

Molecular recognition in the binding of vitamin B₁₂ by the cobalamin-specific Intrinsic Factor

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Equilibrium constants (given as $\log K/M^{-1}$) have been determined at pH 7.4 and 4°C for binding by porcine Intrinsic Factor (B₁₂-binding protein from the gut, specific for the 'cobalamin' series of Co corrinoids) of vitamin B₁₂ or cyanocobalamin (10.5), cyanocobinamide, α -ribazole and α -ribazole-phosphate (main fragments produced by cleaving off the 'cobalamin' side-chain, all ≤ 3), and cyanocobinamide in the presence of $\geq 10^{-4}$ M ribazole (5.6 and independent of ribazole concentration), i.e. ribazole catalyses the binding of the cobinamide. It is proposed that the specificity of Intrinsic Factor for the cobalamins depends on the presence of the ribazole fragment in the cobalamin side-chain to promote an essential change in conformation before the corrinoid fragment can be bound.

Intrinsic Factor; Vitamin B₁₂; Cobalamin; Ribazole

1. INTRODUCTION

Coenzymes offer an unusually good opportunity for establishing the contribution of different parts of the molecule to the overall kinetics, energy and specificity of binding to macromolecules because of their ease of dissection into well-defined fragments. In spite of the current intense interest in molecular recognition, however, only two such examples appear to have been studied in detail. Comparison of the binding of the complete NAD and of 6 fragments of varying length to the apo-form of L-lactate dehydrogenase revealed a mechanism of molecular recognition in which the binding of AMP (and longer fragments) triggers a conformation change and nicotinamide mononucleotide can only bind in the presence (i.e. after the binding) of AMP [1,2]. Similar studies on the binding of NADPH and of several fragments to apo-dihydrofolate reductase also suggested that reduced nicotinamide mononucleotide could bind only after a change in conformation induced by some other fragment (not identified, but larger than AMP) [3].

Vitamin B₁₂ or cyanocobalamin (Fig. 1) is a member of the 'cobalamin' series of Co corrinoids, which all possess the nucleotide side-chain terminating in dimethylbenzimidazole as well as the amide side-chains shown in Fig. 1. The nucleotide side-chain can be cleav-

ed from B₁₂ at the point indicated to give cyanoaquacobinamide or Factor B, which exists as a mixture of 2 isomers involving interchange of the axial ligands [4], together with α -ribazole (i.e. the base + ribose) and α -ribazole-phosphate [5]. The single peptide glycoprotein Intrinsic Factor (IF), which binds B₁₂ in the gut (for attachment to, and transport through, the gut wall), is apparently unique amongst the mammalian B₁₂-binding proteins in its specificity for the cobalamin series [6]; it binds one B₁₂ per peptide [7] with a very high pH-independent (4.5–11) binding constant of 10^9 – 10^{10} M⁻¹ [8–11]. The nucleotide side-chain clearly plays an important role in the mechanism of molecular recognition of the cobalamin by IF in humans and other mammals, but the basis of such action remains unknown; neither cyanocobinamide [10,11] nor the base dimethylbenzimidazole [7] appear to bind to IF. We report here a comparison of the binding by IF of B₁₂ and of its components cyanocobinamide, α -ribazole and α -ribazole-phosphate, both separately and together, in order to identify the basis of the molecular recognition of cobalamins by IF.

2. MATERIALS AND METHODS

B₁₂ was obtained from Calbiochem, and *B₁₂ (0.0447 μ g and 10.5 μ Ci per ml) from Amersham International. Porcine IF (in vials containing 1000 units, where one unit binds 1 ng B₁₂) was obtained from Sigma; the total contents of each vial were dissolved in 1 ml phosphate-buffered solution pH 7.4 containing 0.1% bovine serum albumin (as recommended by Sigma) to give a stock solution which was stored at -20°C. Cyanocobinamide [12], ribazole and ribazole-phosphate [5] were prepared as previously described.

The very high value of the equilibrium constant for interaction between IF and B₁₂ (ca 10^{10} M⁻¹) necessitates the use of very low con-

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Abbreviations: B₁₂, cyanocobalamin or vitamin B₁₂; *B₁₂, ⁵⁷Co-labelled B₁₂; IF, Intrinsic Factor

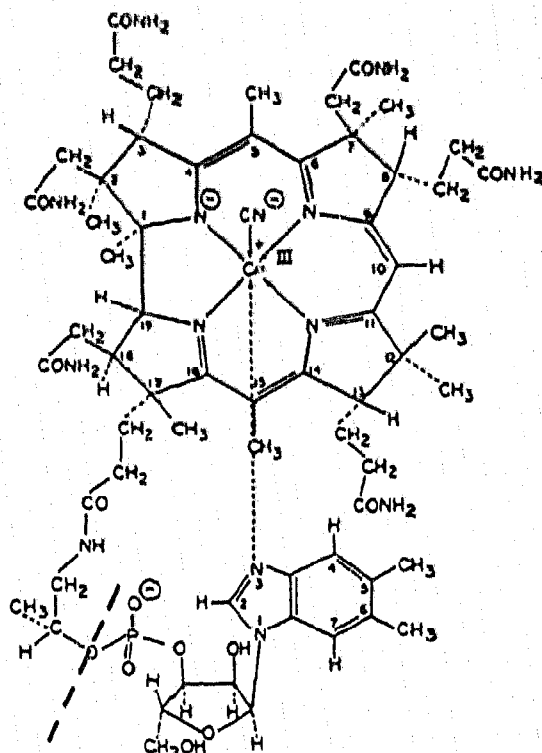
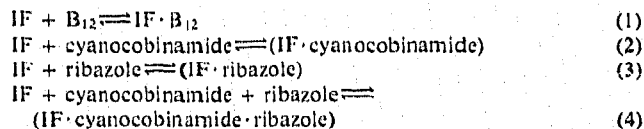


Fig. 1. Molecular structure of B₁₂, indicating (---) the point of cleavage of the side-chain to form cyanocobinamide and ribazole-phosphate.

centrations and analysis by radio-counting (of *B₁₂). The assay [13,14] is based on the fact that, after equilibrium between IF and *B₁₂, the addition of dextran-coated charcoal will rapidly adsorb any unbound B₁₂ and can be removed by centrifugation, and the concentration of IF-bound *B₁₂ in the supernatant determined by counting. The binding constants of other corrinoids (including 'cold' B₁₂) and fragments (such as ribazole) can then be determined by competition with *B₁₂ for IF. Each assay solution of 250 µl contained 3.04 pmol IF, 0.07 pmol *B₁₂ and varying concentrations (10⁻⁹–10⁻² M) of the components to be tested and were allowed to equilibrate for 1 h. All experiments were carried out in air at 4°C. In the following potential equilibria (with equilibrium constants numbered accordingly) it was assumed (cf. [7]) that each protein molecule bound one B₁₂ or cyanocobinamide and/or one ribazole (± phosphate):



3. RESULTS

Experiments involving the direct equilibration of IF with *B₁₂ gave a value of $K_1 = 3.3 \times 10^{10} \text{ M}^{-1}$ or $\log K_1 = 10.5$. Further studies using the competition assay with 1, 2, 5 and $10 \times 10^{-9} \text{ M}$ added 'cold' B₁₂ led to self-consistent values for 'cold' B₁₂ of $K_1 = 3.3, 3.2, 3.9$ and 3.2 ; av. $3.4 \times 10^{10} \text{ M}^{-1}$, also $\log K_1 = 10.5$. Full details will be given elsewhere.

Initial studies on the binding of the component parts of B₁₂ showed that neither cyanocobinamide, ribazole nor ribazole-phosphate alone at concentrations up to 10⁻³ M caused any significant displacement of *B₁₂ from IF, i.e. K_2 and $K_3 < 10^3 \text{ M}^{-1}$. Displacement was, however, caused by 1:1 mixtures of both cyanocobinamide + ribazole and cyanocobinamide + ribazole-phosphate with 50% displacement occurring at 5.5 and $4.6 \times 10^{-4} \text{ M}$ respectively, i.e. at $5 \times 10^{-4} \text{ M}$ (within experimental error) in both cases. Very surprisingly, the amount of displacement did not vary with the square of the concentration of the 1:1 mixture as expected from equation (4), but linearly with concentration. This unexpected result was explored by varying the 2 components separately with the results shown in Table 1. The data show that for 10⁻³, 10⁻⁴ and 10⁻⁵ M cyanocobinamide, the displacement of *B₁₂ is independent of ribazole concentration, at least down to 10⁻⁹ M ribazole; studies at lower concentrations were invalidated by the increasing errors involved. The % binding depends only on the concentration of cyanocobinamide and the 4 results at 10⁻⁴ and 10⁻⁵ M cyanocobinamide and ribazole yield values of $K_2 = 5.3, 3.0, 3.9$ and 2.6 ; av. $3.7 \times 10^3 \text{ M}^{-1}$ or $\log K_3 = 5.6$.

4. DISCUSSION

Our value of $K_1 = 3.3 \times 10^{10} \text{ M}^{-1}$ ($\log K_1 = 10.5$) for binding of B₁₂ to porcine IF at 4°C compares well with the value expected by extrapolation of the values reported for human IF over the range from 38°C down to 22°C, viz. 0.14 to $1.3 \times 10^{10} \text{ M}^{-1}$ [9].

Table 1

Binding of cyanocobinamide and ribazole to Intrinsic Factor in competition with radio-labelled *B₁₂

Conc./M	% *B ₁₂ bound to IF in the presence of a fixed concentration of one component (given below) and varying concentrations (see left) of the second component (cyanocobinamide + ribazole)		
	Cyanocobinamide 10 ⁻⁴ M	Ribazole 10 ⁻⁵ M	Ribazole 10 ⁻⁴ M
10 ⁻⁹	27	100	100
10 ⁻⁸	29	94	100
10 ⁻⁷	31	99	99
10 ⁻⁶	32	94	100
10 ⁻⁵	42	88	84
10 ⁻⁴	39	42 ^a	39 ^a
10 ⁻³		28 ^a	28 ^a
10 ⁻²			2

^a Used for calculating equilibrium constants (see text)

The assay (see section 2) involved competition between *B₁₂ and (cyanocobinamide + ribazole) for IF. The results (average of 3 experiments at each concentration) are given in the form of *B₁₂ bound to IF in the presence of cyanocobinamide + ribazole as a percentage of the *B₁₂ bound in their absence. (Actual concentrations of cyanocobinamide and of ribazole were 1.2 and $1.4 \times 10^{-11} \text{ M}$ respectively; here all taken as $1.0 \times 10^{-11} \text{ M}$ for simplicity.)

Our results confirm that cyanocobinamide alone does not displace $^*B_{12}$ from IF (cf. [10,11]) and in addition show (a) that ribazole and ribazole-phosphate alone will not do so, i.e. IF will not bind alone to either of the 2 complete fragments (cyanocobinamide, ribazole-phosphate) which together (with elimination of H_2O) form B_{12} ; (b) that cyanocobinamide and ribazole (or ribazole-phosphate) together will do so, i.e. there is some form of cooperative interaction; and (c) that ribazole and ribazole-phosphate have virtually the same effect, i.e. the phosphate does not play any significant additional role. If one assumes that this cooperative binding of the 2 fragments with IF involves the same sites as with B_{12} itself, then the inability of either fragment alone to displace $^*B_{12}$ can be used to derive values of $\log K_2$ and $\log K_3 < 3$ for binding cyanocobinamide and ribazole separately at the relevant sites. We have further shown that, in the presence of some minimum concentration (10^{-9} M) of ribazole, the observed binding depends only on the concentration of added cyanocobinamide according to equation (2) with $\log K_2 = 5.6$.

We suggest that, in the binding of cyanocobinamide with ribazole to IF, the first step involves the binding of ribazole which causes a change from an inactive conformation P_1 to the active P_2 ; this is able to bind cyanocobinamide, giving P_3 and followed by the dissociation of ribazole (at least at the concentrations used). In other words, ribazole catalyses a conformation change. Certain enzymes are reported to require an analogous ligand-induced preconditioning or maturation before the substrate can be bound [15,16], though no example appears to have been reported hitherto among metalloenzymes [17]; an instructive example is phosphofructokinase, where ATP reversibly catalyses the inter-conversion of inactive and active conformations [15]. By analogy we suggest that, in the binding of B_{12} to IF, the first step involves binding the nucleotide side-chain (containing ribazole) which causes the change in conformation and allows the corrinoid part to be bound, accompanied by a further change in conformation; the ribazole, being part of the covalently attached side-chain, remains bound to IF. It is, in fact, likely that most of the enhanced binding constant of B_{12} ($\log K_1 = 10.5$) compared to that of cyanocobinamide in the presence of ribazole ($\log K_2 = 5.6$) would be contributed by the additional binding of ribazole ($\log K_3 \leq 3$ when free) which could be significantly enhanced when incorporated into the nucleotide side-chain of B_{12} . This two-step binding could explain the report that the binding of B_{12} to IF shows saturation kinetics [10], as well as inducing a significant conformation change in IF [18].

There is an obvious parallel between the binding of NAD by lactate dehydrogenase (see section 1) and of B_{12} by IF. Both coenzymes consist of a functional fragment (nicotinamide, corrinoid) and a recognition frag-

ment (AMP, ribazole), and the functional fragment can bind only to a conformation induced by the prior binding of the recognition fragment. The difference between the catalytic function of ribazole and the stoichiometric binding of AMP may be more apparent than real; binding of ribazole is likely to be observed at higher concentrations (and the limit of $K_3 < 10^3$ allows for a binding constant comparable to that of AMP), and no tests have been made for catalysis by lower levels of AMP.

Our results show that IF exhibits a specificity of $\log K \geq 7.5$ for the cobalamin B_{12} over the cobinamide (cf. $\log K = 10.5$ and ≤ 3 respectively) and that this specificity can be ascribed to the role of the nucleotide side-chain in promoting or catalysing a key conformation change required before the main functional (i.e. corrinoid) part of the molecule can be bound. Analogous mechanisms have now been found in all 3 examples of coenzyme binding so far studied (involving NAD [1], NADPH [3] and now B_{12}) and may therefore represent a fairly common device used in the process of molecular recognition of coenzymes in general.

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