pH-absorbance profiles at two wavelengths (B). Acyl-Cbls (9–18 \( \mu \)M) in H\(_2\)O (solid line), in 0.1 N HCl (dashed line), and in 0.1 M sodium citrate buffer adjusted to the indicated pH values. (a) Formyl-Cbl, (b) acetyl-Cbl, (c) propionyl-Cbl.

**Fig. 3.** Photo-sensitivity of acyl-Cbls. Photo-irradiation was carried out for the indicated time periods with a 200-W tungsten light bulb from a distance of 1 m, and the extents of photolysis (decomposition to aqCbl) were monitored by the increase of absorbance at 350 nm. Formyl-Cbl (solid line); acetyl-Cbl (dashed line); propionyl-Cbl (dash-dot line).
the Co-C bond cleavage at 100˚C for 2 min (Fig. 4A). In contrast, acetyl-Cbl was decomposed at a very slow rate under the same conditions, and propionyl-Cbl was quite stable to the heat treatment at least within 20 min.

Upon the heat treatment at 100˚C in 0.1 N HCl, formyl-Cbl underwent the cleavage of its Co-C bond, forming aqCbl. The decomposition followed a pseudo-first-order kinetics with a rate constant \( k \) of 0.054 min\(^{-1}\) (Fig. 4B). Again, acetyl-Cbl and propionyl-Cbl were decomposed much more slowly under the same conditions. Since the half life of formyl-Cbl in 0.1 N HCl was more than six-times longer than at pH 7.0, it is likely that acid markedly stabilizes the Co-C bond of formyl-Cbl, possibly by decreasing the contribution of the hydrated form of the -CHO group.

**Stability to cyanide in unbufferized solution and at pH 8.0**

Upon the treatment with 0.1 M KCN at 25˚C in unbufferized solution, propionyl-Cbl and acetyl-Cbl underwent marked spectral changes to that of (CN)\(_2\)-Cbl (Fig. 5A). This is consistent with the previous finding of other investigators that the Co-C bond of acetyl-Cbl is heterolytically cleaved by nucleophilic agents (8, 10, 11). In contrast, when the Co-C bond cleavage was monitored by the increase of \( A_{367} \), i.e., the formation of (CN)\(_2\)-Cbl, formyl-Cbl was essentially unsusceptible to KCN under the same conditions (Fig. 5A).

On the contrary, no spectral changes were observed at 25˚C at least within 1 d when acyl-Cbls were treated with 0.1 M KCN in potassium phosphate buffer (pH 8.0) (data not shown). It is thus evident that the Co-C bond of all acyl-Cbls is stable toward KCN at pH 8.0. One possibility is that this is due to a much lower concentration of CN\(^-\) at this pH (pK\(_a\) for HCN is 9.2), and these results might indicate that the Co-C bond of propionyl-Cbl and acetyl-Cbl is sensitive to the nucleophile CN\(^-\), but not to HCN. The other possibility is that their Co-C bond is cleaved not by CN\(^-\) but by OH\(^-\) that is generated from KCN, as in the decomposition of \( \beta \)-cyanoethylcobalamin (16). The latter possibility is more likely, because acetate is formed upon the alkaline cyanide decomposition of acetyl-Cbl (11). The stability of formyl-Cbl in unbuffered KCN solution may indicate that the carbonyl character of the -CHO group in formyl-Cbl is strong—in other words, its carbonyl group exists mainly in the hydrated form as in formaldehyde.

As shown by Eq. 1, acyl-Cbls can be considered to decompose through their hydrated forms. However, formyl-Cbl was much more stable in unbuffered KCN (weakly alkaline) solution, although it exists mainly in the hydrated form. Hence, it is likely that the rate of decomposition of acyl-Cbl is determined by the stability, rather than the abundance ratio, of its hydrated form.
The assumption that the -CHO group in formyl-Cbl exists mainly in the hydrated form as in formaldehyde would be reasonable, because the electron-withdrawing effect or electronegativity of the Cbl moiety seems to be almost comparable to that of a hydrogen atom, as judged from similar chemical shifts of $^{19}$F in CF$_3$-Cbl and CF$_3$H (17).

Stability in alkaline solution

The Co-C bond of acetyl-Cbl is cleaved by the strong nucleophile OH$^-$, forming hydroxocobalamin (OH-Cbl) under aerobic conditions (10). When the formation of OH-Cbl was monitored by the increase of $A_{357}$, acyl-Cbls were rapidly decomposed in 0.2 N NaOH at 25˚C at a rate increasing in the order: propionyl<formyl<acetyl (Fig. 5B). The higher reactivity of acetyl-Cbl than propionyl-Cbl can be explained by its stronger carboxyl character, i.e., by the increasing $\sigma$-donor strength in the order: H$<CH_3$<CH$_2$H. In contrast, the lower reactivity of formyl-Cbl than acetyl-Cbl would be accounted for by the stability of their hydrated forms.

To date, no papers have appeared which report evidence for the occurrence of acyl-Cbls in nature. We recently discussed about the mechanism of inactivation of AdoCbl-dependent diol dehydratase by glycerol (18, 19) and the possible involvement of formyl-Cbl in the inactivation process (20). Theoretical calculations suggest that glycerol inactivation of this enzyme begins with an intramolecular hydrogen transfer from the glycerol 3-OH group to C1 of the substrate radical, forming an O3-centered radical that decomposes to formaldehyde and a glycol radical (20). The formaldehyde produced might react with cob(II)alamin (Cbl$^\text{II}$) and the glycol radical, forming ethylene glycol and an inactive cobalamin like formyl-Cbl. This hypothesis is attractive, because an alkylcobalamin-like spectrum is actually observed upon glycerol inactivation (21) and gradually changes to an OH-Cbl-like one upon dialysis (T. Toraya and T. Tobimatsu, unpublished results). The biochemical examination of this possibility is under current investigation.

REFERENCES