The riboflavin precursor, L-3,4-dihydroxy-2-butanoic acid 4-phosphate, is formed from d-ribulose 5-phosphate by a single 24-kDa enzyme. Studies with various specifically labeled d-ribulose 5-phosphates as substrate showed that the carbon atoms 1–3 of the enzyme product correspond to carbon atoms 1–3 of the substrate, whereas C-4 of the product stems from C-5 of the substrate. Carbon atom 4 of the substrate is released as formate together with the hydrogen atom attached to it. The skeletal rearrangement which leads to the loss of C-4 and the direct linkage between C-3 and C-5 of the substrate is an intramolecular reaction. The hydrogen atom at C-3 of the enzyme product is introduced from solvent water. A reaction mechanism which is in agreement with all experimental data is proposed.

The initial steps in the biosynthesis of riboflavin (5) yield 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (2) from the precursor, GTP (1) (Scheme I; for review see Refs. 1–3). The pyrimidine 2 reacts with a 4-carbon unit from formation of the riboflavin precursor, 6,7-dimethyl-8-ribityllumazine (6) (1–3). The structure and origin of the 4-carbon unit was unknown until quite recently. Various hypotheses involving the involvement of diacetyl or other intermediates of the butanediol pathway (4–6) as well as different carbohydrate-type precursors (7–10) have been proposed (for review see Ref. 1).

Alworth et al. (11) obtained indirect evidence for the incorporation of C-1 of ribose into the lumazine 6 by studies on the biosynthesis of the vitamin B12 component, 6,7-dimethyl-8-ribityllumazine (6) (1–3). The structure and origin of the 4-carbon unit was unknown until quite recently. Various hypotheses involving the involvement of diacetyl or other intermediates of the butanediol pathway (4–6) as well as different carbohydrate-type precursors (7–10) have been proposed (for review see Ref. 1).

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The first in vitro evidence for the formation of the lumazine 6 from a carbohydrate phosphate was obtained by studies performed in the laboratory of Shavlovsky (15, 16) with cell extracts of the yeast, Candida (Pichia) guilliermondii. In line with the in vivo studies, the enzyme-catalyzed reaction involved the loss of C-4 of the pentose/pentulose precursor (17). Subsequent studies with mutants of C. guilliermondii demonstrated the formation of a carbohydrate phosphate, Compound X, from a pentose or pentulose phosphate (18). Compound X serves as the committed precursor for 6,7-dimethyl-8-ribityllumazine (6) (19).

Compound X was recently identified as L-3,4-dihydroxy-2-butanoic acid 4-phosphate (4), and the committed substrate of L-3,4-dihydroxy-2-butanoic acid 4-phosphate synthase was identified as d-ribulose 5-phosphate (3) (20–22). The formation of L-3,4-dihydroxy-2-butanoic acid 4-phosphate from ribulose phosphate is catalyzed by a monomeric 24-kDa enzyme (22). This paper describes studies on the mechanism of this unusual reaction.

EXPERIMENTAL PROCEDURES

Materials—The following materials were purchased from the suppliers indicated: D-[2-14C]glucose from Omicron, Ithaca, NY, D-[6-14C]glucose and D-[U-14C]glucose from ICN, Innerberg, Switzerland. D-[2,3,5-13C3]Ribose 5-phosphate was a gift of H. Floss, Seattle, Washington.

Enzymes—L-3,4-Dihydroxy-2-butanoic acid 4-phosphate synthase was purified to homogeneity from C. guilliermondii as described earlier (22). The activity of the enzyme was measured as described (22). One enzyme unit catalyzes the formation of 1 nmol of L-3,4-dihydroxy-2-butanoic acid 4-phosphate from d-ribulose 5-phosphate per hour at 37 °C. D-Ribulose 5-phosphate, pentose phosphate isomerase, and alkaline phosphatase were purchased from Sigma or Boehringer Mannheim.

13C-Labeled D-Glucose 6-Phosphate—The reaction mixtures contained D-glucose appropriately labeled with 13C (9 mg), 55 mM ATP, 100 mM dithiothreitol, 20 mM MgCl2, 200 mM Tris hydrochloride, pH 7.8, and 25 units of hexokinase in a total volume of 2.5 ml. The mixture was incubated at 37 °C for 1 h. A solution of barium chloride (1 M, 200 μl) was added, and the resulting precipitate was removed by centrifugation. Ethanol was added to a final concentration of 80%, and the mixture was kept at −20 °C overnight. The precipitate was harvested by centrifugation and washed twice with 80% ethanol.

13C-Labeled D-Ribulose 5-Phosphate—The barium salt of D-glucose 6-phosphate was suspended in 3 ml of a solution containing 200 mM Tris hydrochloride, pH 7.8, 13 mM MgCl2, 67 mM NaCl, 1.3 mM NADP, 53 mM a-ketoglutarate, 67 mM dithiothreitol, 4.2 units of glucose-6-phosphate dehydrogenase, and 2 units of phosphogluconate dehydrogenase, and 3.7 units of glutamate dehydrogenase. The mixture was centrifuged, and the supernatant was incubated at 37 °C for 3.5 h. A solution of barium chloride (1 M, 200 μl) was added, and the resulting precipitate was removed by centrifugation. Ethanol was added to a final concentration of 80%, and the mixture was kept at −20 °C overnight. The precipitate was harvested by centrifugation and washed twice with 80% ethanol.

Enzyme Reactions—Reaction mixtures contained 5 mM 13C-labeled d-ribulose 5-phosphate, 100 mM potassium phosphate, pH 7, 10 mM MgCl2, and 250 units of L-3,4-dihydroxy-2-butanoic acid 4-phosphate synthase in a total volume of 0.5 ml. The reaction mixtures were incubated at room temperature for 24 to 48 h. Prior to NMR measurements, D2O was added to a final concentration of 10%. Some experiments were performed in D2O as solvent. For these experiments, the enzyme was dialyzed five times against deuterated 0.1 M potassium phosphate, pH 7, in D2O. pH values are uncorrected glass electrode readings. In experiments using D-ribose 5-phosphate as substrate, the following enzymes were used: glucose-6-phosphate dehydrogenase, malate dehydrogenase, and NADH. The reaction mixtures were incubated at 37 °C for 1 h. A solution of barium chloride (1 M, 200 μl) was added, and the resulting precipitate was removed by centrifugation. Ethanol was added to a final concentration of 80%, and the mixture was kept at −20 °C overnight. The precipitate was harvested by centrifugation and washed twice with 80% ethanol.
substrate, 1 unit of pentose phosphate isomerase was added to the reaction mixture.

NMR Spectroscopy—

* $^{13}$C NMR spectra were measured at 90.6 MHz using an AM 360 NMR spectrometer (Bruker). The spectral parameters were as follows: pulse angle, 30° (2 μs); repetition rate, 2.5 s; 16,000 data points zero-filled to 32,000; composite pulse $^1$H decoupling. Spectral simulation was performed using the program PANIC (Bruker).

Fluoroacetylation of 3,4-Dihydroxybutanone—Reactions mixtures containing L-3,4-dihydroxy-2-butanoic acid-4-phosphate were treated with alkaline phosphatase (6 units/ml) at 37°C for 3.5 h. The
mixtures were lyophilized and the residue was extracted with methylene chloride. The solution was evaporated to dryness. The residue was dissolved in 200 μl of methylene chloride. N-Methylbistrifluoroacetamide (Fluka, 300 μl) was added, and the mixture was incubated at 65 °C for 90 min.

Gas Chromatography and Mass Spectrometry—Experiments were performed with a gas chromatograph 4160 (Carlo Erba) equipped with a DB1 column (ICT, Frankfurt) which was connected to a mass spectrometer MAT 1125 (Varian). Helium was used as carrier. Samples were applied to an injector preheated to 180 °C. The column was heated from 30 to 150 °C at a rate of 6 °C/min. Chemical ionization was performed with isobutane.

RESULTS

The specific origin of each of the carbon atoms of L-3,4-dihydroxy-2-butanone 4-phosphate was analyzed by experiments with specifically 13C-labeled D-ribulose 5-phosphates. The labeled carbohydrate samples were treated with L-3,4-dihydroxy-2-butanone 4-phosphate synthase of C. guilliermondii, and the reaction mixture was analyzed by 13C NMR spectroscopy. The 13C NMR signals of the enzyme product, L-3,4-dihydroxy-2-butanone 4-phosphate, had been assigned earlier and are summarized in Table I.

An experiment with D-[1-13C]ribulose 5-phosphate as substrate yielded L-[1-13C]3,4-dihydroxy-2-butanone 4-phosphate as shown by a strong 13C NMR signal at 28.2 ppm (Scheme II, top). It follows that C-1 of the product is derived from C-1 of the substrate. Similarly, D-[5-13C]ribulose 5-phosphate yielded a product characterized by a 13C NMR signal at 67.3 ppm, thus indicating that C-4 of L-3,4-dihydroxy-2-butanone 4-phosphate is derived from C-5 of the substrate (Scheme II, bottom).

An experiment using D-[2,3,5-13C3]ribulose 5-phosphate (17) (Scheme III) as substrate is shown in Fig. 1. The enzyme substrate was generated in situ from D-[2,3,5-13C3]ribose 5-phosphate by treatment with pentose phosphate isomerase. It should be noted that the sequence of labeled carbon atoms in the multiply labeled substrate is interrupted by the unlabeled C-4. In the product, the signal of C-2 appears as a doublet due to coupling to C-3 (Fig. 1A). C-4 appears as a doublet of doublets due to coupling to C-3 and to phosphorus. C-3 appears as a pseudotriplet of doublets with an intensity distribution of 1:2:2:1:1. The pseudotriplet signature is a consequence of the superposition of two doublets originating by the coupling of C-3 to both C-2 and C-4, since the 13C couplings constants for these respective bonds are virtually identical (about 40 Hz). Thus it follows that C-3 has 13C-labeled neighbors at both C-2 and C-4. The simulated 13C NMR spectrum for [2,3,4-13C3]3,4-dihydroxy-2-butanone 4-
The biosynthesis of riboflavin was studied using 13C NMR spectroscopy. Simulated spectra (Fig. 2) and experimental spectra (Fig. 3) were compared. The experimental spectrum of 3,4-dihydroxy-2-butanone 4-phosphate (Fig. 2A) corresponded to the simulated spectrum, while the enzymatically generated sample showed additional features (Fig. 2B). Treatment of the enzymatic product with alkaline phosphatase gave a spectrum similar to the simulated one (Fig. 1B). The NMR spectra of 3,4-dihydroxy-2-butanone 4-phosphate obtained enzymatically from D-[U-13C5]ribulose 5-phosphate (A) and from an equimolar mixture of D-ribulose 5-phosphate (natural 13C abundance) and D-[U-13C5]ribulose 5-phosphate in 0.1 M phosphate pH 7 (B) were measured. D2O was added to a final concentration of 2% prior to the NMR measurements. The experimental spectrum shows some fine structure which is not accounted for by the simulation. These additional features result from the fact that the enzyme experiment had been carried out in buffer containing 30% D2O. As shown below, this leads to partial deuteration of the enzyme product at C-1 and C-3 with the consequence of long range coupling and isotope shift effects.
Fig. 4. Simulated $^{13}$C NMR spectra. A, [U-$^{13}$C]$^{13}$C-dihydroxy-2-butanone 4-phosphate (4a); B, [1,2,3$^{13}$C]$^{13}$C-dihydroxy-2-butanone 4-phosphate (4b); C, [3,1$^{13}$C]$^{13}$C-dihydroxy-2-butanone 4-phosphate (4c); D, equimolar mixture of the molecular species in 4a–4d. The simulations were performed with the coupling constants shown in Table I.

Scheme IV
Biosynthesis of Riboflavin

C-2 formate C-3 C-4 C-1

A

B

FIG. 5. $^{13}$C NMR spectra of the products ([U-$^{13}$C]3,4-dihydroxy-2-butanone 4-phosphate and formate) generated enzymatically from [U-$^{13}$C]ribulose 5-phosphate in water (A) and in D$_2$O (B).

The data show conclusively that the enzyme product was contiguously labeled at C-2, C-3, and C-4. On the other hand, the formate released enzymatically from D-[2,3,5-$^{13}$C$_3$]ribulose 5-phosphate is not labeled as shown by the absence of a signal at 173 ppm in Fig. 1. It follows that L-3,4-dihydroxy-2-butanone 4-phosphate is formed by elimination of C-4 from D-ribulose 5-phosphate.

The skeletal rearrangement involved in the formation of L-3,4-dihydroxy-2-butanone 4-phosphate could occur as an intramolecular or as an intermolecular process. In order to distinguish between these alternatives, we performed two experiments using as substrates (i) D-[U-$^{13}$C$_5$]ribulose 5-phosphate (Fig. 3A) and (ii) an equimolecular mixture of D-[U-$^{13}$C$_5$]ribulose 5-phosphate and D-ribulose 5-phosphate with natural $^{13}$C abundance (Fig. 3B). The simulated spectrum of L-[U-$^{13}$C$_3$]3,4-dihydroxy-2-butanone 4-phosphate is shown in Fig. 4A and is virtually identical with the experimental spectrum of the enzyme product obtained from pure D-[U-$^{13}$C$_5$]ribulose 5-phosphate shown in Fig. 3A. All $^{13}$C NMR signals appear as complex multiplets. Specifically, C-1 shows coupling to C-2 and C-3. C-2 is coupled to C-1 and C-3. Since the respective coupling constants are virtually identical, a pseudotriplet signature is observed. C-3 is coupled to all other carbon atoms (i.e., C-1, C-2, and C-4) and to $^{31}$P. C-4 is coupled to C-3 and to $^{31}$P.

Starting with a mixture (1:1) of unlabeled D-ribulose 5-phosphate and D-[U-$^{13}$C$_5$]ribulose 5-phosphate, an intermolecular reaction should yield the species 4a–4d shown in Scheme IV in equal amounts. In contrast, the intramolecular reaction yields only the species 4a and 4d. The simulated coupling patterns for each of the molecular species 4a–4c are shown in Fig. 4, A–C. All carbons of the unlabeled species 4d in Scheme III are unlabeled and would contribute no significant $^{13}$C NMR signal intensity. A hypothetical mixture of equal amounts of 4a–4d should therefore yield the simulated spectrum shown in Fig. 4D.

The experimental $^{13}$C NMR spectrum of the enzyme product obtained from the mixture of totally $^{13}$C-labeled and unlabeled substrate (Fig. 3B) is virtually identical with the result obtained with pure D-[U-$^{13}$C$_5$]ribulose 5-phosphate (Fig. 3A). No contributions by the hypothetical species 4b and 4c (Scheme IV, Fig. 4, B and C) can be observed. It follows that only one $^{13}$C-labeled species, i.e., species 4a in Scheme IV, was
formed, and that the enzyme-catalyzed reaction is an intramolecular rearrangement.

$^{13}$C NMR spectra of L-$\text{[U-}$^{13}$C$_2$$]$3,4-dihydroxy-2-butanone 4-phosphate synthesized enzymatically from L-$\text{[U-}$^{13}$C$_2$$]$ribulose 5-phosphate in buffer containing H$_2$O and D$_2$O, respectively, are shown in Fig. 5. Whereas the NMR signatures of C-2 and C-4 appear virtually identical in both experiments, the signals of C-1 and C-3 are absent in the experiment performed in D$_2$O. This indicates that deuterium atoms have been incorporated at C-1 and C-3 of the product. This was confirmed by mass spectrometry analysis of 3,4-dihydroxy-2-butanone 4-phosphate generated enzymatically in D$_2$O as solvent. The enzyme product was dephosphorylated and derivatized with N-methylbistrifluoroacetamide (Scheme V). The resulting 3,4-bistrifluoroacetoxy-2-butanone (7) was analyzed by gas chromatography/mass spectrometry. 7 is characterized by a molecular ion with $m/e = 297$ (M + 1) and a fragment at $m/e = 183$ resulting from the loss of one trifluoroacetyl group (Fig. 6A). In the sample obtained enzymatically in D$_2$O as solvent, the masses of both these fragments are increased by up to 4 units as shown in Fig. 6B. Thus, the sample from the experiment in D$_2$O as solvent had incorporated up to four deuterium atoms/molecule. Since no deuterium incorporation occurs at C-4 as shown by the NMR data, it follows that all three methyl protons and the hydrogen atom at C-3 can be derived from the solvent under the experimental conditions (Scheme VI).

The observed deuterium incorporation could occur by enzyme catalysis or by spontaneous hydrogen exchange. More specifically, incorporation of deuterium could occur (i) at the level of the substrate prior to the reaction, (ii) during the enzymatic reaction, and (iii) at the level of the product after the reaction. In order to explore these possibilities, model experiments were performed by incubating D-ribulose 5-phosphate and L-3,4-dihydroxy-2-butanone 4-phosphate, respectively, in D$_2$O-buffer at pH 7 at room temperature. The samples were monitored at intervals by $^1$H and $^{13}$C NMR spectroscopy. The results of these experiments were as follows. (i) Deuterium is incorporated by spontaneous exchange at C-1 but not at C-3 of D-ribulose 5-phosphate. (ii) Deuterium is incorporated by spontaneous exchange at C-1 but not at other positions of L-3,4-dihydroxy-2-butanone 4-phosphate.

In light of the model experiments, the incorporation of deuterium into the methyl group during enzymatic formation of L-3,4-dihydroxy-2-butanone 4-phosphate could have occurred by spontaneous deuterium exchange of the substrate and/or the product. Thus, it is impossible to decide from the available data whether the enzyme reaction catalyzed by L-3,4-dihydroxy-2-butanone 4-phosphate synthase involves the introduction of deuterium into the methyl group of the product.

On the other hand, it follows that the incorporation of deuterium in position 3 must have specifically occurred during the enzyme-catalyzed reaction, since it could have occurred neither with the substrate nor with the product.

The signal at 173 ppm in Fig. 5 is due to formate produced as the second enzyme product. Apparently, the size of this singlet is the same if the enzyme reaction is performed in H$_2$O and D$_2$O, respectively. It follows that the hydrogen atom attached to the formate carbon is not derived from the solvent and must therefore be derived from the substrate, D-ribulose 5-phosphate.

**DISCUSSION**

The reaction catalyzed by L-3,4-dihydroxy-2-butanone 4-phosphate synthase involves a major reorganization of the substrate molecule. We have shown that formate is specifically formed from C-4 of the substrate, D-ribulose 5-phosphate, whereas the other carbon atoms of the substrate are incorporated into the product, L-3,4-dihydroxy-2-butanone 4-phosphate. This pattern is in line with *in vivo* studies on the biosynthesis of riboflavin in fungi and bacteria (12-14).

The skeletal rearrangement which results in the elimination of C-4 as formate is an intramolecular process. The hydrogen atom attached to the second product, formate, stems from the organic substrate and not from solvent, *i.e.* carbon 4 of the

![Diagram](image)

**Scheme VI**

$\text{CH}_2\text{O}$ $\text{D}_2\text{O} \rightarrow \text{CD}_3$ $\text{C}_3\text{O} + \text{HCOOH}$
substrate is released together with the hydrogen atom bound to it.

The hydrogen atom bound to C-3 of L-3,4-dihydroxy-2-butanone 4-phosphate is derived from the bulk solvent. However, it remains unknown whether the hydrogen atom introduced at C-1 in the course of the enzyme reaction is derived from substrate or solvent.

A mechanism which is consistent with all experimental details is shown in Scheme VII. We propose that the initial step consists in an isomerization reaction by which the carbonyl group is shifted from C-2 to C-3 of the substrate molecule. The elimination of water could then yield a diketone-type compound.

Studies on the decomposition of ribulose 1,5-bisphosphate in neutral or alkaline solution gave indirect evidence for the formation of 8 and 9 by elimination of phosphate (23, 24). Whereas the phosphate present at C-1 of the precursor in the model reaction is a better leaving group than the hydroxyl group at C-1 of the enzyme substrate, the formation of 9 from ribulose 5-phosphate appears mechanistically plausible.

The key event in the sequence of reactions proceeding to the formation of 4 is the proposed formation of the branched carbohydrate 10 from 9 which could occur by anionotropic migration of C-5 of 9 together with the attached phosphate moiety. The reaction could proceed in a hydrophobic cavity where the reaction intermediate is not accessible to the solvent water.

The terminal phase of the reaction would then imply the release of formate which appears mechanistically straightforward. This reaction would imply that the hydrogen atom attached to C-4 of the substrate becomes part of the formate moiety, whereas a proton has to be introduced at C-3 of the newly formed product. Indeed we have found that the proton attached to C-3 of the product stems from the bulk solvent.

The enzyme reaction yields the L-enantiomer of 4 rather than a racemate. This implies that the isomerization of the proposed enolate intermediate (11) occurs at the active site of the enzyme and not in the bulk solvent.

In a formal sense, the enzyme reaction involves a reduction of C-1 of the substrate from a hydroxymethyl to a methyl group. The proposed mechanism does not involve electron transfer reactions but relies on group and proton transfer steps. In line with this hypothesis, no redox cofactor seems to be required for the reaction. It is assumed that the various proton transfer steps are mediated by acidic and basic side chains of the enzyme. We have shown that the hydrogen atom at C-3 of the product stems from the solvent and not from the substrate. It is unknown whether other proton transfer steps implied in the proposed mechanism proceed by intramolecular exchange or by exchange with the solvent.

L-3,4-Dihydroxy-2-butanone 4-phosphate synthase catalyzes a highly complex reaction despite its small size of only about 200 amino acids. A more detailed understanding of this complex reaction will depend on the elucidation of the structure and properties of the enzyme.

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