The Synthesis of a 5'-Deoxyadenosylcobalamin-agarose Adsorbent and Its Utility in the Purification of Ribonucleotide Reductase*

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SUMMARY

A 5'-deoxyadenosylcobalamin-agarose adsorbent has been prepared for the purification by affinity chromatography of enzymes which require 5'-deoxyadenosylcobalamin as a coenzyme. The synthesis involved the covalent attachment of cyanocobalamin to agarose through a 12 carbon chain. This hydrocarbon chain was attached to the corrin nucleus by reacting diaminododecane with cyanocobalamin a-carboxylic acid in the presence of water-soluble carbodiimide. The substituted cobalamin was then coupled to agarose “activated” by cyanogen bromide. The complex was reduced with chromous chloride and reacted with 5'-O-p-tolylsulfonyl-adenosine to yield the desired adsorbent.

This adsorbent has been successfully used in the purification of ribonucleotide reductase from *Lactobacillus leichmannii*, which requires 5'-deoxyadenosylcobalamin as a coenzyme. Ribonucleotide reductase is retained by the adsorbent if it is applied in the presence of a dithiol and an allosteric effector. The enzyme is readily eluted with a solution containing only buffer and dithiol.

**EXPERIMENTAL PROCEDURES**

**Materials**

Nucleotides were purchased from P-L Biochemicals. Cyanocobalamin, dithiothreitol, 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide hydrochloride and Sepharose 4B-200 were obtained from Sigma. Cyanogen bromide and dodecylamine hydrochloride were purchased from Eastman, 1,12-diaminododecane from Aldrich, and 5'-O-p-tolylsulfonyl-adenosine from Waldhof. Ribonucleotide reductase from *Lactobacillus leichmannii* was kindly supplied by Dr. R. L. Blakley.
Methods

Ribonucleotide reductase activity was determined by the method of Yamada et al. (8), using dimethylgluturate buffer instead of phosphate buffer. Protein concentration was determined by either the procedure of Lowry et al. (9) or the biuret method (10). Thin layer chromatography was carried out on Eastman Kodak precoated 6060 silica gel or 6065 cellulose sheets. The following solvent systems were used: Solvent I, sec-butyl alcohol-acetic acid-water (127:1:50, v/v); Solvent II, water saturated n-butyl alcohol-acetic acid (100:1, v/v); Solvent III, isopropyl alcohol-ammonium hydroxide-water (85:1.3:15, v/v); Solvent IV, isobutyralcohol-10% aqueous Na2HPO4 (1:1, v/v). Absorption spectra were recorded with a Cary model 15 spectrophotometer; other visible and ultraviolet spectral measurements were made with a Zeiss PMQII spectrophotometer.

Synthesis of \( \alpha-(5,6\text{-dimethylbenzimidazolyl})\)-Co-5'-deoxyadenosylcobamnic acid abed-\( \delta \)-pentamidine e-(12-aminonon-dodecyl)amide agarose (deoxyadenosylcobalamin-agarose)

Cyanocobalamin monocarboxylic acids—Cyanocobalamin (370 \( \mu \)moles) was dissolved in 150 ml of 0.5 M HCl and incubated at 37\(^\circ\) for 3 hours. The solution was then neutralized and the cobamides were desalted by extraction into phenol and applied to a column of Dowex 1 X2 (acetate form, 200 to 400 mesh, 2.5 \( \times \) 50 cm). The column was washed with water to remove untreated cyanocobalamin and then developed with 0.04 M sodium acetate (pH 4.67). The first monocarboxylic acid was eluted as a single peak, and was desalted by extraction into phenol and crystallized from aqueous acetone. The second peak contained two carboxylic acids; these were also desalted by extraction into phenol and the aqueous solution was evaporated to dryness. The residue was dissolved in n-butyl alcohol-water-acetic acid (75:15:1, v/v) and applied to a column of cellulose powder (Cellex N, 2.5 \( \times \) 50 cm). Elution with the same solvent separated the two carboxylic acids (II and III). Both carboxylic acids were crystallized from aqueous acetone after evaporation of the solvent. (The yield of II was 26 \( \mu \)moles; of II, 47 \( \mu \)moles; of III, 29 \( \mu \)moles.) Each isomer behaves as a monocarboxylic acid on paper electrophoresis in 0.5 M sodium acetate (pH 9.2). However, the monocarboxylic acids could be readily distinguished by thin layer chromatography on silica gel sheets in Solvent I. \( R_{f} \)Cyanocobalamin for I was 1.5; for II, 1.3; for III, 0.88 (Table I). Chromatography on Whatman No 3MM paper with Solvent II separated I and II from III but not from each other. These results and those obtained by Armitage et al. (6) and Bernhauer et al. (7) identify isomers I, II, and III as the \( b \)-, \( e \)-, and \( d \)-monocarboxylic acids of cyanocobalamin, respectively.

\( \alpha-(5,6\text{-dimethylbenzimidazolyl})\)-cobamic acid abed-pentaamid e\( \delta \)-(12-aminonon-dodecyl)amide cyanamide—A reaction mixture containing 45 \( \mu \)moles of cyanocobalamin \( e \)-carboxylic acid and 2.2 \( \mu \)moles of daminododecan in 13 ml of water was adjusted to pH 6.0 with 1 N HCl. The solution was then treated with 463 \( \mu \)moles of 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride and stirred at room temperature. After 22 hours an additional 420 \( \mu \)moles of the carbodiimide was added and the solution was stirred for 2 hours. Thin layer chromatography of the reaction mixture with Solvent I showed that all the carboxylic acid had reacted to yield three new compounds (\( R_{f} \), 0.3, 0.4, and 2.5). (Control experiments showed that both slow running corrinoids were formed also when cyanocobalamin \( e \)-carboxylic acid was treated with the carbodiimide in absence of the diamine. On the other hand, no products were formed when cyanocobilamin was treated with daminododecan in the presence of carbodiimide.) The reaction mixture was evaporated to dryness, the residue dissolved in a small volume of sec-butyl alcohol-water (10:3, v/v), and the solution applied to a column of cellulose powder (Cellex N, 2.5 \( \times \) 40 cm), which was developed with the same solvent. The first red compound was desalted by extraction in phenol and evaporated to dryness. This cobamide behaved as a cation on paper electrophoresis in 0.5 M acetic acid, pH 2.5. Its visible and ultraviolet absorption spectra were almost identical with that of cyanocobalamin. Furthermore, treatment of the cobamide with sodium acetate and carbodiimide converted it to an electrophoretically neutral compound, which on thin layer chromatography in Solvent I showed a single spot with \( R_{f} \)cyanocobalamin = 3.4. These properties are consistent with those expected for the desired product (yield 15.6 \( \mu \)moles, 35%).

\( \alpha-(5,6\text{-dimethylbenzimidazolyl})\)-cobamic acid abed-pentaamid e\( \delta \)-(12-aminonon-dodecyl)amide cyanamide-agarose (cyanocobalamin-agarose)—Sepharose 4B-200 (15 ml) was activated with 3.8 \( g \) of cyanogen bromide as described by Cuatrecasas (14). The activated agarose was treated with 10 \( \mu \)moles of cyanocobalamin \( \delta \)-aminododecyamine in 15 ml of 0.1 M sodium carbonate buffer, pH 10. The reaction was allowed to proceed at 4\(^\circ\) F (20 hours) and the substituted agarose was then washed with copious amounts of water.

5'-Deoxyadenosylcobalamin agarose—The substituted agarose was suspended in 30 ml of 0.1 M EDTA buffer, pH 9.5, and nitrogen was bubbled through the suspension for 30 min to ensure complete removal of oxygen. The suspension was then treated with 130 \( \mu \)g of chromous chloride dissolved in 30 ml of deoxygennated 0.1 M EDTA solution, pH 9.5. The second solution was added through a fritted glass funnel under nitrogen to remove insoluble impurities. After 15 min, 40 mg of 5'-O-tosyladenosine dissolved in 0.5 ml of deoxygenated dimethylsulfoxide was added under nitrogen in dim light. The reaction mixture was stirred at room temperature in the dark for 4 hours and then filtered on a Buchner funnel. The desired agarose derivative was washed with water to remove soluble reaction components.

\( \delta \)-Deoxyadenosylcobalamin-monocarboxylic acids—Separate solutions containing 15 \( \mu \)moles of cyanocobalamin \( b \)-, \( d \)-, and \( e \)-carboxylic acid were reduced with chromous chloride and then treated with 20 \( \mu \)g of 5'-O-tosyladenosine. The deoxyadenosylcobalamin carboxylic acids were purified on Dowex 50W X2 (200 to 400 mesh) and desalted by extraction with phenol (3).
Yields were 10.6, 8.1, and 9.7 μmoles of 5'-deoxyadenosylcobalamin b-, d-, and e-carboxylic acid, respectively.

5'-Deoxyadenosylcobalamin e-carboxylic acid was treated with diamino-2-dodecene and carbodiimide as described above. The desired product was purified by chromatography on cellulose powder with sec-butyl alcohol-water-acetic acid (127:50:1, v/v/v) as the solvent. The absorbance spectrum was similar to that of 5'-deoxyadenosylcobalamin (maxima at 260, 375, and 522 nm). Thin layer chromatography on silica gel sheets in Solvent 1 showed that the product was homogeneous (Re= 2.3).

5'-Deoxyadenosylcobalamin e-carboxylamidine was prepared from cyanocobalamin e-carboxylic acid, n-decylamine, and carbodiimide as described above. The yield was 3.7 μmoles from 23 μmoles of carboxylic acid. This cobamide was chromatographically pure (Re = 3.5, silica gel sheets, Solvent 1) and was converted to the 5'-deoxyadenosyl derivative as outlined above. The product was purified by chromatography on cellulose powder (Cellulose N, 2.5 × 50 cm); the starting material was first eluted with sec-butyl alcohol-water (5:1, v/v) and the product was then eluted with sec-butyl alcohol-water (5:2, v/v). The yield was 0.35 μmole (Re = 2.7, silica gel sheets, Solvent 1).

**RESULTS**

**Properties of Deoxyadenosylcobalamin-agarose—**As would be expected, the visible and ultraviolet spectra of the cobalamin-agarose complex could not be determined, however, in neutral and alkaline solution, the complex was red in color whereas, in acidic solution (0.01 N HCl), the color changed to yellow. Such a color change is typical of 5'-deoxyadenosylcobalamin (11). This color change in acid was not observed when the agarose suspension was first exposed to light, an indication of photodecomposition of the 5'-deoxyadenosylcobalamin moiety to aquocobalamin. Furthermore, treatment of the complex with 0.1 M KCN in the dark caused a color change from red to purple. This suggests the formation of a dicyanoaminocobamide. Although both deaminations by light and by cyanide altered the cobalamin moiety, these treatments did not remove the cobalamin from the agarose. Treatment of 5'-deoxyadenosylcobalamin-agarose with 0.1 M KCN released adenine, which was identified by its ultraviolet spectrum in acid and in alkaline solution and by its behavior on thin layer chromatography on cellulose sheets in two solvent systems: Solvent III; Rf = 0.44; Solvent IV; Rf = 0.31. Treatment of 0.3 ml of the complex with 0.1 M KCN released 0.084 μmole of adenine determined from the absorbance and a molar extinction coefficient of 13.6 × 10³ M⁻¹ cm⁻¹ at 260.5 nm. When the complex was treated with 6 M HCl at room temperature for 12 hours, the cobalamin moiety was removed from the agarose. The liberated corrinoids could be quantitated after conversion to the dicyano derivatives in 0.1 M KCN. Treatment of 0.3 ml of the complex with acid released 0.087 μmole of dicyanoaminocorronoids determined from the absorbance and a molar extinction coefficient of 30.8 × 10³ M⁻¹ cm⁻¹ at 368 nm. These results indicated that 0.28 μmole of 5'-deoxyadenosylcobalamin were bound per ml of packed agarose.

**Coenzymatic Properties of Cobamide Analogues—**In order to establish which of the cobamides are able to bind to ribonucleotide reductase, the deoxyadenosyl derivatives of the cobamide analogues were tested for their ability to act as coenzymes or inhibitors in the enzyme system of L. leichmannii. Although the deoxyadenosyl derivatives of the monocarboxylic acids do not function as coenzymes, they are nevertheless able to bind to the enzyme since they are able to inhibit the reduction of ATP (Table II). These results are at variance with those of Morley et al. (13), who reported that the e-carboxylic did not bind to the enzyme. When the negatively charged carboxylate ion is blocked by a long hydrocarbon chain, binding becomes tighter as indicated by a lower Kᵢ value for deoxyadenosylcobalamin e-dodecylamide. Furthermore, when the negatively charged carboxylate ion is blocked and replaced by a positively charged group, the cobamide is able to function as a coenzyme. When used at very high concentrations, the 5'-deoxyadenosylcobalamin monocarboxylic acids were able to act as coenzymes; at 80 μM they showed 1 to 4% of the activity observed for 8 μM deoxyadenosylcobalamin. The low solubility of deoxyadenosynlcobalamin e-dodecylamide precluded the testing of this analog at high concentrations; however, at the concentrations tested (~12 μM), this analog showed approximately 6% of the activity observed for 8 μM deoxyadenosylcobalamin.

**Purification of Ribonucleotide Reductase by Affinity Chromatography on Deoxyadenosylcobalamin-agarose Columns—**The results obtained for the chromatography of partially washed ribonucleotide reductase on deoxyadenosylcobalamin-agarose columns are shown in Figs. 1 and 2. On control columns of unmodified agarose, no retention of the enzyme occurred and the activity-protein elution profiles were coincident. When the enzyme preparation was applied to the affinity column in buffer, slight retention of enzyme activity occurred but no significant purification could be achieved (Fig. 1A). Attempts to improve retention of the enzyme by applying the enzyme preparation in the presence of a substrate (Fig. 1B), an allosteric effector (Fig. 1C), or a dithiol were not successful. However, when the enzyme preparation was applied to the affinity column in the presence of both a dithiol and an allosteric effector, virtually quantitative adsorption occurred (Fig. 1D). Subsequent investigation demonstrated that the enzyme activity could be readily eluted with a solution containing only buffer and dithiol.

In the experiment shown in Fig. 1D, ribonucleotide reductase activity was eluted between 40 and 52.5 ml, yielding a 6-fold purification with a recovery of 53%. Only the deoxyribonucleo-
Fin. 1. Affinity chromatography of partially purified ribonucleotide reductase on 5'-deoxyadenosylcobalamin-agarose columns. The columns (0.0 X 22 cm) were equilibrated with 0.1 M Tris-phosphate buffer, pH 7.2, containing the following components: A, none; B, 2 mM ATP; C, 0.1 mM dGTP; D, 10 mM dithiothreitol and 0.1 mM dGTP. A solution containing 2.3 mg of protein (specific activity 21.7 units per mg) in 0.25 ml of the same buffer was applied to each column. Each column was run at 4°C in the dark; the flow rate was about 7 to 9 ml per hour; 2.5-ml fractions were collected. The column developed with buffer containing dithiothreitol was flushed with nitrogen to keep the system free of oxygen. Elution was attempted by varying the conditions as indicated by the arrows: A, 0.2 M Tris-phosphate buffer, pH 7.2; B, 0.1 M Tris-phosphate buffer, pH 7.2; D, 0.1 M Tris-phosphate buffer, pH 7.2, containing 10 mM dithiothreitol. The protein concentration was determined by the Lowry method (9) using a highly purified ribonucleotide reductase preparation (130 units per mg) as standard. The protein concentration of the standard was determined by the biuret method (10). The fractions were first dialyzed against 0.05 M phosphate buffer, pH 7.2, to remove the Tris buffer, which interfered with the protein determination.

tide effectors such as dGTP and not the ribonucleotide substrates were able to improve retention of reduced ribonucleotide reductase. Furthermore, combinations of substrates and effectors do not improve retention of the reduced enzyme by the affinity column. When the enzyme preparation was applied to the adsorbent in a lower buffer concentration (0.05 M Tris-phosphate, pH 7.2, 10 mM dithiothreitol, and 0.1 mM dGTP), elution of the enzyme with the same solution without dGTP was slow and it was convenient to increase the buffer concentration to 0.1 M to yield a concentrated enzyme solution. At lower buffer concentration more unspecific binding appeared to occur and the change to a higher buffer concentration also resulted in the release of some nonenzyme protein. For example, when the less purified reductase preparation was used, the enzyme activity was completely retained if the preparation was applied to the column in the presence of a low concentration buffer, a dithiol, and dGTP (Fig. 2). The enzyme could be eluted by increasing the buffer concentration and omitting the dGTP. In this experiment, ribonucleotide reductase was eluted between 54 and 66 ml with a recovery of 56% and a 6-fold purification.

After each experiment the columns were readily regenerated by washing the cobalamin-agarose adsorbents with 20 volumes of 1 M ammonium hydroxide, 20 volumes of 0.05 M hydrochloric acid, and then with 20 volumes of water. With the use of this regeneration system, the affinity columns have been used for at least 20 experiments without any detectable loss of activity. Because the affinity columns are photolabile, all operations were done in the dark or in dim light.

**Fig. 1.** Affinity chromatography of partially purified ribonucleotide reductase on 5'-deoxyadenosylcobalamin-agarose columns. The columns (0.0 X 22 cm) were equilibrated with 0.1 M Tris-phosphate buffer, pH 7.2, containing the following components: A, none; B, 2 mM ATP; C, 0.1 mM dGTP; D, 10 mM dithiothreitol and 0.1 mM dGTP. A solution containing 2.3 mg of protein (specific activity 21.7 units per mg) in 0.25 ml of the same buffer was applied to each column. Each column was run at 4°C in the dark; the flow rate was about 7 to 9 ml per hour; 2.5-ml fractions were collected. The column developed with buffer containing dithiothreitol was flushed with nitrogen to keep the system free of oxygen. Elution was attempted by varying the conditions as indicated by the arrows: A, 0.2 M Tris-phosphate buffer, pH 7.2; B, 0.1 M Tris-phosphate buffer, pH 7.2; D, 0.1 M Tris-phosphate buffer, pH 7.2, containing 10 mM dithiothreitol. The protein concentration was determined by the Lowry method (9) using a highly purified ribonucleotide reductase preparation (130 units per mg) as standard. The protein concentration of the standard was determined by the biuret method (10). The fractions were first dialyzed against 0.05 M phosphate buffer, pH 7.2, to remove the Tris buffer, which interfered with the protein determination.

**Fig. 2.** Affinity chromatography of partially purified ribonucleotide reductase. The deoxyadenosylcobalamin-agarose column (0.4 X 35 cm) was equilibrated with 0.05 M Tris-phosphate buffer, pH 7.2, containing 10 mM dithiothreitol and 0.1 mM dGTP. A protein solution (specific activity 9.2 units per mg) containing 10.25 mg in 1 ml of the same buffer was applied to the column. The column was run at 4°C in the dark; the flow rate was 3.5 ml per hour; 2.5-ml fractions were collected. Elution was started (arrow) with 0.1 M Tris-phosphate buffer, pH 7.2, containing 10 mM dithiothreitol.

**Discussion**

A 5'-deoxyadenosylcobalamin-agarose adsorbent for the affinity chromatography of enzymes which require 5'-deoxyadenosylcobalamin as coenzyme has been prepared by the reaction sequence outlined in Scheme 1. This support has several advantages over the hydroxocobalamin-albumin-cellulose support described by Olesen et al. (2). Cuatrecasas (14) has shown that the interposition of a hydrocarbon chain between the ligand and the agaroase support eliminates any steric restrictions that may prevent enzyme-ligand interaction. He also indicated that by using agarose instead of cellulose as the support a higher degree of ligand substitution is possible. In addition to these advantages the attachment to the corrin nucleus of the cobalamin rather than to the axial nucleotide or nucleoside is an advantage because the corrin nucleus can be substituted quite drastically at the propionamide side chains without loss of...
coenzyme activity. For instance, Morley et al. (13) have shown that even the 2,4-dinitroanilide and anilide of 5'-deoxyadenosylcobalamin e-carboxylic acid are active as coenzymes in the ribonucleotide reductase reaction.

Furthermore, the penultimate agarose complex, cyanocobalamin-agarose (Scheme 1) can be readily converted to specific supports for the affinity chromatography of other cobalamin-binding proteins. The cyanocobalamin-agarose support can be converted to an hydroxocobalamin-agarose support by reduction of cyanide. Such an adsorbent as well as the cyanocobalamin-agarose support should also be useful in the purification of methyltetrahydrofolate-homocysteine transmethylase apoenzyme. The 5'-deoxyadenosylcobalamin-agarose support should also be useful in the purification of dehydroxynase Toraya et al. (15) have shown recently that holopropyanediol dehydroxynase can be completely resolved into apoenzyme and cobamide by gel filtration on Sephadex G-25 in the absence of potassium ion. Incubation of apoenzyme with deoxyadenosylcobalamin and potassium phosphate yielded holoenzyme in nearly quantitative yields. These properties suggest that apopropanediol dehydroxynase would bind to the affinity column in the presence of potassium phosphate and that it could be eluted by a buffer lacking potassium ions.

The behavior of ribonucleotide reductase on the affinity column, in particular the good retention in the presence of dithiothreitol and dGTP, is consistent with the properties of this enzyme described before. It has been shown (16) that ribonucleotide reductase catalyzes tritium exchange between 5'-deoxyadenosylcobalamin and solvent only in the presence of a dithiol and either a ribonucleoside triphosphate or a deoxyribonucleoside triphosphate, dGTP being the most effective deoxyribonucleotide. Furthermore, Yamada et al. (8) have reported more recently that binding of cob(II)alamin to the enzyme was markedly affected by dGTP or ATP. The presence of either nucleotide increased cobalamin-enzyme interaction by 2 orders of magnitude. It is at present unclear why ATP in the presence of a dithiol is ineffective in promoting the retention of the enzyme to the affinity column.

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REFERENCES

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