Regulation of Enzymes for Erythrose 4-Phosphate Synthesis in *Brevibacterium flavum*

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Partially purified FBPase of *B. flavum* exhibited Michaelis-Menten kinetics, with K_m of 22 μM for F1, 6P₂, but was inhibited by its substrate, F1, 6P₂, at more than 30 μM . The inhibition reached 76% at 1.0 mM. FBPase was also competitively inhibited by AMP, S1, 7P₂, and S7P, the inhibitor constants being 32, 23, and 170 μM , respectively. The presence of S1, 7P₂ in *B. flavum* was suggested by the presence of ATP-dependent S7P kinase and S1, 7P₂ aldolase activities, which corresponded to 27 and 89% of the 6PFK and F1, 6P₂ aldolase activities, respectively. Transaldolase activity involved in S7P formation was also detected in cell-free extracts. Partially purified PGI showed K_m s of 1.4 and 0.54 mM in the forward and reverse directions, respectively and was inhibited by E4P. The inhibition was of the mixed type with respect to G6P, with K_i and K_i' of 32 and 260 μM , respectively. None of the metabolites tested inhibited TK. Whereas the specific activities of PGI and 6PFK were not affected by carbon sources in the culture medium, those of FBPase, 1PFK, and TK were elevated 2.6, 2.6, and 1.7-fold on growth on acetate, fructose, and citrate, respectively.

The regulatory mechanism for the synthesis of E4P, a biosynthetic precursor of aromatic amino acids, is interesting, especially in *Brevibacterium flavum*, since some mutants of this bacterium produce large amounts of various aromatic amino acids.^{1,2)}

E4P is synthesized through the TK reaction from F6P, an intermediate in glycolysis (Fig. 1). In the preliminary experiment, TK activity was not significantly affected by E4P or related metabolites, suggesting the possible regulation of PGI, which catalyzes the formation of F6P in glycolysis. The synthesis of E4P via the pentose phosphate pathway and its regulation were also possible, although this is a minor pathway in the glucose metabolism in this bacterium.³⁾ However, previous studies^{4,5)} showed that G6PDH and 6-phosphogluconate dehydrogenase in this pathway are mainly regulated by NADPH, phosphoribosylpyro-

phosphate, and some of the glycolytic intermediates.

The synthesis of E4P from fructose requires

Fig. 1. Pathway for and Regulation of Erythrose 4-Phosphate Synthesis in *Brevibacterium flavum*.

(1), PGI; (2), TK; (3), FBPase; (4), 6PFK; (5), 1PFK. $\rightleftharpoons$, inhibition.

Abbreviations: FBPase, fructose bisphosphatase; PGI, phosphoglucoisomerase; TK, transketolase; 1 or 6PFK, 1- or 6-phosphofructokinase; G6PDH, glucose-6-phosphate dehydrogenase; F1 or 6P, fructose 1- or 6-phosphate; F1,6P₂, fructose 1,6-bisphosphate; G6P, glucose 6-phosphate; S7P, sedoheptulose 7-phosphate; S1,7P₂, sedoheptulose 1,7-bisphosphate; E4P, erythrose 4-phosphate; Xu5P, xylulose 5-phosphate; R5P, ribose 5-phosphate; PEP, phosphoenolpyruvate.

FBPase, in addition to TK, for the conversion of F1, 6P₂ to F6P, since fructose is converted to F1, 6P₂ via F1P but not via F6P in its central metabolism.⁶⁾ The same enzyme is also required for synthesis from acetate, citrate, and other organic acids.

This paper deals with the regulatory properties of TK, PGI, and FBPase in *B. flavum*, and reports feedback inhibition of PGI by E4P, and of FBPase by S7P and S1, 7P₂, as well as strong inhibition of FBPase by AMP.

Materials and Methods

Organism and culture media. *Brevibacterium flavum* No. 2247 (ATCC 14067) was used.

The compositions of Medium 40⁴⁾ and 41⁷⁾ were given in previous papers.

Chemicals. F1, 6P₂, F6P, G6P, NADP, G6PDH (yeast), PGI (yeast), and a mixture of triosephosphate isomerase and glycerol-3-phosphate dehydrogenase (rabbit muscle) were purchased from Boehringer Mannheim. S1, 7P₂, S7P, AMP, and E4P were from Sigma Chemical Co. Other chemicals used, see Table I, were purchased from Sigma Chemical Co., Boehringer Mannheim, or ICN Pharmaceuticals.

Enzyme preparations. FBPase: cells of strain No. 2247 cultured at 30°C for 24 hr in Medium 40 containing acetic acid as a carbon source were harvested, washed twice with 0.2% KCl, suspended in 0.1 M Tris-HCl buffer, pH 7.5, and then sonically disrupted at 10 kc for 20 min. The supernatant solution of the sonicate obtained on centrifugation at 100,000×g for 60 min was applied on a hydroxyapatite column (1.5×15 cm; Bio-Rad Bio-Gel HT) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, containing 1 mM MnCl₂ and 10 mM MgCl₂. The column was washed with 25 ml of the same buffer. FBPase was eluted with a potassium phosphate concentration gradient, which was formed with 110 ml of the same buffer and that containing 0.2 M potassium phosphate. Fractions of 2 ml were collected. Fraction Nos. 56 to 58 were pooled and passed through a Sephadex G-25 column (1.5×10 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, containing 1 mM MnCl₂ and 10 mM MgCl₂, in order to remove inorganic phosphate.

PGI: The supernatant solution of the sonicate obtained above was applied on a DEAE cellulose column (2.5×21 cm; Whatman DE-52) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5. The column was washed with 100 ml of the same buffer. The enzyme was eluted with a KCl concentration gradient, which was formed with 500 ml of the same buffer and that containing 0.5 M KCl.

Fractions of 8 ml were collected. Fraction Nos. 82 to 84 were pooled.

TK: The supernatant solution of the sonicate prepared from cells of strain No. 2247 grown for 24 hr in Medium 40 containing citric acid as a carbon source was passed through a Sephadex G-25 column (1.5×10 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, and used as the TK preparation.

Enzyme assays. FBPase was assayed by measuring F6P formed, as described previously.⁶⁾ The reaction mixture contained 0.05 M Tris-HCl buffer, pH 8.5, 0.03 mM F1, 6P₂, 15 mM MgCl₂, 0.5 mM NADP⁺, 5 μg of G6PDH, 10 μg of PGI, and the enzyme, in a total volume of 0.5 ml. The reaction was initiated by the addition of the enzyme and the formation of NADPH was followed by measuring the increase in absorbance at 340 nm with a Gilford 2600 Spectrophotometer at room temperature (ca. 23°C). FBPase was assayed by measuring the liberation of inorganic phosphate from F1, 6P₂ using the same reaction mixture except for the omission of NADP⁺ and coupling enzymes when indicated. The reaction was stopped after 10 min incubation at 30°C by the addition of 0.25 ml of 10% trichloroacetic acid and the precipitate was removed by centrifugation. The amount of inorganic phosphate in the supernatant was determined by the method of Chen *et al.*⁸⁾

PGI in the forward direction (from G6P to F6P) was colorimetrically assayed by determining F6P formed in the reaction mixture. The reaction mixture contained 0.05 M Tris-HCl buffer, pH 8.0, 4 mM G6P, and the enzyme, in a total volume of 0.5 ml. The reaction was stopped after 5 min incubation at 30°C by the addition of 2.0 ml of the reagent of Roe.⁹⁾ After 10 min incubation at 80°C, the absorbance at 405 nm was measured. PGI in the reverse direction (from F6P to G6P) was assayed spectrophotometrically by following the reduction of NADP⁺ at 340 nm in the presence of G6PDH. The reaction mixture was the same as that for the forward direction except that 4 mM F6P, 5 mM MgCl₂, 0.5 mM NADP⁺, and 5 μg of G6PDH were added instead of G6P. The reaction was started by the addition of the enzyme and the formation of NADPH was monitored at 340 nm.

TK was assayed by measuring glyceraldehyde 3-phosphate formed. The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, 0.24 mM NADH, 0.01 mM thiamine pyrophosphate, 1.0 mM MgCl₂, 0.5 mM Xu5P, 0.5 mM R5P, 20 μg of the mixture of triosephosphate isomerase and glycerol-3-phosphate dehydrogenase, and the enzyme, in a total volume of 0.5 ml. The reaction was started by the addition of the enzyme and the decrease in NADH was monitored at 340 nm.

Transaldolase was assayed by measuring glyceraldehyde 3-phosphate formed. The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, 5 mM EDTA, 0.5 mM E4P, 4.0 mM F6P, 0.2 mM NADH, 20 μg of the mixture of triosephosphate isomerase and glycerol-3-phosphate dehy-

drogenase, and the enzyme, in a total volume of 0.5 ml. The reaction was carried out as for the TK assay.

1PFK, 6PFK, and the amount of protein were assayed by the methods described previously.⁶⁾

Results

General properties of fructose bisphosphatase, phosphoglucisomerase, and transketolase

FBPase activity was almost completely lost during DEAE-cellulose column chromatography under similar conditions to those for PGI purification given later. This seems due to the action of KCl in the column buffer, which strongly inhibited FBPase activity. However, when FBPase was stored in Tris-HCl buffer, pH 7.5, at a low concentration (1.5 mg protein/ml) at 5°C for 24 hr, it was fairly stable and only 9% of the original activity was lost. It was further stabilized by the presence of 1 mM Mn^{2+} and 10 mM Mg^{2+} . The addition of ammonium sulfate and KCl enhanced the inactivation during storage. Therefore, the enzyme was partially purified, 4.6-fold with a 43% yield, by hydroxyapatite column chromatography in the presence of Mn^{2+} and Mg^{2+} . The resultant preparation did not contain aldolase, PGI, or TK activity at all. On the other hand, PGI activity was quite stable in the presence of KCl. The enzyme was partially purified by DEAE-cellulose column chromatography, in which it was eluted with 0.27 M KCl and purified 4.3-fold, with a 31% yield. This preparation did not contain aldolase, FBPase, or TK activity. The properties of both the enzymes were examined using these partial purified preparations.

FBPase required Mg^{2+} as a cofactor for its activity. The activity became maximum at more than 5 mM Mg^{2+} . PGI did not require any cofactor.

The optimum pHs of the FBPase and PGI reactions were 8.5 with Tris-HCl buffer and 8.0 with either Tris-HCl or TES-NaOH buffer, respectively.

Figure 2 shows double reciprocal plots of the FBPase reaction rate against the substrate concentration. The plots gave a straight line at lower substrate concentrations, indicating that

Fig. 2. Double Reciprocal Plots of the Fructose Bisphosphatase Reaction Rate against the F1, 6P₂ Concentration.

FBPase was assayed under the standard conditions except that Tris-HCl buffer, pH 8.0, and the indicated concentrations of F1, 6P₂ were used.

Fig. 3. Double Reciprocal Plots of the Phosphoglucisomerase Reaction Rate against the Substrate Concentration.

PGI was assayed in the forward (A) and reverse (B) directions under the standard conditions except that Tris-HCl buffer, pH 7.5, and the indicated concentrations of G6P or F6P were used.

the reaction follows Michaelis-Menten kinetics. K_m with respect to FBP was estimated to be 22 μM . The activity was inhibited by the substrate at more than 30 μM , 76% at 1 mM F1, 6P₂, which was estimated by extrapolation of the linear plots at lower concentrations.

Figure 3 shows double reciprocal plots of the PGI reaction rate against the substrate concentration. The plots in both the forward and reverse directions gave straight lines. K_m s with respect to G6P and F6P were estimated to be 1.4 and 0.54 mM, respectively, showing the higher affinity of the enzyme for F6P.

Double reciprocal plots of the TK reaction rate against the substrate concentration gave a

straight line and apparent K_m s with respect to Xu5P and R5P were estimated to be 0.11 and 1.2 mM in the presence of 1.2 mM R5P and

2 mM Xu5P, respectively.

Table I. EFFECTS OF METABOLITES ON FRUCTOSE BISPHOSPHATASE, PHOSPHOGLUCOISOMERASE, AND TRANSKETOLASE ACTIVITIES

FBPase was assayed under the conditions given in Fig. 2, except that the concentration of F1,6P₂ was 0.02 mM. PGI was assayed under the conditions in Fig. 3, except that the concentration of G6P or F6P was 1.4 or 0.54 mM, respectively. TK was assayed under the standard conditions, except that the concentrations of Xu5P and R5P were 0.11 and 1.2 mM, respectively.

Addition (1 mM)	Relative activity (%)		
	FBPase	PGI	TK
None	100	100	100
Glucose 1-phosphate	—	95	—
G6P	88 ^b	—	98
Fructose 1-phosphate	93	88 ^c	101
F6P	44 ^b	—	87
F1, 6P ₂	24 ^a	91 ^c	87
Fructose 2,6-bisphosphate	72	98 ^c	—
Glyceraldehyde 3-phosphate	50	94	—
2-Phosphoglycerate	85	97	—
3-Phosphoglycerate	85	95	92
PEP	89	97	107
Pyruvate	92	99	97
Acetyl-coenzyme A	83	86	101
Citrate	85	95	103
Oxaloacetate	86	96	102
6-Phosphogluconate	70	55	113
R5P	83	100	—
Ribulose 5-phosphate	89	63, 61 ^c	117
Xu5P	90	95, 91 ^c	—
E4P	44	7.4, 5.0 ^c	145
S7P	1.9	75, 94 ^c	81
S1, 7P ₂	1.6	77, 97 ^c	83
Phosphoribosylpyrophosphate	63	98	104
Phenylalanine	109	92	—
ATP	83	102	—
ADP	79	93	—
AMP	0.9	92	—
GTP	28	100	—
GDP	33	100	—
GMP	62	98	—
ITP	81	99	—
UTP	79	100	—
CTP	78	99	—

^a Estimated by dividing the activity at 1 mM F1, 6P₂ by that extrapolated to 1 mM of the plots in Fig. 2.

^b Assayed by measuring the liberation of inorganic phosphate.

^c Assayed in the reverse direction.

Regulation of fructose bisphosphatase, phosphoglucosomerase, and transketolase activities

The effects of various metabolites on the FBPase, PGI, and TK activities were examined using modified assay systems with the same substrate concentrations as their K_m values. As shown in Table I, FBPase was inhibited by S7P and E4P, intermediates in the pentose phosphate pathway, AMP, an indi-

Fig. 4. Effects of the Inhibitor Concentrations on the Fructose Bisphosphatase and Phosphoglucosomerase Activities.

FBPase (A) and PGI in the forward direction (B) were assayed under the same conditions as those in Table I in the presence of inhibitors. —○—, AMP; —▲—, S1, 7P₂; —□—, S7P; —●—, E4P.

Fig. 5. Double Reciprocal Plots of the Fructose Bisphosphatase and Phosphoglucosomerase Reaction Rates against the Substrate Concentrations in the Presence and Absence of Inhibitors.

FBPase (A) and PGI in the forward direction (B) were assayed under the same conditions those in Table I except for the substrate concentrations and the following additions. To the assay mixture, 0.5 mM S7P (A, —●—), 0.05 mM S1, 7P₂ (A, —□—), 0.05 mM AMP (A, —△—), 0.1 mM E4P (B, —▲—), or nothing (—○—) was added.

cator for the energy charge, F6P, the reaction product, and S1, 7P₂. Among them, AMP, S1, 7P₂, and S7P showed strong inhibition, 99, 98, and 98%, respectively. It was also inhibited by glyceraldehyde 3-phosphate, GTP, and GDP. On the other hand, PGI was inhibited 93% by 1 mM E4P. Among other metabolites tested, ribulose 5-phosphate and 6-phosphogluconate showed weak inhibitory effects.

Figure 4 shows the effects of the inhibitor concentrations on the enzyme activities. FBPase was completely inhibited by AMP, S1, 7P₂, and S7P at more than 1 mM, and their concentrations giving 50% inhibition were estimated to be 45, 60, and 370 μ M, respectively. On the contrary, the inhibition of PGI by E4P was not complete, but reached 95% at maximum. The concentration of E4P giving 50% inhibition was 90 μ M. These results suggest the operation of these inhibitions *in vivo*. From double reciprocal plots of the reaction rate against the substrate concentrations in the

presence and absence of the inhibitors (Fig. 5), the inhibition of FBPase by S1, 7P₂, AMP, and S7P was shown to be competitive with respect to F1, 6P₂, the inhibitor constants being 23, 32, and 170 μ M, respectively. The inhibition of PGI by E4P was of the mixed type with respect to G6P, the inhibitor constants, K_i and K_i' , being 32 and 260 μ M, respectively.

S7P seems to be present *in vivo*, since the pentose phosphate pathway has been shown to operate in this bacterium.³⁾ In fact, the activity of TK, which catalyzes the formation of S7P from R5P and Xu5P, and that of transaldolase, which catalyzes the formation of S7P from E4P and F6P, were observed, the specific activities being 48 and 87 nmol/min/mg protein, in cell-free extracts of *B. flavum* No. 2247, respectively.

S1, 7P₂ is not an intermediate of the pentose phosphate pathway. However, as shown in Table II, S7P-dependent ADP formation from ATP was observed in cell-free extracts, suggesting the presence of S7P kinase activity, which forms S1, 7P₂ from S7P and ATP. The activity corresponded to 27% of the F6P-dependent 6PFK activity. The activity of aldolase, which catalyzes the reversible S1, 7P₂ formation from E4P and triose phosphate, was

Table II. SEDOHEPTULOSE 7-PHOSPHATE KINASE AND SEDOHEPTULOSE 1, 7-BISPHOSPHATE ALDOLASE IN *Brevibacterium flavum*

The supernatant solution of the sonicate prepared from cells of strain No. 2247 grown for 24 hr in Medium 41 was passed through a Sephadex G-25 column (1.5 $\times$ 10 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, (buffer A) for assay II and with Buffer A containing 0.1 M ammonium sulfate, 10 mM MgCl₂, and 2 mM EDTA (Buffer B) for assay I, and then used as the enzyme. The reaction mixture for assay I was the same as that for the 6PFK assay, except that the concentrations of S7P or F6P and ATP were 1.0 mM, respectively. The reaction mixture for assay II contained 50 mM Tris-HCl buffer, pH 7.5, 4 mM S1, 7P₂ or F1, 6P₂, 0.2 mM NADH, 20 μ g of a mixture of glycerol-3-phosphate dehydrogenase and triose phosphate isomerase, and the enzyme, in a total volume of 0.5 ml.

Assay	Substrate	Product measured	Specific activity (nmol/min/mg protein)
I	S7P, ATP	ADP	34
	F6P, ATP	ADP	124
II	S1, 7P ₂	Triose phosphates	22
	F1, 6P ₂	Triose phosphates	246

Table III. EFFECTS OF CARBON SOURCES IN THE CULTURE MEDIUM ON THE ACTIVITIES OF FRUCTOSE BISPHOSPHATASE, PHOSPHOGLUCOISOMERASE, TRANSKETOLASE, 6-PHOSPHOFRUCTOKINASE, AND 1-PHOSPHOFRUCTOKINASE

The enzymes were prepared from cells of strain No. 2247 grown for 24 hr in Medium 40 containing glucose, fructose, acetate, or citrate as a carbon source by the method described in Table II. The buffers used for Sephadex column equilibration were Buffer A for the FBPase, PGI, TK, and 1PFK assays, and Buffer B for the 6PFK assay.

Carbon source	Growth ($A_{562\text{ nm}}$)	Specific activity ^a				
		FBPase	PGI	TK	6PFK	1PFK
Glucose	0.405	41	814	42	234	116
Fructose	0.358	34	965	48	205	298
Acetate	0.425	108	799	64	280	113
Citrate	0.360	—	—	72	—	—

^a nmol/min/mg protein.

also detected on measuring the formation of triose phosphate from S1, 7P₂, and corresponded to 8.9% of the aldolase activity for F1, 6P₂ as a substrate. These results suggest the presence of S1, 7P₂ *in vivo* and, furthermore, that S1, 7P₂ is a regulator of FBPase activity. On the other hand, the FBPase preparation used in this study also exhibited inorganic phosphate-liberating activity for S1, 7P₂, which corresponded to 87% of that for F1, 6P₂, suggesting that S1, 7P₂ acts as a substrate analog for FBPase.

As shown in Table I, TK activity was not significantly inhibited by any of the metabolites tested. The activity was slightly enhanced by E4P, suggesting that the enzyme catalyzed the formation of glyceraldehyde 3-phosphate from E4P as a substrate.

Regulation of enzyme synthesis

Table III shows the effects of carbon sources in the medium on the activities of FBPase, PGI, TK, 6PFK, and 1PFK. The specific activities of PGI and 6PFK were scarcely affected by the carbon sources. However, when grown on acetate or fructose as a carbon source, FBPase and 1PFK showed 2.6- and 2.6-fold higher activity than when grown on the other carbon sources, indicating the induction of these enzymes by acetate and fructose, respectively. This also suggests that FBPase and 1PFK play important roles in gluconeogenesis and fructose metabolism, respectively. TK activity was also elevated 1.5 and 1.7-fold on growth on acetate and citrate, respectively.

Discussion

FBPase of *B. flavum* was found to be strongly inhibited by AMP, F1, 6P₂, S7P, and S1, 7P₂ in the present study (Fig. 1). Since the concentrations of the former two inhibitors seem to be increased by operation of glycolysis, FBPase activity is being under depression by these inhibitions when glycolysis is operating. As 6PFK is not susceptible to the regulation reported in other microorganisms

and has a high Michaelis constant for F6P,³⁾ it can be concluded that a futile cycle between F6P and F1, 6P₂, which is formed by 6PFK and FBPase, is regulated by the inhibition of FBPase during glycolysis and the high *K_m* of 6PFK during gluconeogenesis. Inhibition by AMP and F1, 6P₂ has been reported in various microorganisms.¹⁰⁾ In particular, the *Bacillus* enzyme^{11,12)} is highly sensitive to AMP inhibition (*K_i*, 2 μ M). Therefore, since it was completely inhibited by AMP under various physiological conditions, the inhibition by AMP does not seem to be actually effective in the regulation of FBPase activity. The overcoming effect of PEP as to the AMP inhibition has been reported to play rather an important role in the regulation mechanism. In *B. flavum*, PEP neither activates FBPase nor overcomes the AMP inhibition. However, the *B. flavum* enzyme was less sensitive to the inhibition than the *Bacillus* enzyme. This suggests that the inhibition by AMP acts as a regulation mechanism in itself.

The inhibition of FBPase by S7P and S1, 7P₂ seems to be a means of feedback control for the biosynthesis of E4P and other intermediates in the pentose phosphate pathway in the metabolism of fructose, acetate, and other organic acids. Whereas inhibition by S1, 7P₂ has been reported in a photosynthetic bacterium, *Rhodospseudomonas palustris*,¹³⁾ the effect of S7P has not been examined in any other microorganisms.¹⁰⁾

On the other hand, the inhibition of PGI by E4P would be regarded as a means of feedback control of E4P biosynthesis in glucose metabolism. This inhibition does not seem to disturb the central metabolism of glucose, if F6P, the product of the PGI reaction, is in equilibrium with E4P *via* the TK reaction. PGI of yeast¹⁴⁾ has been reported to be inhibited by E4P. Moreover, 6-phosphogluconate, ribulose 5-phosphate, and R5P are also potent inhibitors in this microorganism. Their inhibitions were competitive with respect to the substrate, unlike that of the *B. flavum* enzyme, and the yeast enzyme was highly sensitive to the E4P inhibition (*K_i*, 2 μ M).¹⁵⁾

The production of L-phenylalanine by a *B. flavum* mutant was seriously reduced when it was cultured on fructose as a carbon source, compared with on glucose, whereas that of L-glutamic acid was not.^{16,17)} This seems due to the different regulation of the synthesis of E4P, one of the precursors of L-phenylalanine biosynthesis in fructose metabolism. Contrary to this, L-glutamic acid biosynthesis does not require E4P as a precursor. Thus, the reduction in L-phenylalanine production on cultivation on fructose can be attributed to the following. When fructose as well as glucose is actively catabolized, the intracellular concentrations of AMP and F1, 6P₂ seem to be elevated. As they are strong inhibitors of FBPase, it is very likely that the concentration of F6P stays at a low level and the metabolic flow from F6P towards E4P is reduced.

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