THE ROLE OF ATP IN THE BIOSYNTHESIS OF COENZYME B_{12}

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We have recently described the conversion of vitamin B_{12} to its coenzyme form in cell-free extracts of Clostridium tetanomorphum (7). A similar conversion takes place in extracts of Propionibacterium shermanii (3). The requirements for the activation of the vitamin include glutathione, DPNH, a flavin, $MnCl_2$ and ATP (3).

The B_{12} coenzyme contains an adenine nucleoside linked to reduced cobalt (2,5,6). The carbohydrate component of this nucleoside is not one of the more common sugars; its exact structure has not yet been reported. We have previously shown that the adenine moiety of ATP is the precursor of the adenine component of the adenine nucleoside in the B_{12} coenzyme (7). We now wish to report that the sugar moiety of this adenine nucleoside also originates in ATP.

Vitamin B_{12} was incubated with the enzyme system from Cl. tetanomorphum in the presence of randomly-labelled C^{14}-ATP under the conditions previously described (7). The B_{12} coenzyme was separated from residual ATP and other adenine derivatives by successive passages through columns of Dowex-1-formate and then Dowex-50-Na^+ (1,7). Enzymatic assay (1,7) for B_{12} coenzyme in the Dowex-50 effluent indicated that 21.5 mμmoles had been formed during the incubation. The radioactivity in this fraction corresponded to an incorporation of 21.0 mμmoles of ATP, calculated on the basis that the radioactivity in both the adenine and ribose portions of ATP was utilized. This stoichiometric relationship suggests that both the base and pentose moieties of ATP serve

as precursors of the adenine nucleoside fragment of coenzyme B_{12}. Parallel experiments using ATP-8-C^{14} have shown that one equivalent of adenine is incorporated into vitamin B_{12} during its conversion to coenzyme B_{12} (7).

The incorporation of radioactivity from randomly labelled ATP into both the adenine and sugar moieties of the coenzyme nucleoside was verified by degradation of the enzymatically labelled coenzyme B_{12} with subsequent localization of the radioactivity. Several methods were used to cleave the nucleoside component of the B_{12} coenzyme into adenine (which is adsorbed on Dowex-50 Na^+) and a sugar (not adsorbed on Dowex-50 Na^+), as follows: when an aliquot of the partially purified coenzyme B_{12} was passed through Dowex-50 Na^+, no radioactivity was retained on the column (Table I, A). Hydrolysis of the coenzyme in 0.2 N HCl for one hour at 100°, followed by passage over Dowex-50 led to retention of about 60% of the radioactivity on the column, while the remainder was in the effluent (Table I, B). A similar result was obtained when the coenzyme was treated with cyanide (6) (Table I, C), or if the intact nucleoside was isolated after photolysis (Table I, D; Dowex-50 eluate), and then hydrolyzed to the sugar and base (D, acid-hydrolyzed) (6). Cyanide degradation of coenzyme B_{12} labelled by ATP-8-C^{14} results in complete retention of the radioactivity on the Dowex-50 column (7); this indicates that the radioactivity in the Dowex-50 effluent in these experiments, using randomly labelled ATP, must be derived from the ribose moiety of ATP. Acid hydrolysis of the randomly labelled ATP used in this experiment, followed by similar fractionation on Dowex-50, established the ratio of radioactivity in the adenine and ribose moieties (Table I, E). It can be seen that the distribution of radioactivity in the adenine nucleoside of the B_{12} coenzyme corresponded closely with that of the ATP. From this data, it seems clear that the adenosine fragment of ATP is a direct precursor of the coenzyme adenine nucleoside. Thus, the transformation of the ribose moiety of ATP to the sugar characteristic of B_{12} coenzyme probably occurs while still bound to adenine.

Table 1

Localization of Radioactivity from ATP-C^{14} in B_{12} Coenzyme

Treatment	cpm Dowex-50 Effluent	cpm Dowex-50 Eluate	Ratio Effluent / Total
A - Untreated Coenzyme	185	9	
B - Acid-hydrolyzed Coenzyme	120	142	0.46
C - Cyanide-treated Coenzyme	108	148	0.44
D - Photolyzed Coenzyme	0	250	
D, Acid-hydrolyzed	107	195	0.35
E - Acid-hydrolyzed ATP-C^{14}	1366	1919	0.42

The incubation mixture contained, in a volume of 1 ml: 0.4 ml of Cl. tetanomorphum extract (20 mg protein/ml), 0.12 μmoles of vitamin B_{12}, 10 μmoles of glutathione, 2 μmoles of $MgCl_2$, 50 μmoles of sodium phosphate buffer, pH 7.4, 10 mg of Difco yeast extract and 0.2 μmoles of randomly-labelled ATP-C^{14} (Schwarz BioResearch) (58,000 cpm/μmole). After incubation at 37° for 4 hours, the mixture was treated as described in the text. Radioactivity was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer, using 2 ml of aqueous sample and 10 ml of the scintillator mixture reported by Bray (4).

The data above indicates that the sugar moiety of the coenzyme B_{12} adenine nucleoside is derived from ATP, and not, as has been suggested, from FMN (3). Table II demonstrates that the requirement for yeast extract (7) for coenzyme B_{12} biosynthesis can be replaced by a combination of DPNH and FMN, in accordance with the studies of Brady and Barker (3). However, ethanol flavin*, a compound in which the ribityl moiety of riboflavin is replaced by an ethylene glycol moiety, also satisfies the flavin requirement. This experiment further excludes the flavin as the precursor of the carbohydrate fragment of the coenzyme B_{12} adenine nucleoside.

* This compound, prepared by the Upjohn Company, Kalamazoo, Michigan, was a gift from Dr. Lin Tsai of the National Heart Institute.

Table II

Replacement of FMN by Ethanol Flavin for Coenzyme B_{12} Biosynthesis

System	Coenzyme B_{12} formed (mμmoles/ml)
Complete	16.5
Yeast extract replaced by DPNH	0
Yeast extract replaced by DPNH + FMN	15.5
Yeast extract replaced by DPNH + ethanol flavin	19.5

Incubation conditions were identical with those in Table I, except that unlabelled ATP was used. Where indicated, 0.1 μmole of FMN, 0.1 μmole of ethanol flavin or 1 μmole of DPNH was used. After a 30 minute incubation period, 0.1 ml of the incubation mixture was removed for assay of coenzyme B_{12} (7).

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