The Formation of α-Glycosidic 5'-Nucleotides by a Single Displacement Trans-N-glycosidase*

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Among the vitamins, the members of the B12 group have the most complex structure. Biosynthetic studies have thus far been concerned with labeling experiments designed to determine the origin of the corrin nucleus (1-3), the associated methyl groups (3), and the aminopropanol moiety (4), and with enzymatic studies on the formation of the coenzyme form of the molecule (5-9). The present work was carried out to gain some insight into the origin of the unusual N-α-glycosidic bond found in the vitamin between the ribose and the benzimidazole-like base, such as 5,6-dimethylbenzimidazole.

In a previous publication from this laboratory (10), it was shown that Propionibacterium shermanii converts free 5,6-dimethylbenzimidazole or benzimidazole to the corresponding N-1-substituted nucleosides and excretes them into the medium. The present paper presents evidence to show that these are α-glycosidic nucleosides, that they are derived from the corresponding 5'-nucleotides by dephosphorylation, and that these nucleotides are, in turn, formed by the action of a trans-N-glycosidase. This enzyme catalyzes an exchange between benzimidazoles and the nicotinic acid of N,MN. Since an inversion of configuration occurs, the reaction probably involves a single displacement.

EXPERIMENTAL PROCEDURE

Chemicals—Bi12-2-14C was synthesized from α-phenylendiamine and formic acid.14C (10, 11) or was obtained from the California Corporation for Biochemical Research.

Ribazole phosphate (α-DBI-R-3'P') was prepared by a modification of the method of Kaczka and Folkers (12). Vitamin B12 (10 mg) (Sigma Chemical Company) was treated with 8 ml of 6 N HCl for 13 hours at 30°. Most of the free HCl was removed under reduced pressure at room temperature, and the solid residue was taken up in a small volume of water. Following electrophoresis on paper in 0.5 M acetic acid, the band fluorescing in ultraviolet light (253.7 mp) was eluted with water.

N,MN was obtained from the Sigma Chemical Company. The following chemicals were kindly provided as gifts: β-N,MN by Dr. F. N. Minard (13), β-N,MN free from detectable α-N,MN by Dr. N. O. Kaplan (14), and β-BI-R and β-DBI-R by Dr. G. B. Brown (15). Glass beads (Superbrite No. 100; diameter, 0.2 mm) were obtained from the Minnesota Mining and Manufacturing Company.

Growth Conditions—In the initial experiments P. shermanii (ATCC 9614) was grown without aeration at 30° on a medium similar to that used by Bernsauer, Deuel, and Willians (16), except that Difco tryptone and Difco Casamino acids were used instead of hydrolyzed casein which was prepared locally. No cobalt was added to the medium. In later experiments, frozen cells of P. shermanii grown anaerobically on a cornsteep liquor-glucose-calcium carbonate medium were used. These were the kind gift of Dr. D. Perlman (cf. Reference 17). For the purpose of the present study, there appeared to be no significant differences in the bacteria grown under these conditions. Lactobacillus delbrueckii (ATCC 9649) was grown at 37° as described by Takagi and Horecker (18); the cells were packed by centrifugation and stored at -15°.

Cofactors—In early experiments, material essential for the enzymatic reaction was obtained by repeated extraction of P. shermanii with 2 ml portions of 30% pyridine per g of bacteria. The pyridine was removed by lyophilization or by flash evaporation at 30°. As knowledge of the enzyme system developed, known nucleotides, notably N,MN or N,N,MN, were used instead.

Cell-free Extract of P. shermanii and Assay of Enzyme—Details of the preparation and purification of the enzyme and of the composition of the assay mixture are given in the following paper (19). Here it suffices to state that the crude enzyme was obtained by grinding the bacteria in a Waring Blender with glass beads (20) and that the assay mixture contained enzyme, N,MN or other cofactor, a benzimidazole, and buffer at an alkaline pH. The final volume was usually 50 μl.

Electrophoresis—Following the enzymatic reaction, 30 μl of the mixture were applied to strips of Whatman No. 1 paper (4 X 15.5 cm) in a suitable solvent and subjected to electrophoresis for about 1 hour with a gradient of 23.5 volts per cm in the Kensington Scientific Corporation model 50 paper electrophoresis apparatus. The solvent used in preliminary experiments was 0.5 M acetic acid. The pH of this medium is low enough to cause the compounds of interest to migrate to the cathode.
(Table I), to inactivate the enzyme directly, and to permit the bluish fluorescence of BI or DBI and their nucleosides and nucleotides to be observed directly under ultraviolet illumination (filter transmitting light with a wave length of 253.7 m). However, in this liquid the paper is easily overloaded, with a resulting blurring of the bands. When Tris-borate buffer, pH 8.6 (2.0 M Tris, 25 ml, and 0.25 M borate acid, 125 ml) was used, overloading was not encountered, and much sharper bands were obtained. In this system the mixture is applied near the cathode end of the paper, and the substances under study move toward the anode, with the lowest mobility exhibited by free BI (Table I). Inactivation by heat was not necessary for preliminary experiments, but was always used for quantitative studies (19).

**Counting of Radioactivity on Paper**—Radioactive bands were located by means of the automatic graphical record given by an Actigraph II strip counter (Nuclear-Chicago) with 2σ geometry. The radioactivity in different bands was compared by positioning them under the Geiger tube and feeding the output directly into a decade scaler for a given time. This method proved precise and reproducible and was more convenient than the counting of eluates on planchets.

**Other Methods**—Ultraviolet absorption spectra of the benzimidazoles were recorded with the Bausch and Lomb Spectronic 505 spectrophotometer or obtained with the aid of a Beckman DU spectrophotometer with photomultiplier attachment, useful for accurate readings of the sharp maxima characteristic of the benzimidazoles (see Fig. 2). Absorption cells of 10-mm light path were used.

Phosphate was determined by the sensitive method of Kuttner and Cohen (21) in the elegant modification of Hurst (22), which uses hydrazine sulfate to stabilize stannous chloride, the reducing agent.

Free ribose was determined by the method of Nelson (23). Bound ribose was determined by the spectrophotometric periodate consumption method of Dixon and Lipkin (24) with the necessary correction for material eluted from the paper.

*Escherichia coli* alkaline phosphatase (chromatographically pure, Worthington Biochemical Corporation) was used as a reagent to dephosphorylate the benzimidazole nucleotides to the corresponding nucleosides. Incubations were carried out at pH 8.6 and 30°.

Snake venom (Crotalus adamanteus) obtained from Sigma Chemical Company was used as a source of 5'-nucleotidase (25). Purification was not necessary since the venom had no activity on adenosine 3'-phosphate or α-D-glucose 3'-phosphate.

In ascending paper chromatography, the Partridge solvent (26) as modified by Smith (27) (90 volumes of 1-butanol, 15 volumes of acetic acid, and 25 volumes of water) was run on Whatman No. 1 paper for 6½ hours.

**RESULTS**

The previous study (10) had indicated that pyridine extracts of washed cells of *P. shermanii* (grown with cobalt) contained three radioactive substances derived from BI-2-14C, separable by electrophoresis in 0.5 M acetic acid (Table I). One of these proved to be the nucleoside of the base, and the others were tentatively identified as benzimidazolylhydroxocobamide and benzimidazolylcobamide coenzyme.

**Initial Demonstration of Further Benzimidazole Derivative**—Similar extracts from cells grown without cobalt show two spots in addition to BI-2-14C: the nucleoside and a second compound which moves more slowly toward the cathode in 0.5 M acetic acid. The electrophoretic mobility of this compound was indistinguishable from that of benzimidazolylhydroxocobamide (Table I). Since, however, no cobalt had been added and the pink color expected of cobamides was absent, it seemed improbable that the slow moving component was benzimidazolylhydroxocobamide. Its identification thus became a matter of considerable interest.

The low mobility of the unknown compound toward the cathode in 0.5 M acetic acid compared to the nucleoside suggested the presence of a single compensatory negative charge on the molecule. It therefore seemed possible that this compound was a mononucleotide of BI. When the reaction mixture was incubated with the *E. coli* phosphatase, the suspected nucleotide disappeared and a component with the electrophoretic mobility and acid fluorescence of the nucleoside appeared. A typical experiment obtained with a partially purified enzyme is given below (Fig. 1). Since the *E. coli* phosphatase hydrolyzes nucleoside monophosphates and polyphosphates (28, 29) but cannot hydrolyze the pyrophosphate bond in dinucleotides, the supposition that the unknown compound extracted from intact *P. shermanii* is a BI mononucleotide was tentatively confirmed. It appeared plausible that this compound is a precursor of the ribonucleoside to which it is converted by the action of an endogenous phosphatase, or that it is derived from the nucleoside by action of a kinase.

**Initial Enzyme Experiments**—To test these alternatives it seemed simplest to demonstrate the appropriate reaction in cell-free systems. Initial experiments were far from promising, but when a cell-free extract of *P. shermanii* was fortified with a pyridine extract of this organism and incubated with BI-2-14C at pH 8.6 for several hours, two new radioactive bands appeared in electrophoretograms with the mobilities of BI-R and the presumptive nucleotide. The latter compound is converted to the nucleoside by treatment with the *E. coli* phosphatase. In the crude enzyme system (without exogenous phosphatase), both nucleotide and nucleoside are formed in this order. When fluoride, a common inhibitor of phosphatase (30), is added to the crude *P. shermanii* enzyme system, only the nucleotide is formed. Hence, it appears that the nucleotide is formed first and then is converted by action of an endogenous phosphatase to BI-R.

**TABLE I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>0.5 M acetic acid (migration to cathode)</th>
<th>Tris-borate (migration to anode)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzimidazole nucleoside</td>
<td>0.69</td>
<td>3.74</td>
</tr>
<tr>
<td>Benzimidazolylcobamide coenzyme</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Benzimidazolylhydroxocobamide</td>
<td>0.18</td>
<td>8.23</td>
</tr>
</tbody>
</table>

* See “Experimental Procedure.”
† Compound identified by criteria discussed below and elsewhere (10, 19).
†† Tentative identification by criteria discussed in detail elsewhere (10).
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FIG. 1. Action of phosphatase on product of enzymatic reaction. BI-2-14C and N,MN were incubated at pH 8.6 as described in the text and subjected to electrophoresis in Tris-borate buffer, pH 8.6. The curves represent tracings of strip counter records of the electrophoretograms obtained with the reaction mixture: A, before; B, during; and C, after action of added chromatographically pure E. coli alkaline phosphatase. For results obtained with snake venom α-nucleotidase, see Fig. 3.

TABLE II
Conversion of benzimidazole (BI) to its nucleotide (BI-R-P) and nucleoside (BI-R)

Crude P. shermanii enzyme containing 1.35 mg of protein was incubated with 0.6 μmole of MgSO₄, 80 μmole of BI-2-14C (10.4 c.p.m. per μmole), and 2 μmole of N,MN and sodium hydroxide to a pH of about 9 at 30° in a total volume of 90 μl. After the stated intervals, aliquots were applied to Whatman No. 1 paper in 0.5 N acetic acid, subjected to electrophoresis, and counted as described in the text (“Experimental Procedure”).

<table>
<thead>
<tr>
<th>Time</th>
<th>BI</th>
<th>BI-R-P</th>
<th>BI-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
<td>21</td>
<td>12</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>13</td>
<td>82</td>
</tr>
</tbody>
</table>

Cosubstrate—Before attempting enzyme purification, it seemed useful to establish the nature of the active compound or compounds in the pyridine extract. Since the active material could be adsorbed on charcoal and eluted with pyridine, the participation of a nucleotide was suspected. While neither adenosine 2'-phosphate nor adenosine 3'-phosphate was active, several nucleoside 5'-phosphates (AMP, GMP, CMP, and UMP as well as 3',5'-cyclic-AMP) showed slight activity. Any one of these nucleotides (2 μmoles) was about as effective as the pyridine extract from 200 mg of P. shermanii. However, none of the foregoing nucleotides had any activity with the partially purified enzyme. On the other hand, N,MN is an effective co-factor (Table II) and shows activity with the partially purified enzyme. Subsequent experiments proved that N,MN is still more active (19). Since N,MN was available in limited amounts, it could be used in only the most critical experiments, and N,MN was chosen to aid in the purification of the enzyme and the study of some of its properties. These aspects of the investigation are reported elsewhere (19). Here it may be noted that the partially purified enzyme is free of phosphatase activity.

These observations suggest the presence of enzymes catalyzing the following two reactions in the crude P. shermanii extract. The reactions are written in terms of the most probable natural substrates, N,MN and DBI, although most of the supporting data have been obtained with N,MN and BI.

$$\text{DBI} + \beta\text{-N,MN}^+ \rightarrow \alpha\text{-DBI-R-5'-P} + \text{nicotinate} + \text{H}^+ \quad (1)$$

Identification of Product as α-Glycosidic 5'-Nucleotide—To obtain sufficient product for detailed analysis, 0.57 μmole of BI-2-14C (8.7 × 10⁴ c.p.m. per μmole), 0.45 μmole of N,MN, and 40 μmole of Tris buffer, pH 8.6, were incubated with 30 μl of partially purified enzyme (10) in a total volume of 70 μl at 30° for 17 hours. Electrophoretic separation of a small aliquot on

FIG. 2. Absorption spectra of benzimidazole and derivatives. A, BI in 0.1 N HCl; B, β-BI-R in 0.1 N HCl; C, α-BI-R purified from P. shermanii medium and eluted from a Dowex 50W column with HCl (10); D, α-BI-2-14C-R-5'-P prepared enzymatically by reaction between BI-2-14C and N,MN. See text for further details about isolation procedure and analytical data on this sample. The pH of this solution was about 4. Values below 200 μμ are not plotted because of considerable end absorption. Curves A, B, and C were obtained with a Bausch and Lomb recording spectrophotometer (Spectronic 505); Curve D was plotted from readings in a Beckman DU spectrophotometer equipped with photomultiplier attachment. Absorption maxima for the free base were found at 261 to 261.5, 267, and 273.5 μμ, and for the nucleosides and nucleotide at 262, 268.5, and 275 μμ.
paper in 0.5 N acetic acid and scanning in the strip counter revealed 57% conversion of the BI-2-MC to the product. All of the incubation mixture was then applied to a number of acid-washed Whatman No. 1 paper strips in 0.5 N acetic acid and subjected to electrophoresis under the usual conditions. The strips were dried thoroughly in a stream of air at room temperature to remove excess acetic acid, and the region showing the characteristic mobility and fluorescence of the product was cut out. On elution with water by descending chromatography, a concentrated solution of product was obtained.

This material has the absorption spectrum (Fig. 2D) characteristic of a benzimidazole derivative. Table III shows that it contains equimolar amounts of benzimidazole, ribose, and phosphate.

Ribose is strongly bound to benzimidazole (31, 32). The value in Table III is based on periodate consumption and not on a direct estimation of the sugar. The presence of two vicinal hydroxyl groups is assumed in calculating the amount of pentose. The results are consistent with the inference that the molecule contains a ribofuranoside phosphorylated at position 5. Vicinal hydroxyls would, however, also be present in a ribopyranoside phosphorylated at position 2 or 4. The use of snake venom 5'-nucleotidase (Fig. 3A) and chromatography (Fig. 3B) confirmed the conclusion that the product was a 5'-nucleotide.

The configuration of the N–C glycosidic bond in the nucleotide remained to be determined. For this purpose, two specific β-nucleosidases were used. One such enzyme occurs in L. delbrueckii (18); it does not attack α-nucleosides (18, 33). A comparable enzyme is also present in P. shermanii. The former enzyme has a preference for purine nucleosides over pyrimidine nucleosides (18), while the reverse holds for the P. shermanii enzyme (Table IV). Both enzymes attack β-D-BI-R and β-BI-R. They do not attack authentic α-DBI-R prepared from vitamin B12 nor the ribonucleosides isolated from intact P. shermanii or obtained by dephosphorylation from the enzymatically prepared nucleotides (Fig. 4). It follows that the ribosidic bond in the compounds under investigation has the

<table>
<thead>
<tr>
<th>Component and method*</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzimidazole</td>
<td>µmole</td>
</tr>
<tr>
<td>Radioactivity</td>
<td>0.265</td>
</tr>
<tr>
<td>Absorption spectrum†</td>
<td>0.243</td>
</tr>
<tr>
<td>Ribose</td>
<td>0.249</td>
</tr>
<tr>
<td>Periodate consumption</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.253</td>
</tr>
<tr>
<td>Total phosphate</td>
<td></td>
</tr>
</tbody>
</table>

* See “Experimental Procedure”.
† See also Fig. 1.

Fig. 3. Identification of product of enzymatic reaction as 5'-nucleotide. A, identification by electrophoretic method; 8 mmoles of each nucleotide, alone or mixed, 4 mmoles of Tris-HCl buffer, pH 8.6, and 5 µl of a 10% aqueous suspension of C. adamantus venom were incubated for 30 minutes at 37° in a total volume of 15 µl. Small aliquots were subjected to electrophoresis for 1 hour on Whatman No. 1 paper in 0.5 N acetic acid (see “Experimental Procedure”). ● represents the positions of the nucleotides or nucleosides as observed by their fluorescence before (upper rectangle of each pair) and after (lower rectangles) treatment with the venom. B, identification by chromatographic method. The authentic α-DBI-R-3'-P, presumptive α-DBI-R-5'-P, and mixture, as well as authentic 3'-AMP and 5'-AMP and mixture, were subjected to ascending chromatography in 1-butanol-acetic acid (see “Experimental Procedure”).
TABLE IV
Specificity of β-nucleoside hydrolase from P. shermanii

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydrolyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-DBI-R*</td>
<td>30%</td>
</tr>
<tr>
<td>α-DBI-R†</td>
<td>(0)</td>
</tr>
<tr>
<td>β-BI-R*</td>
<td>82%</td>
</tr>
<tr>
<td>α-BI-R†</td>
<td>(0)</td>
</tr>
<tr>
<td>Adenosine</td>
<td>18</td>
</tr>
<tr>
<td>Guanosine</td>
<td>21</td>
</tr>
<tr>
<td>Inosine</td>
<td>26</td>
</tr>
<tr>
<td>Uridine</td>
<td>77</td>
</tr>
<tr>
<td>Cytidine</td>
<td>84</td>
</tr>
</tbody>
</table>

* Authentic β-nucleosides of 5,6-dimethylbenzimidazole and benzimidazole obtained from Dr. G. B. Brown (15).
† α-Nucleosides of 5,6-dimethylbenzimidazole and benzimidazole. The former was isolated from vitamin B_{12} as ribose phosphate and dephosphorylated (see text). The latter was obtained from P. shermanii. Data shown are based on results indicated in Fig. 4.

α-glycosidic configuration. Direct polarimetric measurements were not possible with the small amounts of material available.

Other Products of Reaction—If the electrophoretic strips are examined in ultraviolet light or by means of the cyanogen bromide spray reaction (34), it can be shown that nicotinic acid or nicotinamide is formed from the corresponding nucleotides in the absence of BI or DBI. Since there appears to be some hydrolysis of the pyridine mononucleotides at the glycosidic linkage in the absence of BI or DBI, it cannot as yet be determined whether the transfer and hydrolytic activities are due to one or more than one enzyme. No attempt has been made to demonstrate the liberation of hydrogen ion, necessitated by the formation of a tertiary amine at the expense of a quaternary amine. Reaction 1 is essentially irreversible (19).

DISCUSSION

The benzimidazole nucleotides reported above differ from the more familiar nucleotides (AMP, UMP, NMN, etc.) in that the ribosidic bond has the α rather than β configuration.2 They differ from the 3'-nucleotide obtained from vitamin B_{12} by chemical degradation. There is at present no reason to believe that α-DBI-R-3'-P occurs naturally except as a constituent of the cobalamins (12, 37).

It has been shown above that the 5'-α-nucleotides are readily dephosphorylated by an endogenous phosphatase in P. shermanii. In the case of Nocardia rubra, there is good evidence that the α-glycosidic nucleotide of DBI is incorporated into cobalamin (38) by attachment to bound phosphate (39) and that cobalamin phosphate or guanosine diphosphate cobinamide is a likely intermediate (39, 40). Guanosine diphosphate cobinamide has also been found in P. shermanii (41). Hence, it appears that the trans-N-glycosidase, phosphatase, and the products of the reactions are critical for B_{12} biosynthesis. Perhaps the α-δ-nucleoside displaces the GMP of the guanosine diphosphate cobinamide in a reaction analogous to that found by Kennedy and Weiss (42) for the formation of phosphatides, such as lecithin, from D-α,β-diglycerides and cystide diphosphate choline.

The enzyme is also of interest from the point of view of the mechanism of enzyme action. The N-α-glycosidic bond is
formed by displacement of nicotinic acid from N₂MN by a benzimidazole. In the notation of Koehland (43), this is a single displacement reaction. A brief review of the literature on displacement reactions indicates that a single displacement enzyme of this particular type has not been described before.

O-Glycosidic bonds are attacked by phosphorylases and by hydrodrolases in both single displacement reactions (e.g. maltose phosphorylase (44), β-amyloses (45)), and double displacement reactions (e.g. sucrose phosphorylase (46), α-amyloses (47)).

N-Glycosidic bonds, of more significance to the present work, are formed or attacked by phosphorylolytic and nonphosphorylolytic enzymes. The former reactions (for example, nucleoside phosphorylase (48) and nucleotide pyrophosphorylase (49, 50)) proceed by single displacements. Double displacement N-glycoside phosphorylases or pyrophosphorylases have not been reported.

The nonphosphorylolytic reactions, of which the present reaction is an example, are catalyzed by enzymes classified as trans-N-glycosidases. Those hitherto studied appear to proceed by double displacement reactions (nucleoside deoxyribosyltransferase (51), DPNase (52)). The beef spleen enzyme forms numerous analogues of DPN by a double displacement reaction (52), and BI and DBI react in the system to form dinucleotides which are cleaved by potato pyrophosphatase to the mononucleotides (53). In view of the established mechanism of the reaction, these dinucleotides and derived mononucleotides of BI and DBI are presumably the β anomers, but this point has not been investigated. An enzyme from bull semen which catalyzes the hydrolysis of DPN, TPN, NMN, and nicotinamide nucleoside has been purified (54). This enzyme also catalyzes numerous analogues of DPN by a double displacement reaction which are cleaved by potato pyrophosphatase to the mononucleotides (53).

SUMMARY

1. An α-glycosidic ribonucleotide of 5,6-dimethylbenzimidazole was demonstrated in extracts of Propionibacterium shermanii grown in the absence of added cobalt. This nucleotide is found in addition to the nucleoside described before (10). Since the phosphate is attached to carbon atom 5 of the ribose, this compound differs from the 3′-nucleotide isolated from vitamin B₁₂.

2. An enzyme catalyzing the formation of this 5′-nucleotide by reaction between free 5,6-dimethylbenzimidazole and nicotinic acid mononucleotide or nicotinamide mononucleotide was isolated from P. shermanii. The enzyme may be classified as a single displacement trans-N-glycosidase. A similar reaction takes place with benzimidazole.

3. These findings are of importance for an understanding of B₁₂ biosynthesis and of the scope of displacement reactions involving glycosidic bonds.

REFERENCES

The Formation of $\alpha$-Glycosidic 5'-Nucleotides by a Single Displacement Trans-$N$-glycosidase

Herbert C. Friedmann and Daniel L. Harris