Studies on the Mechanism of Cobalamin Binding to Hog Intrinsic Factor*

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SUMMARY

Cobalamin binding to hog intrinsic factor has been studied by affinity chromatography, and the cobalt atom of the cobalamin tested was found to be no more than 5 Å from the surface of the protein. A comparative study of the binding of the corrinoid derivatives versus the amide at C5 of the B-pyrrole ring indicates that a change in charge at this position of the corrin ring has little effect on complex formation with intrinsic factor. Furthermore, the coordination sphere in the region of 5,6-dimethylbenzimidazole must be extremely hydrophobic because CN⁻ will not displace this base from the cobalt atom when cobalamin is bound to intrinsic factor.

Specific binding of corrinoids to intrinsic factor has been the subject of numerous studies which include: (a) binding of different corrinoid derivatives (1-5), (b) pH optima for binding (6, 7), and (c) the effect of different blocking groups on corrinoid-intrinsic factor interactions (8, 9). Cyanocobalamin, aquocobalamin, and even the bulky B'-deoxyadenosylcobalamin have the same binding constant to intrinsic factor which clearly indicates that the substituent at the fifth coordination position is not important in the formation of the cobalamin-intrinsic factor complex (5). Dicyanocobalamin binds less well than cyanocobalamin, and if adenine replaces 5,6-dimethylbenzimidazole as in pseudovitamin B12, then binding to intrinsic factor does not occur (4, 5). The heterocyclic ring structure of 5,6-dimethylbenzimidazole is important; it may be substituted by desdimethylbenzimidazole, 5-hydroxybenzimidazole, trifluoromethylbenzimidazole, or 5-aminobenzimidazole without any decrease in binding capability (1). The B-pyrrole ring lactone of cyanocobalamin binds very poorly by comparison with cyano-cobalamin (5).

Gräsebeck (10) has suggested that cobalamin fits into a "pit" near the surface of intrinsic factor, with substituents in the fifth coordination position pointing out of the surface of the protein molecule. Upon reacting with cobalamin, intrinsic factor has been shown to shrink appreciably according to experiments employing Sephadex G-200 (11) and the ultracentrifuge (12). These experiments indicate an open structure for intrinsic factor until cobalamin binds and the protein then closes around this coenzyme.

In this manuscript we report experiments on the determination of the position and orientation of cobalamins in intrinsic factor. Furthermore, we have discovered pertinent information concerning the role of 5,6-dimethylbenzimidazole in the formation of the cobalamin-intrinsic factor complex.

MATERIALS AND METHODS

Aqublicobalamin was prepared by the method of Penley et al. (13). Methylcobalamin was prepared by the method of Hogenkamp et al. (14). The B-pyrrole ring monocarboxylic acid of cyanocobalamin was prepared by the method of Donnett et al. (15). Three hundred milligrams of cyanocobalamin were dissolved in 1 liter of water, and the pH of the solution was adjusted to 4.0 with acetic acid. Nine milliliters of bromine water were added to the above solution with stirring. The bromine water solution was prepared by dissolving 8 g of bromine in a minimal amount of ethanol and diluting to 1 liter with distilled water. After stirring the reaction mixture for 1 hour, the product was phenol-extracted and lyophilized. The lyophilized powder was dissolved in 0.05 M Tris buffer pH 10.4, and passed down a DEAE-cellulose column (13 × 4 cm). The monocarboxylic acid eluted last from the column. The product was desalted by phenol extraction and lyophilized (yield ∼ 50%). Cyanocobalamin, DEAE- and phosphocellulose were obtained from Sigma Chemical Co. Methyl iodide was purchased from Aldrich Chemical Co. Cyanogen bromide was purchased from Eastman Chemical Co. Crystalline bovine serum albumin was obtained from Reheis Chemical Co. Pharmacia Fine Chemical, Inc. supplied Sepharose 4 B and Sephadex G-25.

Preparation of Cobalamin Affinity-labeled Sepharose—The method of Cuatrecasas (16) was used for the coupling of bromoethylamine to Sepharose. Sepharose-aminobromoethylamine was prepared by the reduction of aquocobalamin to cob(I)alamin (13) followed by the reaction of cob(I)alamin with Sepharose-bromoethylamine. Twenty milligrams of aquocobalamin were dissolved in 10 ml of 10% w/v aqueous NH₄Cl and purged with...
argon in an addition funnel placed on a 50-ml two-neck flask. The Sepharose-bromoethylamine (10 ml) was suspended in 10 ml of water which were placed in the flask and purged with argon. After 30 min the aquocobalamin was reduced with sodium borohydride and added to the Sepharose. After 80 min the product was collected on a glass-Buchner funnel and washed with cold water until the wash water was colorless. The product was exposed only to subdued light, and it was stored at 4° until used.

**Cobalamin Binding to Hog Intrinsic Factor**—Hog intrinsic factor binding to Sepharose-aminocobalamin was assayed by the method of Gottlieb et al. (17). The Sepharose-aminocobalamin was washed with 1 liter of 10% w/v NaCl followed by 500 ml of water to remove any free cobalamin. To ensure that no free cobalamin was present in solution, the last few milliliters of water were collected and assayed. Two milliliters of each of the following materials were placed in separate centrifuge tubes: (a) Sepharose, (b) Sepharose-bromoethylamine, (c) Sepharose-aminocobalamin supernatant (from the above water wash), and (d) Sepharose-aminocobalamin supernatant. Intrinsic factor (1.0 mg) was dissolved in 2.0 ml of water, and 0.2 ml of this solution was added to each centrifuge tube. After incubating for 10 min, the tubes were centrifuged for 10 min at a clinical centrifuge at maximum speed. From each tube 0.5 ml of supernatant was removed, and 0.1 ml of [57Co]cyanocobalamin (1 μCi per ml) was added. After 5 min 1.0 ml of albumin-coated charcoal, prepared by the method of Gottlieb et al. (17), was added and the charcoal was separated by centrifugation after allowing an incubation time of 10 min. The radioactivity of [57Co]cyanocobalamin in both the charcoal precipitate and in the supernatant from each tube was determined.

The binding of the B-pyrole ring monocarboxylic acid of cyanocobalamin to intrinsic factor was assayed in two ways. The first method consisted of incubating intrinsic factor (with a binding capacity of 6.0 ng of cobalamin) with 25 ng of either the monocarboxylic acid of cyanocobalamin or aquocobalamin. After incubating these reaction mixtures for 1 hour, 25 ng of [57Co]cyanocobalamin (0.1 μCi per ng) were added. As a control, identical reactions were set up without monocarboxylic acid of cyanocobalamin or aquocobalamin present. The above reaction mixtures were then passed down a Sephadex G-25 column (1 x 40 cm) at a flow rate of 0.2 ml per min in 0.1 M KH₂PO₄ buffer, pH 7.4, and the amount of each cobalamin bound to intrinsic factor was determined. The albumin-coated charcoal method was used as the second assay procedure to determine the amount of B-pyrole ring monocarboxylic acid of cyanocobalamin bound to intrinsic factor compared to aquocobalamin.

**Instrumentation**—1H nuclear magnetic resonance spectra were run on a Varian HR-200 operating at 290 MHz; deuterium oxide was used as a solvent. Absorption spectra were recorded with a Perkin-Elmer Coleman 124 double beam spectrophotometer. Precise absorbance measurements were made on a Zeiss PMQ II spectrophotometer. γ-Radiation (127Co) was counted in a well-type scintillation counter in connection with a Packard Tri-Carb liquid scintillation spectrometer. Computations involving the percentages of various cobalamins in mixtures were done on an IBM-1800 computer.

**RESULTS**

**Intrinsic Factor Binding to Sepharose-Aminocobalamin**—The position of the cobalamin binding site on intrinsic factor was determined by the use of Sepharose-aminocobalamin. If the cobalamin binding site is near the surface of intrinsic factor, it is to be expected that cobalamin which is covalently attached to Sepharose, should bind intrinsic factor tightly, and no free intrinsic factor should be present in the supernatant from the Sepharose-aminocobalamin. If the cobalamin binding site is deep within intrinsic factor, then intrinsic factor should not bind to Sepharose-aminocobalamin because of the stereochemical problems associated with intrinsic factor moving too close to the Sepharose backbone. Intrinsic factor binding to Sepharose-aminocobalamin would result in the complete absence of intrinsic factor in the supernatant from such a reaction mixture. In this case, the addition of [15Co]cyanocobalamin to the supernatant would result in no [15Co]cyanocobalamin-intrinsic factor complex formation, and the free [15Co]cyanocobalamin could be absorbed by albumin-coated charcoal. In the case of no intrinsic factor binding to Sepharose-aminocobalamin the intrinsic factor would remain in the supernatant and would form the [15Co]cyanocobalamin-intrinsic factor complex which is not absorbed by albumin-coated charcoal. Several important controls were performed concurrently with the assay for intrinsic factor binding to Sepharose-aminocobalamin. It is possible that free aquocobalamin may be present in the Sepharose suspension due to photolysis of the Co—C σ bond, and the resulting aquocobalamin would bind to intrinsic factor to give a false indication of intrinsic factor binding to Sepharose-aminocobalamin. To rule out this possibility the Sepharose-aminocobalamin was washed as described under "Materials and Methods," and the last few milliliters of water wash were collected and assayed. Furthermore, controls were performed to determine whether intrinsic factor binding to Sepharose or to the intermediate Sepharose-bromoethylamine.

The results of this binding experiment are presented in Table I. Intrinsic factor does not bind significantly to either Sepharose or Sepharose-bromoethylamine, nor does the wash from the Sepharose-aminocobalamin contain significant concentrations of aquocobalamin. Intrinsic factor forms such a strong complex with Sepharose-aminocobalamin that no intrinsic factor is detectable in the supernatant. Therefore, these data provide strong evidence that cobalamins bind close to the surface of intrinsic factor.

**Studies on Binding of B-Pyrole Ring Monocarboxylic Acid of Cyanocobalamin to Hog Intrinsic Factor**

Details of the experimental methods used to obtain the above data are given under "Materials and Methods" (100% = 105,000 cpm).

<table>
<thead>
<tr>
<th>Assay mixture</th>
<th>[15Co]Cyanocobalamin in supernatant %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Intrinsic factor + [15Co]Cyanocobalamin</td>
<td>1.0</td>
</tr>
<tr>
<td>2. Sepharose + intrinsic factor + [15Co]Cyanocobalamin</td>
<td>89.9</td>
</tr>
<tr>
<td>4. Sepharose-aminocobalamin supernatant + intrinsic factor + [15Co]Cyanocobalamin</td>
<td>86.1</td>
</tr>
<tr>
<td>5. Sepharose-aminationcobalamin + intrinsic factor + [15Co]Cyanocobalamin</td>
<td>92.5</td>
</tr>
</tbody>
</table>
Cyanocobalamin to Intrinsic Factor—Although binding of a large number of corrinoids to intrinsic factor has been investigated, one derivative which has been ignored is the B-pyrole ring monocarboxylic acid of cyanocobalamin. This derivative is an important one to study because of its structural similarity to both cyanocobalamin and the B-pyrole ring lactone of cyanocobalamin. The only difference between cyanocobalamin and the B-ring carboxylic acid is the conversion of the acetamide side chain at C7 to its corresponding carboxylic acid and the introduction of an hydroxyl group at C4. However, there is a significant alteration in the stereochemistry of the B-ring in this derivative.

The B-pyrole ring monocarboxylic acid of cyanocobalamin was synthesized (15) and characterized by 200 MHz NMR (Fig. 1). It is convenient to compare the NMR spectrum of the monocarboxylic acid to that of cyanocobalamin. The only difference between the two spectra occurs at δ = 6.5 where a doublet appears in the spectrum of cyanocobalamin which is missing in the spectrum of the B-ring monocarboxylic acid. The resonances from both the cyanocobalamin spectrum and the B-ring monocarboxylic acid spectrum were integrated by using the vinyl proton at C10 (δ = 5.9) as a reference for 1 proton. In the region of the spectrum between δ = 5.0 to δ = 9.0, the total number of protons in cyanocobalamin integrated as 19.0 ± 0.2 (Fig. 1a), while for the B-ring monocarboxylic acid the total number of protons integrated as 17.0 ± 0.2. The loss of 2 protons is to be expected for the conversion of the primary amide at C7 to its corresponding monocarboxylic acid. The presence of the free carboxylate group at C7 was confirmed by potentiometric titration. Twenty five milligrams of the B ring monocarboxylic acid derivative were dissolved in a minimum amount of water (4.0 ml), and the pH was raised to 8.0 with 0.1 N KOH. The titration was performed with 0.01 N HCl, and a similar titration was performed with cyanocobalamin as a control. From this experiment a pKₐ of 5.45 was determined for the monocarboxylic acid at C7.

The formation of the B-pyrole ring monocarboxylic acid—intrinisc factor complex was verified by chromatography on Sephadex G-25. A ratio of 4:1 monocarboxylic acid to intrinsic factor was preincubated for 1 hour, and then a 4-fold excess of

![Fig. 1.](a) 220 MHz NMR spectrum of cyanocobalamin in d₆-dimethylsulfoxide. b, 220 MHz NMR spectrum of the B-pyrole ring monocarboxylic acid of cyanocobalamin in d₆-dimethylsulfoxide.

![Fig. 2.](a) Cobalamin binding to intrinsic factor on Sephadex G-25. Twenty-five nanograms of each cobalamin were incubated with intrinsic factor, and 25 ng of [³⁵Co]cyanocobalamin subsequently added. Profiles represent (a) B-pyrole ring monocarboxylic acid of cyanocobalamin (●—●), (b) aquocobalamin (○--○), and (c) with no non-radioactive cobalamin added (○—○).

**TABLE II**

Cobalamin binding to intrinsic factor by albumin-coated charcoal assay

<table>
<thead>
<tr>
<th>Nonradioactive cobalamin added</th>
<th>Radioactivity in ppt</th>
<th>Radioactivity in supernatant</th>
<th>Radioactivity in supernatant normalized to cyanocobalamin</th>
<th>[¹⁰Co] Cyanocobalamin bound to intrinsic factor</th>
<th>Nonradioactive cobalamin bound to intrinsic factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Pyrole ring monocarboxylic acid of cyanocobalamin...</td>
<td>104,152</td>
<td>2,489</td>
<td>2,020</td>
<td>11.9</td>
<td>88.1</td>
</tr>
<tr>
<td>Aquocobalamin...</td>
<td>108,922</td>
<td>1,584</td>
<td>1,176</td>
<td>6.9</td>
<td>93.1</td>
</tr>
<tr>
<td>Cyanocobalamin...</td>
<td>88,254</td>
<td>17,440</td>
<td>17,022</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This reaction mixture was added. This reaction mixture was passed through a Sephadex G-25 column. Since the [¹⁰Co]cyanocobalamin remains in the uncomplexed cobalamin peak, then this experiment shows that the B-pyrole ring monocarboxylic acid forms a strong complex with intrinsic factor (Fig. 2).

The albumin-coated charcoal assay was used to quantitate B-pyrole ring monocarboxylic acid binding to intrinsic factor. The data for this experiment are presented in Table II, and the total radioactivity incorporated is normalized to the nonradioactive cyanocobalamin-intrinsic factor complex. From these data it is clear that the B-pyrole ring monocarboxylic acid of cyanocobalamin binds to intrinsic factor almost as well as aquocobalamin, and this result should be contrasted with the poor binding of the B-pyrole ring lactone of cyanocobalamin to this protein (5).
Aquocobalamin undergoes two reactions in the presence of excess CN\(^{-}\). The first reaction is the displacement of water from the fifth coordination site to form monocyanocobalamin, and the second reaction involves the displacement of 5,6-dimethylbenzimidazole from the sixth coordination site to give dicyanocobalamin. The formation of monocyanocobalamin from aquocobalamin has a log \(K_f\) of 12.0 (18), but the formation of dicyanocobalamin from monocyanocobalamin needs a large excess of CN\(^{-}\) because of the low log \(K_f\) of 3.8 for this reaction (19). The reaction sequence aquocobalamin \(\rightarrow \) monocyanocobalamin \(\rightarrow \) dicyanocobalamin is easily followed by using the different spectral properties of these three cobalamin. Monocyanocobalamin formation can be monitored at 545 nm, and dicyanocobalamin at 580 nm. The spectra of aquocobalamin, monocyanocobalamin, and dicyanocobalamin were digitized, and the percentages of each of these three at different concentrations of CN\(^{-}\) were calculated by a computer program employing a modified least squares fit.

Semilog plots were made of the relative concentrations of aquocobalamin, monocyanocobalamin, and dicyanocobalamin versus the ratio of CN\(^{-}\) to aquocobalamin added in each reaction mixture. This experiment was performed for (a) free aquocobalamin (Fig. 3a), (b) the aquocobalamin-bovine serum albumin complex (Fig. 3b), and (c) the aquocobalamin-intrinsic factor complex (Fig. 3c). The data show that very little dicyanocobalamin is formed in the reaction between CN\(^{-}\) and the aquocobalamin-intrinsic factor complex. Even CN\(^{-}\) concentrations capable of converting all the free aquocobalamin (or the aquocobalamin-bovine serum albumin complex) to dicyanocobalamin did not react with the aquocobalamin-intrinsic factor complex indicating that CN\(^{-}\) does not coordinate to the sixth coordination site in this complex.

**DISCUSSION**

The position of the cobalamin binding site on intrinsic factor was determined by the use of cobalamin affinity-labeled Sepharose. The length of the hydrocarbon arm separating the ligand from the Sepharose support is of great importance in determining the degree of ligand binding by a protein. For example when \(\alpha\)-chymotrypsin is passed through a column with tryptophanmethyl ester attached directly to Sepharose, no retardation of the enzyme is observed (20). However, \(\alpha\)-chymotrypsin will bind strongly to Sepharose-\(\epsilon\)-aminocaproyl-tryptophan-methyl ester. Similar results to the latter example have been obtained in a number of other cases (21).

Hog intrinsic factor does not bind to Sepharose or to 2-bromoethylamine-Sepharose, but it does bind strongly to Sepharose-\(\epsilon\)-aminocaproyl-tryptophan methyl ester. Similar results to the latter example have been obtained in a number of other cases (21). For complete binding, the binding site is definitely located close to the surface of intrinsic factor.

Previous studies concerned with the binding of various corrinoids to intrinsic factor have resulted in the following con-
clusions about the cobalamin-intrinsic factor complex: (a) the group coordinated to cobalt in position 5 has little effect on binding, (b) any changes in the ring portion of 5,6-dimethylbenzimidazole coordinated to the cobalt in position 6 causes nearly complete loss of binding, and (c) the corrin ring (especially the B-pyrrole ring) is extremely important in binding. The latter conclusion was drawn because of the relatively poor binding of the B-pyrrole ring lactone of cyanocobalamin to intrinsic factor (5). This conclusion is questionable because a strong interaction may be altered slightly with little change in affinity for intrinsic factor. However, a strong interaction between benzimidazole and intrinsic factor must occur because of the specificity for this base upon binding.

The failure of CNB to coordinate to position 6 of the cobalt when monocyanocobalamin is bound to intrinsic factor is of great importance. This experiment provides evidence for the coordination sphere in the region of position 6 being in a very hydrophobic environment or else a very tight complex is formed which allows little or no exchange of ions in the interior portion of intrinsic factor.

An over-all picture of the binding of cobalamins to intrinsic factor may now be considered. Complex formation involves corrin binding near the surface of the protein with no more than 5 A penetration. The corrin ring, although most likely of some importance in binding, may be altered slightly with little change in affinity for intrinsic factor. However, a strong interaction between benzimidazole and intrinsic factor must occur because of the specificity for this base upon binding.

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