The Synthesis and Properties of Four Spin-labeled Analogs of Adenosylcobalamin*

(Received for publication, January 18, 1980)

David L. Anton, Pei Kuo Tsai, and Harry P. C. Hogenkamp

From the Department of Biochemistry, Medical School, University of Minnesota, Minneapolis, Minnesota 55455

Four spin-labeled analogs of adenosylcobalamin have been synthesized to aid in the detection and identification of radical intermediates in the adenosylcobalamin-dependent enzymatic reactions and to serve as probes of the coenzyme, substrate, and effector binding sites of the protein. Three isomers of adenosylcobalamin, in which one of the propionamide side chains (b, d, or e) was hydrolyzed, and adenosyllepticobalamin e-carboxylic acid were reacted with 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl in the presence of 1-ethyl-3-(dimethylaminopropyl)-carbodiimide to yield the spin-labeled adenosylcorrinoids. These spin-labeled derivatives of adenosylcobalamin function as coenzymes and/or inhibitors of dioldehydrogenase from Klebsiella pneumoniae and of ribonucleotide reductase from Corynebacterium nephridii. Electron spin resonance has been used to monitor the photolytic cleavage of the carbon-cobalt bond of these analogs.

The enzymatic reactions which require adenosylcobalamin as a coenzyme can be generalized as a migration of hydrogen from a carbon atom to an adjacent one with the concomitant migration of a Group X in the opposite direction (1). In the ribonucleotide reductase reaction, a similar hydrogen transfer takes place.

\[
\begin{array}{cccc}
\text{a} & \text{c} & \text{d} & \text{d} \\
\text{H} & \text{X} & \text{x} & \text{H}
\end{array}
\]

In this reaction, the hydrogen donor and hydrogen acceptor are different compounds (2). Although the mechanism of these reactions has not yet been established, it is generally accepted that the homolytic cleavage of the carbon-cobalt bond of the coenzyme is the prerequisite step in the catalytic process (1). Alternate mechanisms involving the heterolytic cleavage of the carbon-cobalt bond have been postulated by Schrauzer (3) and by Corey et al. (4). Toraya and co-workers (5) have demonstrated that the interaction of the propionamide side chains of adenosylcobalamin with apodioldehydrogenase facilitates the homolytic cleavage of the carbon-cobalt bond and that they contribute to the stabilization of the radical intermediates. All analogs of adenosylcobalamin, in which each one of the propionamide side chains (b, d, or e) is either hydrolyzed or substituted by methyl or methyamine, serve as coenzymes in the dioldehydrogenase system. In the ribonucleotide reduction system, the three adenosylcobalamin b-, d-, and e-carboxylic acids show coenzyme activity only at very high concentrations and are poor inhibitors of ribonucleotide reduction (6). However, removal of the negative charge by amidation even with bulky alky or aryl substituents restores coenzyme activity (6, 7).

Spin-labeled analogs of adenosylcobalamin are of interest because they offer the possibility of detecting the intermediary of free radicals in the adenosylcobalamin-dependent enzymatic reactions. Several years ago, Buckman et al. (8) synthesized a spin-labeled nitroalkylycobalamin by reacting 4-bromocacetamido-2,2,6,6-tetramethylpiperidine-N-oxyl with cobalamin. However, this type of spin-labeled cobalamin or cobinamide is of limited utility as a probe for the adenosylcobalamin-dependent enzymes, because only corrinoids with a 5'-deoxynucleosidyl ligand in the upper coordination position function as coenzymes. The same laboratory (9) prepared a spin-labeled derivative of adenosylcobinamide by coordinating 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl to the cobalt atom in the lower coordination position. Although this analog was reported to be active as a coenzyme in the ethanalamine ammonia-lyase reaction, the conclusions regarding a radical mechanism are open to question because the spin-labeled complexes were not well characterized.

Recently, we have developed more convenient methods for the preparation of the cyanocobalamin-monocarboxylic acids and have demonstrated that the earlier identification of specific propionic acid side chains (b, d, and e) was incorrect (10). These developments led us to synthesize analogs of adenosylcobalamin in which a specific propionic acid side chain is substituted by the spin label 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl. The four spin-labeled corrinoids described in this report are covalent derivatives of adenosylcobalamin; they function as coenzymes and/or inhibitors in dioldehydrogenase and in ribonucleotide reductase.

EXPERIMENTAL PROCEDURES

Materials—Trifluoromethanesulfonic acid, 1-hydroxybenzotriazole monohydrate, and 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl free radical were obtained from Aldrich Chemical Co. 1-Ethyl-3-(dimethylaminopropyl)-carbodiimide HCl was purchased from Pierce Chemical Co. The cyanocobalamin b-, d-, and e-monocarboxylic acids were prepared as described previously (10).

Synthesis of Cyanocobalamin e-Carboxylic Acid—Cyanocobal-
amin e-carboxylic acid (600 mg, 333 \mu mol) was dried in a vacuum oven at 60°C for 5 days. It was then dissolved in 10 g of trifluoro- methanesulfonic acid, and the reaction mixture was incubated at room temperature for 30 min. The solution was added to 150 ml of 1.35 M sodium bicarbonate, and the corrinoids were desalted by phenol extraction. The resulting aqueous solution was concentrated to approximately 150 ml and applied to a column of AG 1 X2 (acetate form, 200 to 400 mesh, 2.5 x 55 cm). The column was washed with water, and the desired corrinoids were eluted with 0.04 M sodium acetate, pH 5.3, onto a second column of the same resin (5 1/2 x 5 cm). The spin-labeled corrinoids were then desalted by phenol extraction and crystallized from aqueous acetone. Yields were: cyanocobalamin e-carboxylic acid, 100 \mu mol, 30%; cyanocobalamin e-carboxylic acid, 176 \mu mol, 43%.

Synthesis of Spin-labeled Analogs of Adenosylcobalamin—Cyanocobalamin monocarboxylic acid isomers (500 mg, 330 \mu mol) were dissolved separately in 50 ml of 0.2 M sodium EDTA, pH 9.5, were reduced with 500 mg of CeCl3 and treated with 500 mg of 5'-chloro-5'-deoxyadenosine dissolved in 10 ml of ethanol. After 1.5 h, the reaction mixtures were desalted by phenol extraction, and the aqueous solutions were applied to columns of AG 1 X2 (acetate form, 200 to 400 mesh, 2.5 x 55 cm). The columns were washed with water, and the adenosylcobalamin monocarboxylic acids were eluted with 0.1 M sodium acetate, pH 4.5. The cobalamin was then desalted by phenol extraction and isolated as glasses (yields, 80 to 90%). Each adenosylcobalamin monocarboxylic acid was dissolved in 20 ml of water containing 50 mg of hydroxybenzotriazole and 300 mg of 4-aminom-2,6,6-tetramethylpiperidine-N-oxyl free radical. The pH of the reaction mixtures was adjusted to 6.0 with 1 M HCl, and 125 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added. The mixtures were stirred at room temperature for 8 to 15 h until all the monocarboxylic acids had reacted, as judged by paper electrophoresis in 0.1 M sodium phosphate buffer, pH 7.0. The spin-labeled corrinoids were desalted by phenol extraction and passed through columns of CM-Sephadex to remove traces of cationic contaminants (yields, 90 to 75%).

Physical and Chemical Properties of the Spin-labeled Corrinoids—The electronic absorption spectra of aqueous solutions of the spin-labeled corrinoids are similar to those of the parent adenosylcorrinoids. In dilute acid solution, the spin-labeled corrinoids change color from red to yellow, indicating that the 5,6-dimethylbenzimidazole ligand is protonated and no longer coordinated to cobalt.

The electron paramagnetic resonance spectra of all four analogs in aqueous solution show the expected nitroxide triplet with a slightly broadened high field line (Fig. 1A). The rotational correlation time (\(\tau\)) of these spin-labeled corrinoids (\(\tau = 3.1 \pm 0.5 \times 10^{-10}\)) calculated from these spectra (13, 14) is very similar to that obtained from the T1 value of the C-10 methine bridge carbon of cyanocobalamin (\(\tau = 4 \times 10^{-10}\)). When these solutions are first purged with nitrogen and then exposed to light, the amplitude of the signal decreases until eventually the ESR signal is almost completely abolished (Fig. 1B). Photoysis under these conditions causes a color change from red to brown, clearly demonstrating that Co(II)-containing corrinoids are formed. Upon oxidation of these Co(II)-containing corrinoids to their respective Co(III) aquo forms, the ESR signal is almost completely abolished (15).

**RESULTS**

### Table 1

**Properties of spin-labeled corrinoids**

<table>
<thead>
<tr>
<th>Compound</th>
<th>HPLC</th>
<th>Paper chromatography</th>
<th>ESR parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solvent System</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>AdoCbl-(d)-COOH</td>
<td>8.7 &amp; 0.35 &amp; 0.66 &amp; 0.62</td>
<td>1</td>
<td>2.0150</td>
</tr>
<tr>
<td>AdoCbl-(l)-COOH</td>
<td>6.8 &amp; 0.30 &amp; 0.55 &amp; 0.50</td>
<td>1.55</td>
<td>2.0151</td>
</tr>
<tr>
<td>AdoCbl-(d)-C</td>
<td>8.2</td>
<td>0.58</td>
<td>0.91</td>
</tr>
<tr>
<td>AdoCbl-(l)-TEMPO</td>
<td>13.3</td>
<td>1.74</td>
<td>1.55</td>
</tr>
<tr>
<td>AdoCbl-(d)-TEMPO</td>
<td>15.0</td>
<td>1.54</td>
<td>1.39</td>
</tr>
<tr>
<td>AdoCbl-(l)-TEMPO</td>
<td>12.6</td>
<td>1.83</td>
<td>1.41</td>
</tr>
<tr>
<td>AdoCbl-(d)-TEMPO</td>
<td>10.5</td>
<td>1.47</td>
<td>1.55</td>
</tr>
</tbody>
</table>

\(a\) Retention times in minutes. A column (4.6 mm x 25 cm) of Ultrasphere ODS (5 \mu, Altex) was eluted for 2 min with 30% methanol in 0.05 M NaH2PO4, followed by a linear gradient from 30 to 60% methanol in 0.05 M NaH2PO4, over 10 min, and finally with 60% methanol in 0.05 M NaH2PO4.

\(b\) Descending paper chromatography on Whatman 1; \(R_{cv} = 1\), 1-butanol:water:NH4OH (50:36:14); II, 1-butanol:ethanol:water (50:15:35); III, 1-butanol:1-propanol:water (37:36:37).

\(c\) Retention times (13, 14) calculated from the ESR spectra.

\(d\) Streaked in this solvent.
forms, the ESR signals are partially generated (Fig. 1C). In contrast, photolysis of the spin-labeled adenosylcorrinoids in the presence of air does not significantly affect either the amplitude or the shape of the nitroxide ESR signal. In control experiments, adenosylcobalamin was photolyzed in the presence of an equimolar amount of piperidine-N-oxyl in the presence or in the absence of oxygen. Photolysis under these conditions did not affect the nitroxide ESR signal, clearly demonstrating that only intramolecular radical coupling between the paramagnetic cobalt atom and the nitroxide radical abolishes the ESR spectrum.

Coenzymatic Properties of the Spin-labeled Corrinoids—In order to establish whether the spin-labeled adenosylcorrinoids are able to interact with the adenosylcobalamin-dependent enzymes, the spin-labeled analogs were tested for their ability to act as coenzymes or inhibitors of dioldehydrogenase and of ribonucleotide reductase. Table II summarizes the kinetic parameters for the four analogs in the dioldehydrogenase reaction. The three spin-labeled analogs derived from the b-, d-, and e-carboxylic acids are able to function as coenzymes, while the analog derived from adenosylepicobalamin e-carboxylic acid is inactive as a coenzyme. As shown by Toraya et al. (5) for the methyl esters and the methylamides of these carboxylic acids, coenzyme activity is clearly dependent on the position of the spin-label; the activity decreases in the order e > d > b. The e isomer shows relatively high activity, while the activity of the b isomer is barely detectable. In contrast these two analogs have very similar apparent $K_m$ values. Toraya and co-workers (5) have demonstrated that coenzyme analogs can be displaced from the enzyme by adenosylcobalamin. Our results (Table III) demonstrate that adenosylcobalamin is also able to displace the spin-labeled adenosylcorrinoids. Preincubation of the enzyme with the spin-labeled isomers b and e causes significant inhibition. The only marginal inhibition by the spin-labeled d isomer is probably a consequence of its high apparent $K_m$ value. The spin-labeled analog of adenosylepicobalamin does not function as a coenzyme; it does, however, interact with the enzyme and acts as an inhibitor.

In the ribonucleotide reductase reaction, only the spin-labeled analog derived from adenosylcobalamin e-carboxylic acid is able to function as a coenzyme. Compared to adenosylcobalamin ($V_{max} = 154$ nmol min$^{-1}$; $K_m = 4.5$ μM), this analog is about 32% as active ($V_{max} = 57$ nmol min$^{-1}$; $K_m = 8.9$ μM). However, the other spin-labeled analogs do interact with the enzyme; preincubation of the enzyme with the analog causes progressive inhibition of the enzyme. Simultaneous incubation of these analogs and adenosylcobalamin with the reductase even with a 15-fold excess of the analog causes little or no inhibition.

**DISCUSSION**

Four analogs of adenosylcobalamin containing a nitroxide free radical have been prepared to aid in the elucidation of the mechanism of the adenosylcobalamin-dependent enzymatic reactions. In three of these analogs, 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl is covalently attached to each of the propionic acid side chains (b, d, and e), which project down from the corrin ring, and thus the spin labels are
Spin-labeled Derivatives of Adenosylcobalamin

positioned close to the 5,6-dimethylbenzimidazole ligand. The fourth analog is derived from adenosyllepicobalamin e-carboxylic acid, in which the e-propionic acid side chain is projecting up from the corrin ring, and thus the spin label is able to approach the methylene carbon of the 5'-deoxyadenosyl ligand. When these four spin-labeled derivatives of adenosylcobalamin interact with two of the adenosylcobalamin-dependent enzymes tested, dioldehydrase and ribonucleotide reductase, they either function as coenzymes or are able to inhibit the reactions in presence of adenosylcobalamin. Photolysis of the carbon-cobalt bond of these four spin-labeled analogs under anaerobic conditions abolishes the ESR signal of the nitroxide, clearly demonstrating a spin-spin interaction between the nitroxide radical and the paramagnetic metal. This effect is an intramolecular spin-spin interaction, because the nitroxide ESR signal is not abolished when equimolar quantities of adenosylcobalamin and 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl are photolyzed under anaerobic conditions. Oxidation of the Co(II)corrinoid to its Co(III) aquo form does not regenerate all of the nitroxide ESR signal, suggesting that irreversible radical coupling between the nitroxide and the nucleosidyl radical or reduction of the nitroxide has occurred. In contrast, photolysis of the spin-labeled analogs of adenosylcobalamin in an excess of oxygen does not significantly effect the ESR signal of the nitroxide, demonstrating that oxygen is a more efficient radical scavenger than the nitroxide.

Many investigators have suggested that the homolytic cleavage of the carbon-cobalt bond of the coenzyme to cob(II)alamin and a 5'-deoxyadenosyl radical is the initial step in all of the adenosylcobalamin-dependent enzymatic reactions. For this homolysis, the photolysis of adenosylcobalamin serves as an ideal radical non-enzymatic model. In the absence of oxygen, cob(II)alamin is stable but the adenosyl radical is very reactive and cyclizes to 5'-deoxy-8,5'-cycloadenosine with the elimination of a hydrogen atom.

In the enzymatic reactions, the 5'-deoxyadenosyn radical abstracts a hydrogen atom from the substrate to form 5'-deoxyadenosine and a substrate radical. The substrate radical then rearranges to a product radical, which in turn abstracts one of the 5'-methyl hydrogens of the nucleoside to yield the product and regenerate the 5'-deoxyadenosyn radical. Thus, at the active site of the adenosylcobalamin-dependent enzymes, the 5'-deoxyadenosyn radical must be positioned far enough from the Co(II) corrinoid to prevent recombination and, furthermore, the active site must shield cob(II)alamin and the other radicals from oxygen and other radical scavengers. It seems reasonable to assume that the coenzyme binding site of the adenosylcobalamin-dependent enzymes is in a rigid region of the protein and that the coenzyme is immobilized on the enzyme surface. Such immobilization of the spin-labeled analogs of the coenzyme will cause drastic changes in the ESR spectrum of the nitroxide, and thus these spin-labeled corrinoids should be very useful in the definition of the active site of these enzymes. Using these spin-labeled analogs, it will also be feasible to establish whether the Co(II)corrinoid is a "conductor" or a "spectator" in the enzymatic reaction (16).

REFERENCES