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Biosynthesis of Riboflavin in Archaea Studies on the Mechanism of 3,4-Dihydroxy-2-butanone-4-phosphate Synthase of *Methanococcus jannaschii**

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The hypothetical protein predicted by the open reading frame MJ0055 of *Methanococcus jannaschii* was expressed in a recombinant *Escherichia coli* strain under the control of a synthetic gene optimized for translation in an eubacterial host. The recombinant protein catalyzes the formation of the riboflavin precursor 3,4-dihydroxy-2-butanone 4-phosphate from ribulose 5-phosphate at a rate of 174 nmol mg^{-1} min^{-1} at 37 °C. The homodimeric 51.6-kDa protein requires divalent metal ions, preferentially magnesium, for activity. The reaction involves an intramolecular skeletal rearrangement as shown by ^{13}C NMR spectroscopy using [U-$^{13}C_5$]ribulose 5-phosphate as substrate. A cluster of charged amino acid residues comprising arginine 25, glutamates 26 and 28, and aspartates 21 and 30 is essential for catalytic activity. Histidine 164 and glutamate 185 were also shown to be essential for catalytic activity.

Flavocoenzymes derived from vitamin B_2 (riboflavin) act as indispensable redox cofactors in all cells (1). They have also been shown to serve a variety of other functions such as DNA photorepair (2), light sensing (3, 4), and bioluminescence (5–9).

The biosynthesis of riboflavin has been studied in considerable detail in eubacteria and fungi (Fig. 1) (for review see Refs. 1 and 10–13). The pyrimidine moiety and the ribityl side chain of the vitamin are derived from GTP (compound **1**), which is converted to 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione 5-phosphate, by a series of three enzyme-catalyzed reactions (14–19). The dephosphorylation of this intermediate by a hitherto unknown enzyme affords 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (compound **2**), which serves as substrate for 6,7-dimethyl-8-ribityllumazine synthase (20–22). The second substrate of that enzyme, 3,4-dihydroxybutanone 4-phosphate (compound **4**), is obtained from ribulose 5-phosphate (compound **3**) by an unusual rearrangement involving the release of carbon atom 4 as formate, which is catalyzed by 3,4-dihydroxy-2-butanone 4-phosphate synthase (22–24). Compounds **2** and **4** are condensed by 6,7-dimethyl-8-ribityllumazine synthase under the formation of the green fluorescent 6,7-dimethyl-8-ribityllumazine (compound **5**), phosphate, and water (22, 25). The lumazine **5** subsequently undergoes a dismutation, affording riboflavin (**6**) and the biosynthetic intermediate **2,** which is recycled in the pathway (26–28). In summary, the formation of one equivalent of riboflavin requires one equivalent of compound **1** and two equivalents of compound **3**.

The biosynthesis of riboflavin in Archaea has not been studied in detail. An *in vivo* study with *Methanobacterium thermoautotrophicum* using [U-$^{13}C_2$]acetate and [1-$^{13}C_1$]pyruvate as tracers indicated that the xylene ring of the vitamin is assembled from two 4-carbon units (29). It was also shown that compound **2** serves as an intermediate in the biosynthetic pathways of riboflavin as well as the 5-deazaflavin (compound **7**) derivative, coenzyme F_{420} (30).

Only one enzyme from the archaeal riboflavin pathway, *i.e.* riboflavin synthase from *M. thermoautotrophicum,* has been reported (31). This paper describes 3,4-dihydroxybutanone-4-phosphate synthase from the Archaeon, *Methanococcus jannaschii*.

EXPERIMENTAL PROCEDURES

Materials—5-Nitroso-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione was synthesized by previously published procedures (32, 33). 5-Amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione was freshly prepared by catalytic hydrogenation of 5-nitroso-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (34). [U-$^{13}C_6$]Glucose was purchased from Isotech (Miamisburg, FL) and was converted to [U-$^{13}C_5$]ribulose 5-phosphate as described earlier (24). Recombinant 6,7-dimethyl-8-ribityllumazine synthase of *Escherichia coli* was prepared as described previously (35). Restriction enzymes were from New England Biolabs (Frankfurt am Main, Germany). T4 DNA ligase was from Invitrogen. Oligonucleotides were synthesized by Interactiva (Ulm, Germany). DNA fragments were purified with QiaQuick Gel Extraction Kit from Qiagen (Hilden, Germany). *Taq* polymerase was from Eurogentec (Seraign, Belgium) and from Finnzyme (Epsoo, Finland). Molecular weight standards were supplied by Sigma.

Strains and Plasmids—Bacterial strains and plasmids used in this study are summarized in Table I.

Restriction Enzyme Digestion of DNA—DNA was digested at 37 °C with restriction enzymes in reaction buffers specified by the supplier. The treated DNA was analyzed by horizontal electrophoresis in 0.8–3% agarose gels.

Estimation of Protein Concentration—Protein concentration was estimated by a dye binding assay (36) or photometrically ($\epsilon_{280\ nm}$ = 10600 $M^{-1}cm^{-1}$).

Construction of Expression Plasmids Used in This Study—Ligation mixtures were transformed into *E. coli* XL1-Blue cells (37). Transfor-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF490541–AF490573 and AF516684.

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mants were selected on LB solid medium supplemented with ampicillin (150 mg/l). The plasmids were reisolated and analyzed by restriction analysis and by DNA sequencing. In all expression plasmids, the genes coding for *ribB* and for mutated *ribB* of *M. jannaschii* are under the control of a T5 promoter and a *lac* operator.

Gene Synthesis and Construction of an Expression Plasmid—A synthetic 109-bp oligonucleotide was prepared by PCR with the overlapping oligonucleotides MJ-MUT-1 and MJ-MUT-2. In a sequence of eight PCR amplifications, the oligonucleotides listed in Table II were used pairwise starting with MJ-MUT-3 and MJ-MUT-4 for the elongation of each prior amplificate. The final PCR product (651 bp) was digested with *Eco*RI and *Bam*HI and was ligated into the plasmid pNCO113 (18, 38), which had been treated with the same restriction enzymes. The resulting plasmid designated pNCO-MJ-MUT-wild type was transformed into *E. coli* XL1-Blue cells.

Site-directed Mutagenesis—Mutations were performed by PCR using synthetic oligonucleotides and pNCO-MJ-MUT-wild type as template. All of the sequences were deposited in the GenBank™ sequence data base (Table III).

DNA Sequencing—Sequencing was performed using the Sanger dideoxy chain termination method (39). Plasmid DNA was isolated from cultures (5 ml) of XL1-Blue strains grown overnight in LB medium containing ampicillin (170 mg/l) using QIAprep spin columns from Qiagen.

SDS-Polyacrylamide Gel Electrophoresis—Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed by previously published procedures (40).

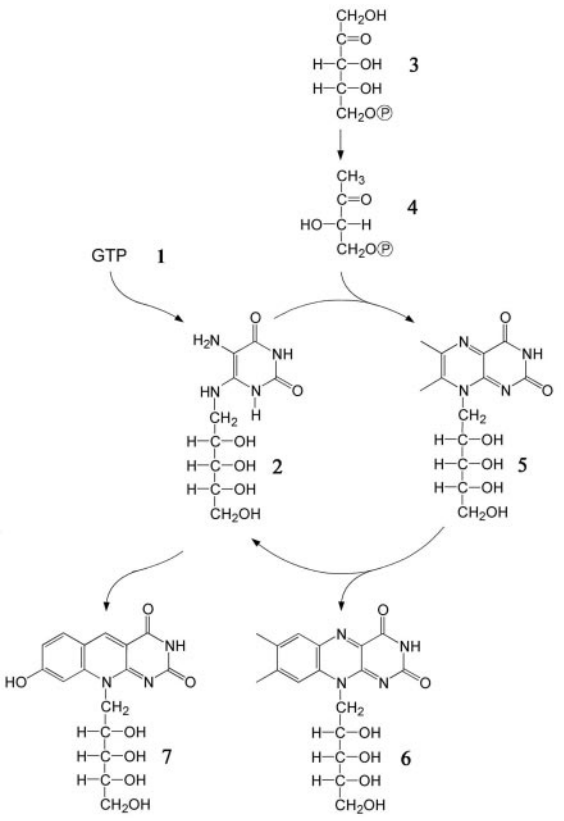

FIG. 1. **Biosynthesis of riboflavin and 8-hydroxy-5-deazariboflavin.**

Polyacrylamide Gel Electrophoresis—Non-denaturating polyacrylamide gels contained 200 mM phosphate, pH 7.2, 4% acrylamide, 0.11% bisacrylamide, and 0.1% tetramethylethylenediamine. Polymerization was started by the addition of 0.05% ammonium peroxodisulfate.

Protein Sequencing—Sequence determination was performed by the automated Edman method using a 471 A Protein Sequencer (PerkinElmer Life Sciences).

Purification of 3,4-Dihydroxy-2-butanone-4-phosphate Synthase—Recombinant *E. coli* strains XL1-*pNCO-MJ-MUT* (wild type or mutated) were grown in LB medium containing ampicillin (170 mg liter^{-1}) at 37 °C. After incubation for 18 h, the cells were harvested by centrifugation (Sorvall GS3 rotor, 5,000 rpm, 15 min, 4 °C). They were washed twice with 0.9% NaCl and frozen at −20 °C.

Frozen cell mass (5 g) was thawed in 40 ml of 30 mM potassium phosphate, pH 7.0. The suspension was cooled on ice and exposed to 60 pulses of a Branson-Sonifier B-12A (Branson Sonic Power Company, Danbury, CT) set to level 5. The suspension was centrifuged (Sorvall SS34 rotor, 15,000 rpm, 15 min, 4 °C). Ammonium sulfate was added to the supernatant to a final concentration of 2 M. After centrifugation (15,000 rpm, 15 min, 4 °C), the protein solution was placed on top of a phenyl-Sepharose SP column (40 ml, Amersham Biosciences), which had been equilibrated with 30 mM potassium phosphate, pH 7.0, containing 1 M ammonium sulfate. The column was developed with 30 mM potassium phosphate, pH 7.0. Fractions were combined, concentrated by ultrafiltration (Ultrafree-15 centrifugal filter device, Millipore, Eschborn, Germany), and placed on top of a hydroxy apatite column (40 ml, Amersham Biosciences), which had been equilibrated with 20 mM potassium phosphate, pH 7.0. The column was developed with a linear gradient of 0–1 M potassium phosphate. Fractions were combined and concentrated by ultrafiltration.

NMR Assay of 3,4-Dihydroxy-2-butanone-4-phosphate Synthase Activity—Assay mixtures contained 100 mM Tris hydrochloride, pH 7.7, 10 mM $MgCl_2$, and 10 mM [U-$^{13}C_5$]ribulose 5-phosphate in 10% D_2O. The reaction was initiated by the addition of protein and was monitored by ^{13}C NMR spectroscopy at 37 °C.

Photometric Assay of 3,4-Dihydroxy-2-butanone-4-phosphate Synthase Activity—Assay mixtures contained 100 mM Tris hydrochloride, pH 7.5, 10 mM $MgCl_2$, 10 mM ribose 5-phosphate, 100 μg of 6,7-dimethyl-8-ribityllumazine synthase of *E. coli*, and 5 units of pentose phosphate isomerase in a total volume of 200 μl. The mixtures were incubated for 5 min at 37 °C to generate ribulose 5-phosphate. A mixture (200 μl) containing 0.75 mM freshly prepared 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione and protein was added. The solution was incubated at 37 °C, and absorbance was monitored at 410 nm. An absorbance coefficient of 12,100 M^{-1} cm^{-1} at 410 nm for 6,7-dimethyl-8-ribityllumazine was used for calculation.

Electrospray Mass Spectroscopy—Experiments were performed as described elsewhere (41).

Analytical Ultracentrifugation—Sedimentation equilibrium experiments were performed with an analytical ultracentrifuge Optima XL-I from Beckman Instruments equipped with absorbance optics. Aluminum double sector cells equipped with quartz windows were used throughout. Protein concentration was monitored photometrically at 280 nm. Protein samples were dialyzed against 50 mM potassium phosphate, pH 7.0. The partial specific volume was estimated from the amino acid composition, affording a value of 0.732 ml g^{-1} (42).

NMR Spectroscopy—^{13}C NMR spectra were recorded at 125.6 MHz using Avance 500 spectrometer from Bruker Instruments (Karlsruhe, Germany).

RESULTS

A hypothetical protein specified by the open reading frame MJ0055 of *M. jannaschii* shows similarity to 3,4-dihydroxy-2-butanone-4-phosphate synthase of *E. coli*. Because open read-

TABLE I
Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source
E. coli strains		
XL1-Blue	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*[*F'*, *proAB*, *lacI^{q}ZΔM15*, *Tn10*(*tet^r*)]	(37)
M15[pREP4]	*lac*, *ara*, *gal*, *mtl*, *recA$^+$*, *uvr$^+$*, *Str^R*, (pREP4:Kan^R, *lacI*)	(38)
Expression plasmids		
pNCO113	*E. coli* expression vector	(18, 38)
pNCO-MJ-MUT-WT	Expression vector for the overexpression of wild type MJ-ribB in *E. coli*	This study

TABLE II
Oligonucleotides used for the construction of the synthetic ribB gene

Designation	Sequence (5′ to 3′)
MJ-MUT-1	gacattccttatgatgaaaaatcatctttctcaattacaattaaccaccgtaagacatttactggtattacag
MJ-MUT-2	cagccaatttttgatagtaaatgcacgatcattatctgtaataccagtaaatgtcttacggtggttaattg
MJ-MUT-3	gcatctcaaaaatttaaagtattgcgtgagctttatccaaatgacattccttatgatgaaaaatcatc
MJ-MUT-4	ccttaccaaagtcattaaaacggccttctttaaccaattcagccaatttttgatagtaaatgc
MJ-MUT-5	atctgcaataaactcggtattccattcatggttgatatcttagaatttgcatctcaaaaatttaaagtattg
MJ-MUT-6	ctgctgcgcgcaagagagttacatggccaggagaacgaaattccttaccaaagtcattaaaacggcc
MJ-MUT-7	aaagacgctggtggcctcatttgcacagctcttcatccggatatctgcaataaactcggtattccattc
MJ-MUT-8	acagtcatttcagtgtgaccttgacgatttttaacaagaccttctgctgcgcgcaagagagttacatg
MJ-MUT-9	gttgcctcccaatttatcactccagagcatattcgtatcatgcgtaaagacgctggtggcctcatttgc
MJ-MUT-10	ttcacaaattgtggtgataggcacgagattggccagctctgccagagctacagtcatttcagtgtgaccttgac
MJ-MUT-11	ttagtttatgactcagatgagcgtgaaggtgaaacggacatggttgttgcctcccaatttatcactccag
MJ-MUT-12	aacgttttgtttcatttttagacatagcattgccatcatcgcccatcatttcacaaattgtggtgataggcac
MJ-MUT-13	aatgtagaaaaagccattgaagcactcaaaaaaggtgaaatcattttagtttatgactcagatgagcgtg
MJ-MUT-14	gatctcttcaccagagagataaatgagattatgtttttcagcataacgttttgtttcatttttagacatag
MJ-MUT-15	ataatagaattcattaaagaggagaaattaactatgaataatgtagaaaaagccattgaagcac
MJ-MUT-16	tattatggatccttaatctttgagatatttatccaaataatagttaatgatctcttcaccagagagataaatgag

TABLE III
Catalytic properties of 3,4-dihydroxy-2-butanone 4-phosphate synthase of M. jannaschii

Protein	V_{max}[a]	V_{max}[b]	K_M[a]	Accession number
	nmol min^{-1} *mg*$^{-1}$	*nmol min*$^{-1}$ *mg*$^{-1}$	*μM*	
wild type	148	174	140	AF490554
D21E	<1	<0.04		AF490552
D21N	<1	<0.04		AF490551
D21S	2	0.50		AF490553
S22A	76	113	437	AF490561
D23S	33		453	AF490550
R25E	3			AF490567
R25K	1.1	0.10		AF490563
E26D	<1	<0.04		AF490557
E26Q	<1	<0.04		AF490572
E26S	<1			AF490570
E28D	<1	<0.04		AF516684
E28Q	6.4	2.30		AF490569
E28S	<1			AF490571
D30E	<1	<0.04		AF490549
D30N	<1			AF490548
D30S	5	0.61		AF490547
C55S	44		191	AF490541
C55G	19		927	AF490542
T112A	3	0.07	1415	AF490562
T115A	38		264	AF490560
D118S	27		253	AF490543
R119S	17		1200	AF490564
T122A	60	70	126	AF490566
H147S	18		41	AF490573
R161S	15		1026	AF490559
H164N	<1	<0.04		AF490556
H164S	<1			AF490555
T165S	54		74	AF490568
T165A	74		1116	AF490565
E166S	39		1054	AF490546
E185D	<1	<0.04		AF490545
E185Q	<1			AF490544
E185S	<1			AF490558

[a] Data from photometric assay.
[b] Data from NMR assay.

ing frame MJ0055 contained numerous codons, which are poorly translated in *E. coli*, we designed a synthetic gene that was optimized for the codon usage of *E. coli*. Approximately 21% (49 of 228) of the codons were replaced, and 10 singular restriction sites spaced at intervals of 10–200 bp were introduced. The DNA sequence was assembled from 16 synthetic oligonucleotides by a sequence of eight PCR steps as described under "Experimental Procedures." The synthetic gene was ligated into the plasmid pNCO113 where it was under the control of a T5 promoter and *lac* operator. The sequence of the synthetic gene was verified by DNA sequencing in both directions and has been deposited in the GenBank™ data base (accession number AF490554). It was transcribed efficiently in a recombinant *E. coli* strain, affording ~30% of cellular protein.

The recombinant protein obtained by heterologous expression of the synthetic gene was purified by the chromatographic procedures described "Experimental Procedures" and was obtained in apparently pure form as judged by sodium dodecyl sulfate polyacrylamide electrophoresis (Fig. 2).

The N-terminal sequence of the recombinant protein was verified by partial Edman degradation affording the sequence MNNVEKAIEALKKGE. A relative mass of 25,799 Da was observed by electrospray mass spectrometry in good agreement with the calculated mass of 25,796 Da.

Sedimentation equilibrium centrifugation of the recombinant *M. jannaschii* protein afforded a molecular mass of 54

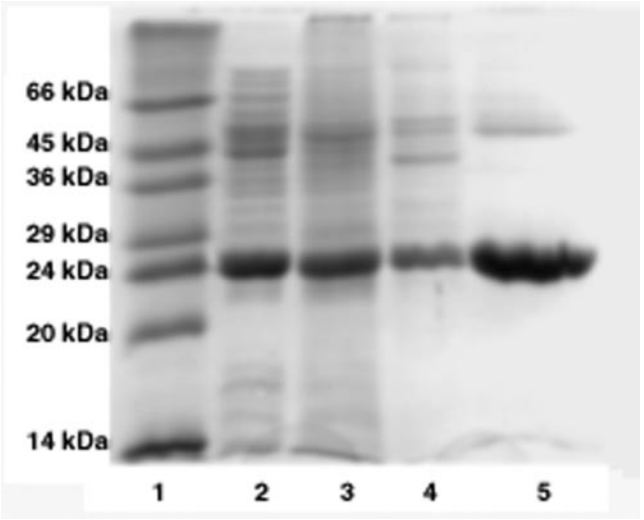

FIG. 2. **Purification of recombinant 3,4-dihydroxy-2-butanone 4-phosphate synthase from *M. jannaschii* as shown by SDS-PAGE.** *Lane 1*, SDS-PAGE size markers; *lane 2*, soluble fraction of cell extract; *lane 3*, protein fraction after precipitation with ammonium sulfate; *lane 4*, protein fraction after phenyl-Sepharose SP chromatography; and *lane 5*, protein fraction after hydroxyapatite chromatography.

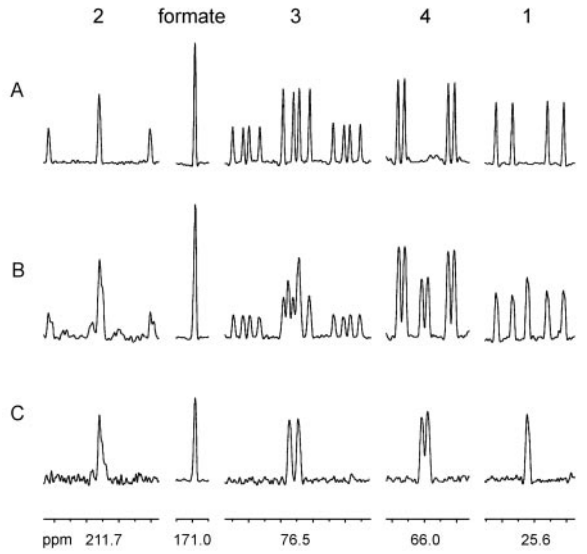

FIG. 3. **^{13}C NMR signals of 3,4-dihydroxy-2-butanone 4-phosphate.** *A*, [U-$^{13}C_4$]3,4-dihydroxy-2-butanone 4-phosphate. *B*, 3,4-dihydroxy-2-butanone 4-phosphate obtained enzymatically from a 1:25 mixture of [U-$^{13}C_5$]ribulose 5-phosphate and unlabeled ribulose 5-phosphate. *C*, unlabeled 3,4-dihydroxy-2-butanone 4-phosphate.

kDa, which indicates a homodimer structure in parallel with the homodimer structure of the *E. coli* enzyme that has been established by analytical ultracentrifugation and by x-ray crystallography (43, 44).

The reaction catalyzed by the recombinant protein was observed by ^{13}C NMR spectrometry in real time using [U-$^{13}C_5$]ribulose 5-phosphate as substrate. The singlet at 170.9 ppm reflects the formate obtained by fragmentation of the substrate. The other four signals are complex multiplets because of $^{13}C^{13}C$ and $^{13}C^{31}P$ coupling. Specifically, the doublet of doublets at 25.7 ppm reflects the methyl group of the enzyme product 3,4-dihydroxy-2-butanone 4-phosphate, which is coupled to the position 2 carbonyl group with a coupling constant of 40.9 Hz and C-3 with a coupling constant of 13.1 Hz. The carbonyl group of **4** resonates at 211.7 ppm and appears as a pseudotriplet because of coupling to C-1 and C-3. The signal of C-3 is a complex multiplet reflecting $^{13}C^{13}C$ coupling to C-2 and C-4 with coupling constants of 41 and 40 Hz, to C-1 with a coupling constant of 13.1 Hz, and to phosphorus with a coupling constant of 7.5 Hz. C-4 of the product also appears as a multiplet because of coupling to C-3 and to phosphorus. The ^{13}C spectrum shown in Fig. 3*A* unequivocally identifies the enzyme product as 3,4-dihydroxy-2-butanone 4-phosphate. The NMR data are summarized in Table IV.

The reaction product obtained from unlabeled ribulose 5-phosphate by the catalytic action of the enzyme is shown in Fig. 3*C* for comparison. The signals of C-3 and C-4 show $^{13}C^{31}P$ coupling. The use of the ^{13}C-labeled substrate results in an ~90-fold sensitivity enhancement for the detection of formate, which was crucial for the detection of residual catalytic activity in certain mutant proteins described below.

The spectrum in Fig. 3*B* was obtained in an enzyme experiment using a mixture of [U-$^{13}C_5$]ribulose 5-phosphate and unlabeled ribulose 5-phosphate proffered at a ratio of 1:25. The spectrum of the product represents a superposition of the spectra in Fig. 3, *A* and *C*. This is specifically demonstrated for C-3 in Fig. 4 by the help of spectral simulation, which accounts rigorously for all observed lines.

The enzyme-catalyzed reaction involves breaking of the bonds connecting C-3 and C-5 to C-4 and the formation of a novel bond between C-3 and C-5 of the substrate. Intermolecular recombination of fragments from labeled and unlabeled substrate would be conducive to partially ^{13}C-labeled products, more specifically, to [1,2,3-$^{13}C_3$]compound **4** and [4-$^{13}C_1$]-compound **4** as shown in Fig. 4, which shows simulated spectra for C-3 of different isotopomers. The ^{13}C spectrum of compound **4** obtained from a mixture of [U-$^{13}C_5$]ribulose 5-phosphate and unlabeled ribulose 5-phosphate is virtually identical with the superposition of spectra for compound **4** obtained from unlabeled ribulose 5-phosphate and [U-$^{13}C_5$]ribulose 5-phosphate. It follows that the reaction proceeds by a strictly intramolecular mechanism.

TABLE IV
^{13}C NMR data of [U-$^{13}C_4$]3,4-dihydroxy-2-butanone 4-phosphate using D_2O as solvent

Position	δ	J_{CC}	J_{CP}
	ppm	*Hz*	*Hz*
1	25.7	40.9 (2) 13.1 (3)	
2	211.7	40.9 (1) 41.0 (3)	
3	76.5	41.0 (2) 40.0 (4) 13.1 (1)	7.5
4	66.0	40.0 (3)	4.9

Because it is known that both enantiomers of 3,4-dihydroxy-2-butanone 4-phosphate can serve as substrates for 6,7-dimethyl-8-ribityllumazine synthase of *Bacillus subtilis* (25), we determined the configuration of the product of *M. jannaschii* 3,4-dihydroxy-2-butanone-4-phosphate synthase. The CD spectrum shown in Fig. 5 is that expected for the L-isomer (23). Hence, the *M. jannaschii* protein converts ribulose 5-phosphate into a mixture of formate and L-3,4-dihydroxy-2-butanone 4-phosphate. It follows that archaea and eubacteria use the same intermediate as precursor for the xylene ring of riboflavin.

Sequence comparison showed that 17 polar amino acids were absolutely conserved in putative orthologs of the 3,4-dihydroxybutanone-4-phosphate synthase from microorganisms and plants. Most notably, a short stretch of charged amino acids extending from position 21 to 30 showed a high degree of overall conservation (Fig. 6).

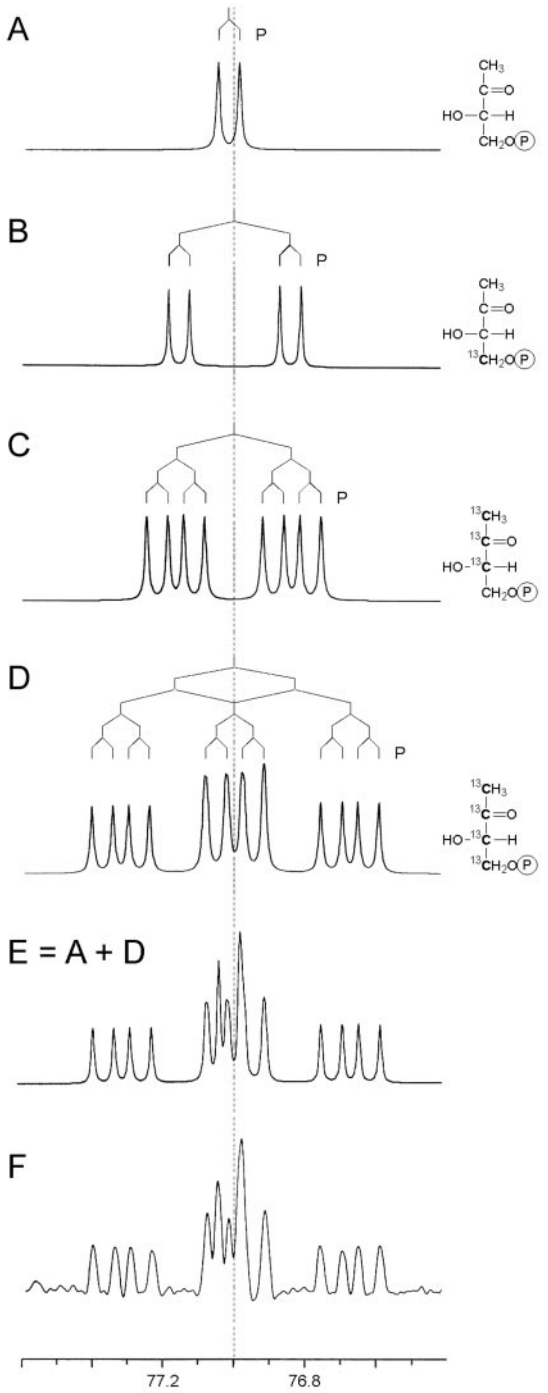

FIG. 4. **Simulated ^{13}C NMR signals for C3 of 3,4-dihydroxy-2-butanone 4-phosphate (compound 4).** *A*, unlabeled compound **4**. *B*, [4-$^{13}C_1$]compound **4**. *C*, [1,2,3-$^{13}C_3$]compound **4**. *D*, [U-$^{13}C_4$]compound **4**. *E*, superposition of spectra *A* and *D*. *F*, experimental ^{13}C NMR signal for C3 of 3,4-dihydroxy-2-butanone 4-phosphate obtained from a 1:25 mixture of [U-$^{13}C_5$]ribulose 5-phospate and unlabeled ribulose 5-phosphate.

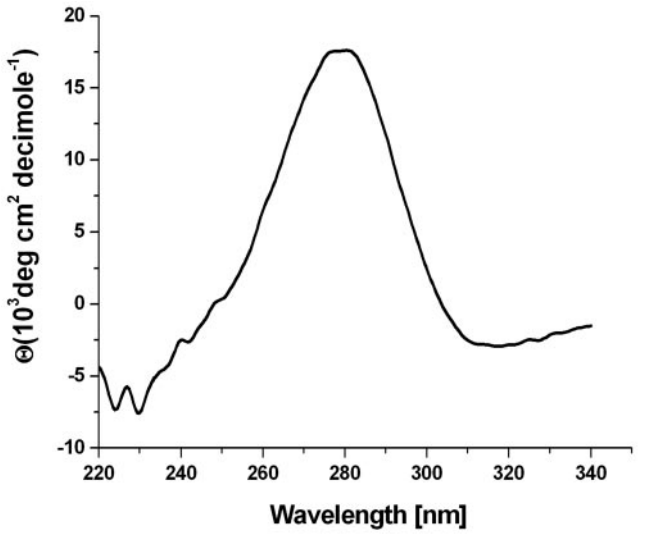

FIG. 5. **CD-Spectrum of 3,4-dihydroxy-2-butanone 4-phosphate enzymatically formed by 3,4-dihydroxy-2-butanone 4-phosphate synthase from *M. jannaschii*.**

Aae	IRQGKMVIVVDDPDRENEGDLVMAAEKVTP
Afu	FRKGSPVLIYDFEDREGETDIAIPAIHVGK
Apl	IRQGKIILVTDDEDRENEGDFICAAEFATP
Ath	IRQGKLVVVVDDENRENEGDLVMAAQLATP
Bsu	LKKGEVIIVVDDEDRENEGDFVALAEHATP
Dra	LRAGRPVILVDDENRENEGDLLMPAATATP
Eco	LREGRGVMVLDDEDRENEGDMIFPAETMTV
Hin	FKNGTGVLVLDDEDRENEGDLIFPAETITP
Hpy	YKNGEMLIVMDDEDRENEGDLVLAGIFSTP
Les	IRQGKMVLVTDDEDRENEGDLVMAASKATP
Mja	LKKGEIILVYDSDEREGETDMVVASQFITP
Mle	IAAGKAVVVIDDEDRENEGDLIFAAEKATL
Mth	LRRGEIVLVFDADNRERETDMIVAAEKIKP
Sce	FKQNKFVIVMDDAGRENEGDLICAAENVST
Spo	FRDGKFLIVLDDETRENEGDLIIAGCKVTT

FIG. 6. **Sequence alignment (positions 11–40) of 3,4-dihydroxy-2-butanone-4-phosphate synthase.** *Aae*, *Aquifex aeolicus*; *Afu*, *Archaeoglobus fulgidus*; *Apl*, *Actinobacillus pleuropneumoniae*; *Ath*, *Arabidopsis thaliana*; *Bsu*, *B. subtilis*; *Dra*, *Deinococcus radiodurans*; *Eco*, *E. coli*; *Hin*, *Haemophilus influenza*; *Hpy*, *Helicobacter pylori*; *Les*, *Lycopersicon esculentum*; *Mja*, *M. jannaschii*; *Mle*, *Mycobacterium leprae*; *Mth*, *M. thermoautotrophicum*; *Sce*, *Saccharomyces cerevisiae*; and *Spo*, *Schizosaccharomyces pombe*. Replacement of the marked amino acids yield in mutant proteins with dramatically decreased activity compared with the wild type enzyme.

The hypothetical reaction mechanism (Fig. 7) suggests a crucial role for acid/base catalysis, and polar ligands are probably involved in the interaction of the protein with the essential divalent metal ion (Mg^{2+} or Mn^{2+}). Preliminary NMR studies on enzyme-substrate complexes had also suggested that the conserved amino acid residues Thr-112, Thr-115, Asp-118, Arg-119, and Thr-122 interact with the substrate (45). The x-ray structure of 3,4-dihydroxy-2-butanone-4-phosphate synthase of *E. coli* in complex with glycerol, which is believed to bind at the active site as a fortuitous substrate analog, is also in line with the hypothesis that the loop comprising the conserved cluster of acidic residues is directly involved in catalysis (46).

The synthetic gene specifying 3,4-dihydroxy-2-butanone 4-phosphate of *M. jannaschii* could be mutagenized conveniently using various experimental techniques. A total of 33 mutant genes could be expressed efficiently in *E. coli*. The recombinant proteins were isolated as described under "Experimental Procedures," and their steady-state kinetic parameters were initially determined by a coupled photometric assay using 6,7-dimethyl-8-ribityllumazine synthase as reporter enzyme (Table III). The replacement of glutamate 26, 28, or 185, aspartate 21 or 30, histidine 164, or arginine 25 afforded proteins with relative activities in most cases of $<0.7\%$. Mutants obtained by the replacement of arginine 119 or 161, threonine 165, or glutamate 166 retained significant enzyme activity, but their K_m values were increased ~10-fold.

The sensitivity of the photometric assay monitoring the formation of 6,7-dimethyl-8-ribityllumazine is limited by the sta-

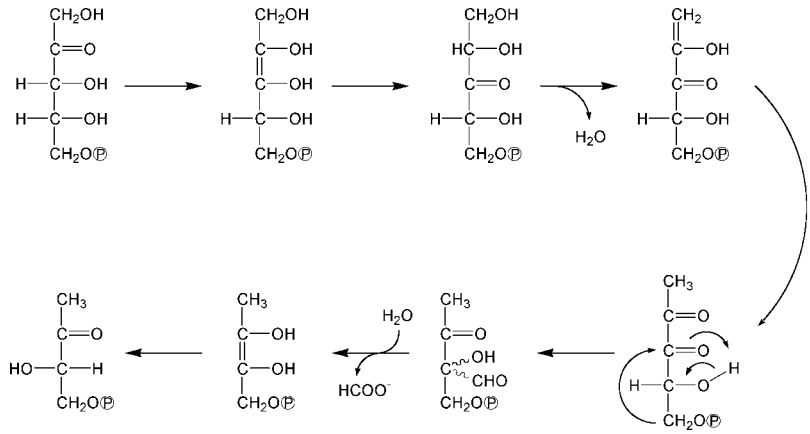

FIG. 7. **Hypothetical reaction mechanism of 3,4-dihydroxy-2-butanone-4-phosphate synthase (22).**

bility of 3,4-dihydroxy-2-butanone 4-phosphate and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione. The relatively large K_m and the relatively low catalytic rate of the auxiliary enzyme, 6,7-dimethyl-8-ribityllumazine synthase, limit the accuracy of the assay still further, particularly in the case of mutants with low residual activity.

Formate, the second product of 3,4-dihydroxy-2-butanone-4-phosphate synthase, has the advantage of virtually unlimited stability. For this reason, we measured the enzyme-catalyzed formation of formate by ^{13}C NMR spectroscopy in real time. To increase the sensitivity of NMR detection, we used [U-$^{13}C_5$]ribulose 5-phosphate as substrate. With the wild type enzyme, this assay recorded an activity of 174 nmol mg^{-1} min^{-1} as compared with 148 nmol mg^{-1} min^{-1} determined with the photometric assay. Energies of activation were 61 and 55 kJ mol^{-1}, respectively, as calculated from the Arrhenius plot (290–330 K) for the NMR and the photometric assay. The two assays also gave similar values with mutant proteins, which display relatively high activities (Table III).

Mutant proteins that had little or no enzyme activity according to the photometric assay were reanalyzed by the NMR assay. To maximize the sensitivity, the assay mixture contained protein in the millimolar range. Operating thus under single turnover conditions, the detection limit was 40 pmol mg^{-1} min^{-1}. That value is equivalent to one molecule of product formed per subunit during a period of 16 h. Even at that level of assay sensitivity, the formation of formate could not be detected with following mutants: D21E, D21N, E26D, E26Q, E28D, D30E, H164N, and E185D.

DISCUSSION

The data reported in this paper show that the formation of the riboflavin precursor, 3,4-dihydroxy-2-butanone 4-phosphate, from ribulose 5-phosphate proceeds similarly in eubacteria, fungi, plants, and archaea. An intramolecular rearrangement precedes the elimination of formate.

The synthetic gene for the expression of the *M. jannaschii* enzyme enabled the production of the protein in high yield in *E. coli*. Because the synthetic gene comprises a closely spaced set of unique restriction sites, it is also ideally suited for mutagenesis by the replacement of short cassettes. This enabled the rapid construction of a large set of mutants addressing all conserved polar amino acid residues, which may be essential for catalysis.

The published assay of 3,4-dihydroxy-2-butanone 4-phosphate activity (47) uses an auxiliary enzyme, 6,7-dimethyl-8-ribityllumazine synthase, to monitor 3,4-dihydroxybutanone 4-phosphate. This assay is suitable for samples with a relatively high catalytic activity but becomes error-prone in the case of mutant proteins with minimum activity.

A novel assay reported in this paper detects the formate formed by 3,4-dihydroxy-2-butanone 4-phosphate by ^{13}C NMR spectroscopy. Formate has the advantage of virtually unlimited stability under the experimental conditions. To enhance the sensitivity of the method, we used [U-$^{13}C_5$]ribulose 5-phosphate as substrate that can be easily obtained from [U-$^{13}C_6$]glucose (24). Using this assay, relative activities of 0.02% as compared with the wild type could be detected.

3,4-Dihydroxy-2-butanone-4-phosphate synthases have a characteristic motif of polar amino acids extending from residue 21 to 30 in case of the *M. jannaschii* enzyme (Fig. 6). Replacement of aspartate 21 or 30 or glutamate 26 or 28 reduces the enzyme activity in most cases to levels of $<0.02\%$ as compared with the wild type enzyme. Outside the patch of charged amino acids, the replacement of threonine 112, histidine 164, or glutamate 185 resulted in the reduction of enzyme activity by at least three orders of magnitude.

The hypothetical reaction sequence in Fig. 7 suggests a large number of proton transfer reactions (around a dozen). Therefore, it is not surprising that charged amino acids could play a major role in such a complex reaction trajectory.

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