ATP FORMATION DURING ENZYMATIC DECOMPOSITION OF COENZYME B_{12}

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The coenzyme forms of vitamin B_{12}[1/] (Barker et al., 1958) contain a 5'-deoxyadenosyl group linked in a covalent bond to the cobalt of the corrin nucleus (Lenhert and Hodgkin, 1961). Enzymatic conversion of vitamin B_{12} to the coenzyme form has been demonstrated in cell-free extracts (Brady and Barker, 1961; Weissbach et al., 1961; Vitols et al., 1966). These studies have established that adenosine triphosphate donates an adenosyl moiety to the vitamin (Peterkofsky et al., 1961) to form the coenzyme in a reaction that leads to the release of inorganic tripolyphosphate (Peterkofsky and Weissbach, 1963) (reaction 1):

$$\text{Vitamin } B_{12} + \text{ATP} \xrightarrow[\text{FMN}]{\text{mercaptoethanol}} \text{adenosyl-}B_{12} + \text{PPPi} \qquad (1)$$

The overall reaction also requires reduced flavin (Brady and Barker, 1961). While the cofactor requirements and products of this biosynthetic reaction have been described, essentially nothing was known about the enzyme mechanism. Some studies are reported here which support the following scheme for the enzymatic reaction:

$$\begin{aligned} &\text{(a)}\quad \text{Enzyme} + \text{ATP} \rightleftarrows \text{adenosyl-enzyme} + \text{PPPi} \\ &\text{(b)}\quad \text{Adenosyl-enzyme} + \text{vitamin } B_{12}\ \text{(reduced)} \rightleftarrows \\ &\qquad \text{adenosyl-}B_{12} + \text{enzyme} \end{aligned} \qquad (2)$$

1/ Vitamin B_{12} is α(5,6-dimethylbenzimidazolyl)cyanocobamide; coenzyme-B_{12} (adenosyl-B_{12}) is α(5,6-dimethylbenzimidazolyl) 5'-deoxyadenosyl cobamide; Pi is inorganic phosphate; PPi is inorganic pyrophosphate; PPPi is inorganic tripolyphosphate.

ATP: cobamide 5'-deoxyadenosyl transferase was partially purified from Clostridium tetanomorphum by a previously described procedure (Peterkofsky and Weissbach, 1963) to yield a preparation that was essentially free of ATP-ase. In the presence of all the components required for the conversion of vitamin B_{12} to coenzyme B_{12}, an exchange of $PPPi^{32}$ into charcoal-adsorbable material took place (Table I). Investigation of the

TABLE I

Requirements for $PPPi^{32}$-ATP Exchange

	cpm incorporated
Complete system	1140
" " (30 min. incubation)	680
" " (.04 ml enzyme)	2670
Omit vitamin B_{12}	1395
" mercaptoethanol	1100
" FMN	1330
" mercaptoethanol + FMN	1100
" ATP	156
" enzyme	143
add PPi	289

Complete incubation mixtures (0.4 ml) contained: potassium phosphate, pH 8.0, 20 µmoles; mercaptoethanol, 20 µmoles; FMN, 0.03 µmoles; $MgCl_2$, 0.05 µmole; ATP, 0.08 µmole; $PPPi^{32}$ (approximately 10^6 cpm/µmole), 0.08 µmole; vitamin B_{12}, 0.03 µmole; enzyme, 163 units/ml (Peterkofsky and Weissbach, 1963), 0.02 ml. Incubation was for 1 hour at 37° in the dark. The reactions were terminated by the addition of 0.05 ml of a suspension of charcoal in $HClO_4$ (Peterkofsky and Weissbach, 1963). The charcoal was then trapped on millipore filters and washed with 1% $HClO_4$. The filters were glued onto planchets and counted in a gas-flow counter.

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requirements for the reaction indicated that the exchange did not require vitamin B_{12}, mercaptoethanol or FMN. However, it was dependent on the addition of enzyme and ATP. Furthermore, the reaction was dependent on time and enzyme concentration. This data suggested that, in the presence of ATP and $PPPi^{32}$, the enzyme preparation was catalyzing the reversible partial reaction (2a), leading to the incorporation of radioactivity into ATP. Table I also shows that the $PPPi^{32}$-ATP exchange reaction was essentially completely inhibited by a concentration of PPi equimolar to that of

the PPPi. The synthesis of B_{12}-coenzyme has also been shown to be inhibited by PPi (Brady et al., 1962; Vitols et al., 1966); this suggests that the $PPPi^{32}$-ATP exchange and B_{12}-coenzyme synthesis may be catalyzed by the same enzyme.

The data of Table II demonstrate another activity catalyzed by the ATP:cobamide 5'-deoxyadenosyl transferase preparation. When the enzyme

TABLE II

Reversal of B_{12}-coenzyme Synthesis

	cpm incorporated
Expt. 1	
complete system	675
omit B_{12}-coenzyme	279
" mercaptoethanol	420
" FMN	468
Expt. 2	
complete system	597
omit B_{12}-coenzyme	240
" $PPPi^{32}$, add PPi^{32}	247
" " " " omit B_{12}-coenzyme	206
Expt. 3	
complete system	476
omit B_{12}-coenzyme	169
omit enzyme	139

Complete incubation mixtures (0.4 ml) contained: potassium phosphate, pH 8.0, 20 µmoles; mercaptoethanol, 40 µmoles; FMN, 0.04 µmoles; $MgCl_2$, 0.05 µmole; $PPPi^{32}$ (1.25×10^6 cpm/µmole), 0.16 µmole; B_{12} coenzyme, 0.054 µmole; enzyme 0.02 ml. Where indicated, PPi^{32} (1.46×10^6 cpm/µmole), 0.16 µmole was included. Incubation was for 1 hour at 37° in the dark. The reactions were terminated as in Table I.

was incubated with $PPPi^{32}$, B_{12}-coenzyme, FMN and mercaptoethanol, there was an incorporation of radioactivity into charcoal-absorbable material. The reaction was dependent on the addition of B_{12}-coenzyme and was somewhat decreased when either mercaptoethanol or FMN was omitted (expt. 1). The enzyme dependence is shown in expt. 3. While there was a B_{12}-coenzyme-dependent incorporation of radioactivity with $PPPi^{32}$, there was no such

incorporation using PPi^{32} (expt. 2). These data suggest that the enzyme preparation catalyzes the reversal of reaction (1). Under these conditions, PPPi would displace vitamin B_{12} from adenosyl-B_{12}, forming ATP, a charcoal-adsorbable product.

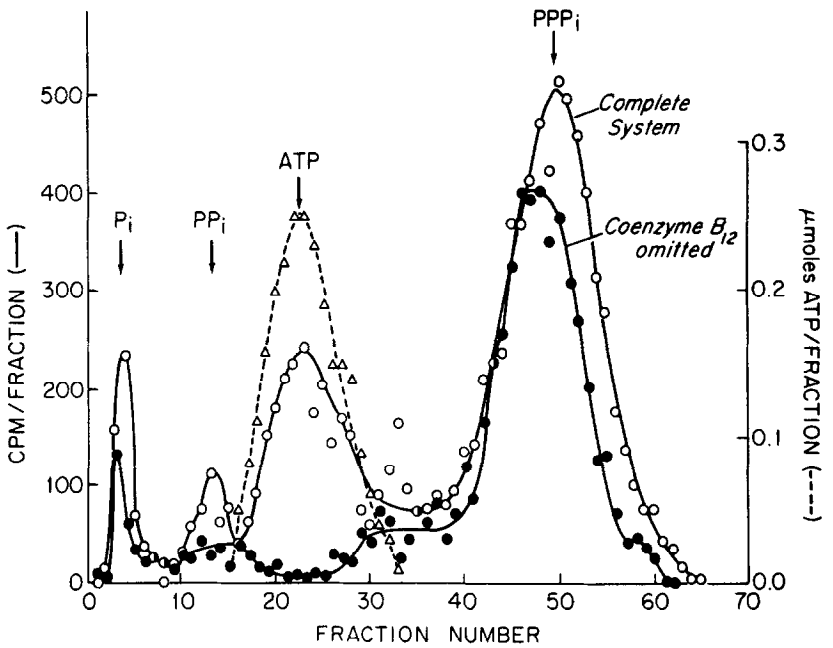

Fig. 1. Two incubation mixtures (0.8 ml volume) were prepared. They contained: potassium phosphate, pH 8.0, 20 μmoles; mercaptoethanol, 40 μmoles; FMN, 0.04 μmoles; $PPPi^{32}$ (2.15×10^7 cpm/μmole), 0.04 μmoles; enzyme, 0.02 ml. One of the two incubations contained coenzyme B_{12}, 0.027 μmole. Incubation was for 1 hr. at 37° in the dark. The reactions were terminated by the addition of 0.1 ml 10% $HClO_4$, then 5 μmoles of carrier ATP were added. One ml of charcoal suspension was added to adsorb the nucleotides. The charcoal was centrifuged and washed with 1 ml of 1% $HClO_4$, then eluted twice with 2 ml aliquots of 50% ethanol containing 0.1 N NH_4OH. After removal of the ethanol and NH_4OH under a stream of air, the radioactive compounds in each incubation mixture were fractionated on columns of Dowex-1 Cl X2 (20 ml bed volume) as previously described (Mudd, 1962; Peterkofsky and Weissbach, 1963). 2 ml fractions were collected. Optical density at 260 mμ was determined on 1:5 dilutions of the fractions to localize the elution peak corresponding to ATP. 1.0 ml aliquots of each fraction were counted with 10 ml of naphthalene-dioxane scintillation solution (Bray, 1960) to determine the distribution of radioactivity.

The experiment detailed in Figure 1 presents evidence that ATP^{32} was formed from $PPPi^{32}$ in the B_{12}-coenzyme-dependent reaction. Enzyme was incubated with $PPPi^{32}$ and the required cofactors in reaction mixtures with

or without B_{12}-coenzyme. The acidified reaction mixture was supplemented with carrier ATP and treated with charcoal. This adsorption step separated the ATP from the bulk of the $PPPi^{32}$ in the reaction mixture. The adsorbed nucleotides and contaminating phosphates were partially eluted from the charcoal with ammoniacal ethanol and fractionated on Dowex-1 chloride under conditions that resolve Pi, PPi, ATP and PPPi (Mudd, 1962; Peterkofsky and Weissbach, 1963). As can be seen in Figure 1, a radioactive peak coinciding with the elution position for ATP is formed in the reaction mixture containing coenzyme B_{12}, but not in the incubation in which this component was omitted. The data indicate, therefore, that reaction (1) is reversible. The failure of Vitols *et al.*, (1966) to demonstrate reversal of the reaction under similar experimental conditions may be related to the difference in sensitivity of the isotope method used here (the whole peak of radioactive fractions in the ATP region in Figure 1 amounts to 0.12 μmoles ATP formed) and the other detection methods.

The data presented in this communication are compatible with the scheme of reaction (2) in which an adenosyl-enzyme is an intermediate in the transfer of the 5'-deoxyadenosyl group of ATP to vitamin B_{12} during the biosynthesis of coenzyme B_{12}.

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