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Key Role for Sulfur in Peptide Metabolism and in Regulation of Three Hydrogenases in the Hyperthermophilic Archaeon *Pyrococcus furiosus*

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The hyperthermophilic archaeon *Pyrococcus furiosus* grows optimally at 100°C by the fermentation of peptides and carbohydrates. Growth of the organism was examined in media containing either maltose, peptides (hydrolyzed casein), or both as the carbon source(s), each with and without elemental sulfur (S^0). Growth rates were highest on media containing peptides and S^0, with or without maltose. Growth did not occur on the peptide medium without S^0. S^0 had no effect on growth rates in the maltose medium in the absence of peptides. Phenylacetate production rates (from phenylalanine fermentation) from cells grown in the peptide medium containing S^0 with or without maltose were the same, suggesting that S^0 is required for peptide utilization. The activities of 14 of 21 enzymes involved in or related to the fermentation pathways of *P. furiosus* were shown to be regulated under the five different growth conditions studied. The presence of S^0 in the growth media resulted in decreases in specific activities of two cytoplasmic hydrogenases (I and II) and of a membrane-bound hydrogenase, each by an order of magnitude. The primary S^0-reducing enzyme in this organism and the mechanism of the S^0 dependence of peptide metabolism are not known. This study provides the first evidence for a highly regulated fermentation-based metabolism in *P. furiosus* and a significant regulatory role for elemental sulfur or its metabolites.

Hyperthermophiles are microorganisms that grow optimally at 80°C and above (46, 47). Virtually all of them are strict anaerobes, and most are heterotrophs. All of the heterotrophs utilize peptides as a carbon source, and most use elemental sulfur (S^0) as a terminal electron acceptor leading to H_2S production. The most studied of the S^0-reducing, heterotrophic hyperthermophiles are species of *Pyrococcus*. Most of these organisms only utilize peptide-related substrates as a carbon source and show no significant growth in the absence of S^0 (9, 12, 19, 36). Notable exceptions are *Pyrococcus furiosus*, *P. woesei*, and *P. glycovorans*, which are capable of metabolizing poly- and oligosaccharides, as well as peptides (2, 4, 10). *P. furiosus* and *P. woesei* can also grow to high cell densities in the absence of S^0.

The pathways of peptide and carbohydrate metabolism have been well studied in *P. furiosus* (1, 7). Glycolysis appears to occur via a modified Embden-Meyerhof pathway (Fig. 1) (22, 35). This pathway is unusual in that the hexose kinase and phosphofructokinase steps are dependent on ADP rather than ATP, and a novel tungsten-containing enzyme termed glyceraldehyde-3-phosphate:ferredoxin oxidoreductase (GAPOR) replaces the expected glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase. Amino acid catabolism in *P. furiosus* is thought to involve four distinct 2-keto acid oxidoreductases that convert transaminated amino acids into their corresponding coenzyme A (CoA) derivatives (Fig. 2) (3, 15, 31, 32). These CoA derivatives, together with acetyl-CoA produced from glycolysis via pyruvate, are then transformed to their corresponding organic acids by two acetyl-CoA synthetases, unique to archaea, with concomitant substrate-level phosphorylation to form ATP (33). Alternatively, it has been postulated (26) that, depending on the redox balance of the cell, 2-keto acids are decarboxylated to aldehydes and then oxidized to form carboxylic acids by a second tungsten-containing enzyme, aldehyde:ferredoxin oxidoreductase (AOR) (34). A third enzyme of this type, termed formaldehyde:ferredoxin oxidoreductase (FOR), is thought to be involved in the catabolism of basic amino acids (42).

During fermentative growth of *P. furiosus* on oligosaccharides such as maltose, the primary end products are H_2, CO_2, and acetate. When S^0 is present in the medium, it is reduced to H_2S, with a corresponding decrease in the amount of H_2 produced (10). However, the precise mechanisms by which H_2 is evolved and S^0 is reduced are not known, as this organism contains two cytoplasmic, NAD(P)H-dependent hydrogenases, both of which can reduce S^0 in vitro (6, 28, 29). In addition, *P. furiosus* contains an H_2-evolving, membrane-bound hydrogenase complex, the function of which is not clear, although it does not reduce S^0 to H_2S in vitro (44). To further complicate

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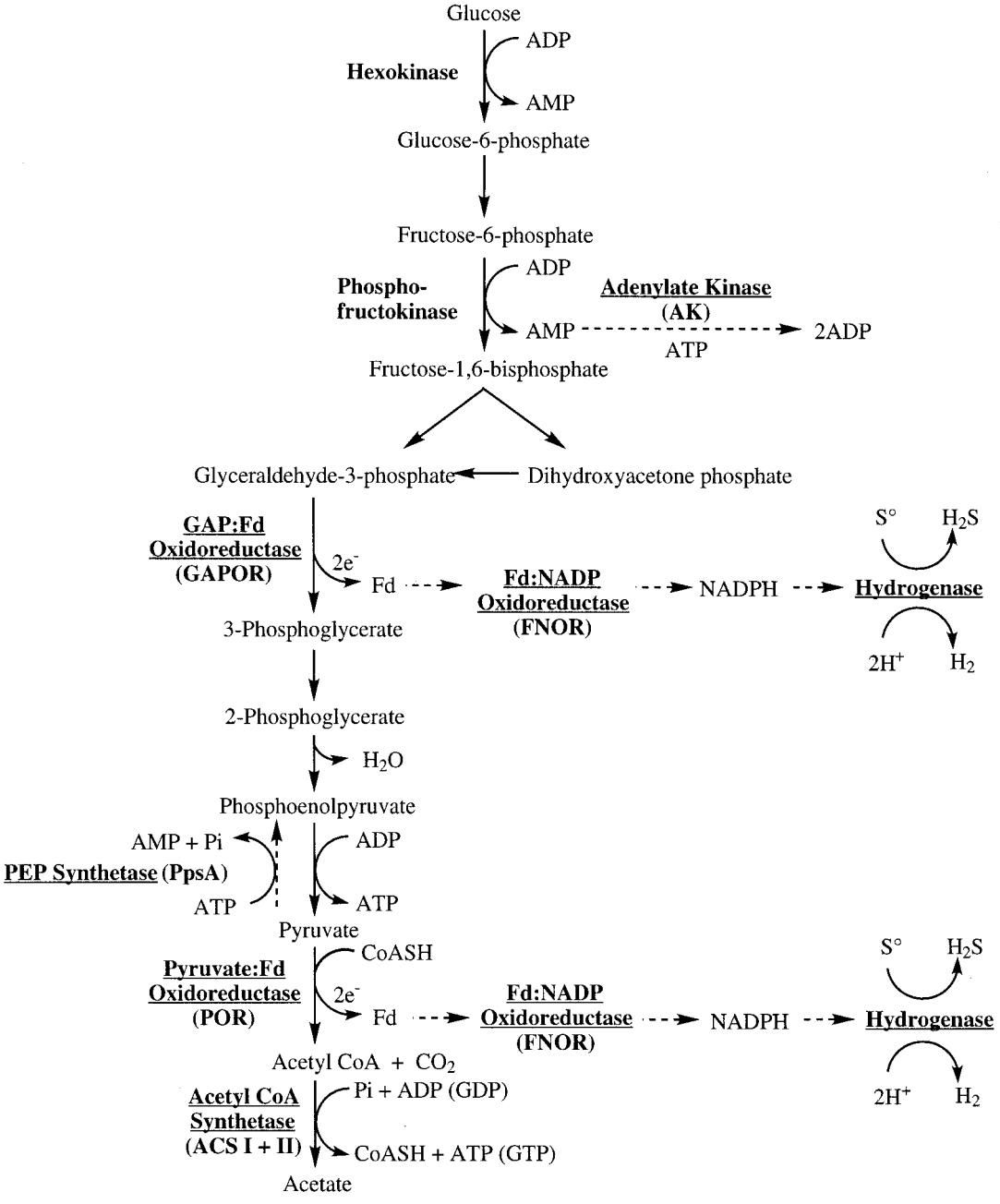

FIG. 1. Proposed glycolytic pathway in *P. furiosus*. The enzymes whose activities were measured in this study are underlined. Fd represents the electron carrier ferredoxin. Modified from reference 35.

matters, the cell yield of *P. furiosus* (dry weight per mole of maltose utilized) increases almost twofold if S^0 is added to a maltose-containing medium (45). It is not known if this organism contains a membrane-bound, respiratory sulfur reductase of the type found in mesophilic S^0-reducing organisms (14).

While the fermentative pathways of *P. furiosus* are reasonably well established, the extent to which they are regulated by the carbon source and by S^0 has not been investigated. Similarly, it is not clear why this organism differs from most other S^0-reducing, heterotrophic hyperthermophiles in being able to grow well in the absence of S^0. Here we report the growth properties of *P. furiosus* grown on various combinations of carbohydrate (maltose), peptides (casein hydrolysate), and S^0. In addition, under each growth condition, the extent of peptide fermentation was assessed by phenylacetate production and the activities of 21 enzymes involved in the fermentative pathways were measured. The results establish a link between S^0 reduction, peptide metabolism, and the activities of several key enzymes, notably the hydrogenases, and readily explain some of the unusual properties of this species of *Pyrococcus*.

MATERIALS AND METHODS

Growth conditions. *P. furiosus* (DSM 3638) was grown in a 20-liter fermentor containing 15 liters of medium, which was prepared as described previously (49).

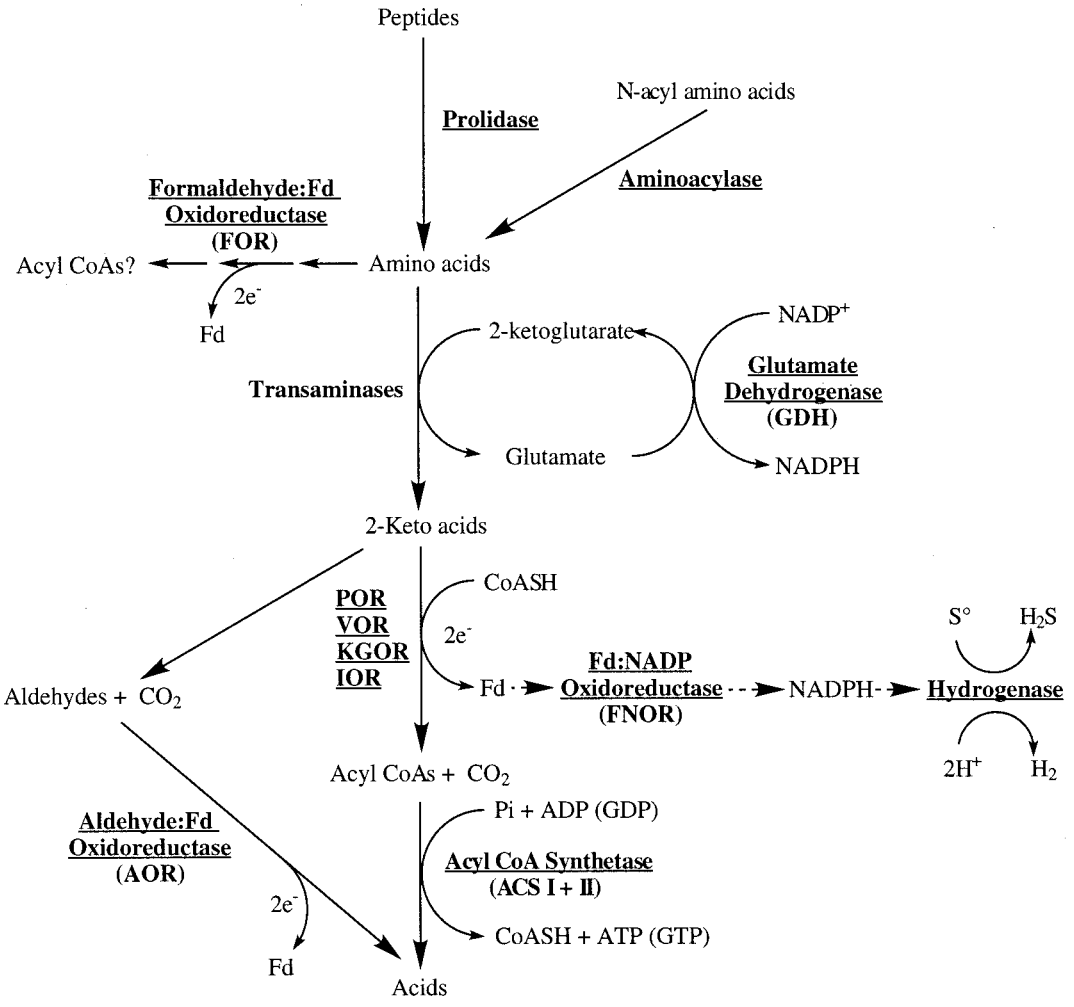

FIG. 2. Proposed peptidolytic pathway in *P. furiosus*. The enzymes whose activities were measured in this study are underlined. FOR is thought to be involved in the metabolism of basic amino acids, although the pathway involved is not known (42). Modified from references 15 and 34.

Medium components were prepared as separate sterile stock solutions and stored at 4°C. Stock solutions were as follows: 5× salts solution, containing, per liter, 140 g of NaCl, 17.5 g of $MgSO_4 \cdot 7H_2O$, 13.5 g of $MgCl_2 \cdot 6H_2O$, 1.65 g of KCl, 1.25 g of NH_4Cl, and 0.70 g of $CaCl_2 \cdot 2H_2O$; 100 mM $Na_2WO_4 \cdot 2H_2O$ (10,000×, containing 33.0 g of $Na_2WO_4 \cdot 2H_2O$ per liter); 1,000× trace minerals solution, containing, per liter, 1 ml of HCl (concentrated), 0.5 g of Na_4EDTA, 2.0 g of $FeCl_3$, 0.05 g of H_3BO_3, 0.05 g of $ZnCl_2$, 0.03 g of $CuCl_2 \cdot 2H_2O$, 0.05 g of $MnCl_2 \cdot 4H_2O$, 0.05 g of $(NH_4)_2MoO_4$, 0.05 g of $AlK(SO_4) \cdot 2H_2O$, 0.05 g of $CoCl_2 \cdot 6H_2O$, and 0.05 g of $NiCl_2 \cdot 6H_2O$; potassium phosphate buffer, pH 6.8 (1,000×), containing 450 ml of 1 M KH_2PO_4 (pH 4.3), to which 1 M K_2HPO_4 was added until the solution reached pH 6.8 (approximately 550 ml); 10% (wt/vol) yeast extract, consisting of 100 g of filter-sterilized yeast extract (Difco) per liter; 10% (wt/vol) casein hydrolysate, consisting of 100 g of filter-sterilized casein hydrolysate (enzymatic; U.S. Biochemicals) per liter; 50% (wt/vol) maltose, consisting of 500 g of filter-sterilized maltose (Sigma) per liter; and resazurin at 5 mg per ml.

Each medium was composed of 1× base salts solution containing, per liter, 800 ml of distilled water, 200 ml of 5× salts, 0.1 ml of 100 mM $Na_2WO_4 \cdot 2H_2O$, 1 ml of 1,000× trace minerals, 0.05 ml of resazurin, and 5 ml of 10% yeast extract (except in the maltose-plus-peptides media; see below). One of three carbon sources (either maltose or peptides, or a combination) was added to the 1× base salts solution. The carbohydrate-based medium contained 0.5% (wt/vol) maltose, and 0.1% (wt/vol) elemental sulfur was added to give the maltose-plus-S^0 medium. The peptides-plus-S^0 medium contained 0.5% (wt/vol) casein hydrolysate (enzymatic) plus 0.1% (wt/vol) sulfur (cultures grew very poorly on casein hydrolysate without sulfur; see below). Cultures grew poorly on acid-hydrolyzed casein and did not grow on Casamino Acids as a peptide source (with or without sulfur). The peptides-plus-maltose medium contained 0.5% (wt/vol) maltose and 0.5% (wt/vol) casein hydrolysate, together with 0.5% (wt/vol) yeast extract. This medium matches that typically used by our laboratory to grow *P. furiosus* in large-scale culture (6). Elemental sulfur (0.1%, wt/vol) was added to give the peptides-plus-maltose-plus-sulfur medium.

The headspace of the fermentor was flushed with N_2–CO_2 (80:20), and 7.5 g each of L-cysteine-HCl $\cdot H_2O$ and $Na_2S \cdot 9H_2O$ were added in that order as reducing agents to remove residual O_2. The pH (measured at room temperature) was adjusted to 6.8 with 1 N NaOH, and 15 ml of 1 M potassium phosphate (pH 6.8) was slowly added. The medium was stirred and heated to 95°C. The pH of the medium at 95°C was 5.9 and was maintained (±0.1 pH unit) by the automatic addition of 5% (wt/vol) $NaHCO_3$. *P. furiosus* was grown under each of the five growth conditions in triplicate.

An exponential-phase culture of *P. furiosus* that had undergone four successive transfers on the experimental medium was used to inoculate the 20-liter fermentor. During growth, 15-ml samples were removed at 1-h intervals from the fermentor and used to measure cell counts, medium pH (at room temperature), and phenylacetate concentration. Cells were counted using a Petroff-Hausser counting chamber and phase-contrast light microscopy. The growth rate was calculated by measuring the slope of a best-fit line through the exponential portion of the growth curve. Cells were harvested in the late-logarithmic phase of growth (1×10^8 to 2×10^8 cells $\cdot$ ml^{-1}). The culture was first cooled to room temperature by pumping 12 liters (at 1 liter $\cdot$ min^{-1}) from the fermentor through a glass cooling coil bathed in an ice-water slurry, and into a stoppered, 20-liter glass carboy flushed with AR. The cooled cells were harvested by centrifugation

at 10,000 × *g* for 15 min (Beckman J2-21 centrifuge, JA-10 rotor) at 4°C, resuspended in 15 to 20 ml of anoxic 50 mM Tris-HCl buffer (pH 8.0) containing 2 mM sodium dithionite (DT) and 2 mM dithiothreitol (DTT) (buffer A) in an anaerobic chamber (VAC Atmospheres), and frozen under Ar at −80°C.

Phenylacetate measurements. Aliquots (1.5 ml) of media from each 1-h sample were spun at 16,000 × *g* for 10 min in a microcentrifuge (Eppendorf). The supernatant was decanted and preserved with 0.1 M H_2SO_4 (final concentration). Phenylacetate concentrations were determined using a Waters 2690 high-performance liquid chromatography (HPLC) separation module equipped with a photodiode array detector. Organic acids were separated on an Aminex HPX-87H column (Bio-Rad) at 60°C using 5 mM H_2SO_4 and acetonitrile (manufacturer's stock solution) as the eluent in the following gradient: 5% acetonitrile, 0 to 5 min; 5 to 25% acetonitrile, 5 to 30 min; 25% acetonitrile, 30 to 35 min. Acetate could not be measured accurately in casein hydrolysate-containing media due to a low signal-to-noise ratio. The specific phenylacetate production rate was calculated by plotting the product of phenylacetate concentration (in nanomoles per milliliter) times growth rate (per hour) divided by 0.693 against cell concentration (cells per milliliter) for each time point sample. The slope of the best-fit line through the points yielded the specific production rate. The production rates were normalized by growth rate to compare the rates from the various growth conditions.

Protein fractionation. All sample transfers and manipulations were carried out in an anaerobic chamber and all buffers were degassed and flushed with Ar and contained 2 mM DT and 2 mM DTT. The cell suspension was thawed, and DNase I in buffer A was added to a final concentration of 0.0002% (wt/vol). The cell suspension was incubated at room temperature with shaking for 30 min. The cells were then disrupted anaerobically by sonication for 30 min (Branson Sonifier 450) by placing the sample vial in an ice-water slurry with the sonicator probe. Cell lysis was verified using phase-contrast microscopy. Debris and unbroken cells were removed by centrifugation (10,000 × *g* for 15 min in a Beckman L8-M ultracentrifuge with a 60 Ti rotor), and a portion of the supernatant was used as the whole-cell extract (WCE). The remainder was centrifuged at 100,000 × *g* for 45 min, and the supernatant was used as the cytoplasmic protein fraction. The membrane pellet was resuspended in buffer A, homogenized using a glass tissue grinder, and then centrifuged at 100,000 × *g* for 45 min. This procedure was repeated three times, and buffer A in the final step contained 4 M KCl. The supernatant from the 4 M KCl wash formed the membrane-associated protein fraction, while the washed membrane pellet was resuspended and homogenized in buffer A, and this formed the membrane-bound protein fraction. Protein fractions that were not used immediately for enzyme assays were frozen in liquid N_2 and stored at −80°C.

Enzyme assays. Activities are expressed in units where 1 U is equivalent to 1 μmol of substrate transformed min^{-1} at 80°C, unless otherwise stated. Protein concentrations were estimated using the Bradford method (5) with bovine serum albumin as a standard.

The following spectrophotometric assays were carried out anaerobically in rubber stopper-sealed glass cuvettes that had been degassed and flushed with Ar. The buffer used was 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (EPPS) buffer (pH 8.4) unless otherwise stated. Aldehyde:ferredoxin (Fd) oxidoreductase (AOR), formaldehyde:Fd oxidoreductase (FOR), and glyceraldehyde-3-phosphate:Fd oxidoreductase (GAPOR) activities were determined by measuring the reduction of 3 mM benzyl viologen (BV) at 600 nm [ε = 7,400 $(M \cdot cm)^{-1}$] using 0.3 mM crotonaldehyde, 50 mM formaldehyde, or 0.4 mM glyceraldehyde-3-phosphate, respectively, as the substrate (34, 35, 42). Formate dehydrogenase (FDH) activity was determined by measuring the reduction of 5 mM BV at 600 nm in 100 mM EPPS (pH 8.4) using 10 mM sodium formate as the substrate (27). The activities of pyruvate:Fd oxidoreductase (POR), 2-ketoglutarate:Fd oxidoreductase (KGOR), indolepyruvate:Fd oxidoreductase (IOR), and 2-ketoisovalerate:Fd oxidoreductase (VOR) were determined by measuring the reduction of 1 mM methyl viologen (MV) at 578 nm [ε = 9,700 $(M \cdot cm)^{-1}$] using 5 mM pyruvate, 2-ketoglutarate, indolepyruvate, or 2-ketoisovalerate, respectively, as the substrate (3, 15, 31, 32). The assay mixtures also contained 2.5 mM $MgCl_2$, 0.4 mM thiamine pyrophosphate (TPP), and 0.1 mM CoA. NADPH:rubredoxin oxidoreductase (NROR) activity was determined by measuring the reduction of 20 μM rubredoxin at 494 nm [ε = 9,220 $(M \cdot cm)^{-1}$] at 25°C in 100 mM EPPS (pH 8.0) using 0.3 mM NADPH as the substrate (25). The combined activities of ferredoxin: NADPH oxidoreductase (FNOR) and NROR were determined by measuring the reduction of 3 mM BV at 600 nm using 0.4 mM NADPH as the substrate (24). The activities of acetyl-CoA synthetases I and II (ACS I and ACS II) in the direction of acetate formation were measured by coupling the reactions to *P. furiosus* POR and IOR, respectively (33). ACS activity was determined by measuring the reduction of 5 mM MV at 600 nm with 5 mM $MgCl_2$, 0.4 mM TPP, 0.025 mM CoA, 1 mM ADP, and 10 mM K_2HPO_4 using 5 mM pyruvate and 40 μg of POR to generate acetyl-CoA or 5 mM indolepyruvate and 40 μg of IOR to generate indoleacetyl-CoA. Hydrogenase activity was determined by following the H_2 evolution rate using 3 mM MV reduced with 30 mM DT as the electron donor (6, 44).

The following enzyme activities were measured under aerobic conditions. Glutamate dehydrogenase (GDH) activity was determined by the reduction of 0.4 mM $NADP^+$ measured at 340 nm [ε = 6,220 $(M \cdot cm)^{-1}$] in 100 mM EPPS buffer (pH 8.4) using 6 mM sodium glutamate as the substrate (40). Superoxide reductase (SOR) activity was determined as apparent superoxide dismutase activity where 1 U is the amount of enzyme required to obtain 50% inhibition of the rate of cytochrome *c* (20 μM) reduction due to superoxide produced aerobically at 25°C by 0.2 mM xanthine and 3.4 μg of xanthine oxidase in 50 mM potassium phosphate buffer (pH 7.8) (20). Adenylate kinase (AK) and guanylate kinase (GK) activities were determined by measuring the rates of ADP and GDP formation, respectively (method modified from that of Rhoads and Lowenstein [38]). For AK, the sample was added to 4 mM AMP, 10 mM $MgCl_2$, and 100 mM KCl and incubated for 2 min. The reaction was initiated by the addition of ATP (4 mM) and quenched by placing the sample on ice. The ADP formed was measured by adding 1 mM phosphoenolpyruvate (PEP), 40 mM NADH, and 5 U each of pyruvate kinase and lactate dehydrogenase (Roche Molecular Biochemicals) and monitoring NADH oxidation at 340 nm [at 25°C; ε = 6,200 $(M \cdot cm)^{-1}$]. GK activity was measured in a similar manner except that 2 mM GMP was substituted for AMP. One unit of AK activity is equivalent to 0.5 μmol of ADP formed min^{-1} (since 2 ADP molecules are produced for each AMP molecule phosphorylated), and 1 U of GK activity equals 1 μmol of ADP formed min^{-1}. PEP synthetase (PpsA) activity was determined by phosphate formation (18). The sample was added to 1 ml of 4 mM pyruvate–10 mM $MgCl_2$–200 mM KCl and incubated for 2 min. The reaction was initiated by adding ATP (4 mM) and quenched after 2 min with 0.2 ml of 5 M H_2SO_4. The amount of phosphate produced was measured spectrophotometrically as described previously (13). Aminoacylase activity was determined by adding the sample to a 500-μl total volume containing 50 mM Bis-Tris HCl (pH 6.5) and 30 mM *N*-acetyl-L-methionine (S. V. Story, A. Grunden, and M. W. W. Adams, submitted for publication). The assay mixture was heated to 100°C for 5 min, mixed with 500 μl of 15% trichloroacetic acid, and then spun at 13,000 × *g* for 5 min. Five hundred microliters of this solution was removed, and 250 μl of ninhydrin reagent (3% ninhydrin in ethylene glycol monomethyl ether) and 250 μl of 0.2 mM sodium acetate cyanide were added. The mixture was heated at 100°C for 15 min. A 1.5-ml volume of 50% isopropanol was then added, and the absorbance of the mixture was read spectrophotometrically at 570 nm. Prolidase activity was determined by measuring the production of proline using the colorimetric ninhydrin method (11, 51) from the hydrolysis of 4 mM Met-Pro dipeptide at 100°C in 50 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.0). One unit of prolidase or aminoacylase activity is defined as the amount of enzyme that liberates 1 μmol of amino acid min^{-1} at 100°C.

Statistical analyses. The culture growth rate data, the phenylacetate production rates, and each enzyme activity measurement were subjected to statistical analyses as described previously (52). The triplicate growth rate and enzyme activity data from the five growth conditions were first compared by an analysis of variance (ANOVA) test and then by a Tukey test (α = 0.05, or a 95% confidence interval). Individual groups of data for each condition are reported as means ± 1 standard deviation (SD). The results of the Tukey test are presented in Tables 1 and 2. The phenylacetate production rates were compared using linear regression analysis, analysis of covariance (ANCOVA), and a Tukey test (α = 0.05).

RESULTS

***P. furiosus* growth versus carbon source.** The growth rates (doubling times ± SDs) for *P. furiosus* under each growth condition are summarized in Table 1. All growth curves demonstrated that the cultures were in exponential growth phase throughout the experiment and that there was no diauxic growth or cultures reaching stationary growth phase (data not shown). Growth was most rapid when cultures were grown on peptides plus S^0 (both with and without maltose). The growth rates in media containing maltose, maltose plus S^0, and maltose plus peptides were not significantly different from each other but were much lower than the growth rates in peptides-plus-S^0 media with and without maltose. On a small scale (50

TABLE 1. Growth rates of *P. furiosus* on various media

Sulfur (S^0)	Doubling time (min)[a] in medium with:		
	Maltose	Peptides	Peptides plus maltose
Present	65.1 ± 1.6	**40.5 ± 4.8**	**47.6 ± 1.4**
Absent	63.3 ± 0.8	No growth	64.1 ± 5.8

[a] Results are means ± SDs. Boldfaced values are significantly different ($P < 0.05$) from all values that are not boldfaced (see Materials and Methods).

ml of medium in 120-ml bottles), growth was extremely poor when cultures were grown in the peptide medium without S^0 after the first transfer, and no significant growth occurred after a second transfer. No attempt was made to grow *P. furiosus* in the fermentor using this medium. These data suggested that S^0 was required for growth of *P. furiosus* on peptides but not on maltose.

To measure the extent to which peptides were being fermented during the growth of *P. furiosus* under the various conditions, we used phenylacetate production as an indicator. Phenylacetate is readily determined in complex mixtures and is the specific product of phenylalanine fermentation via phenylpyruvate and phenylacetyl-CoA (Fig. 2). As shown in Fig. 3, phenylacetate is produced by cultures grown on the peptides-plus-S^0 medium at a rate of 1.34 ± 0.14 nmol $(h \cdot 10^6 \text{ cells})^{-1}$ (±95% confidence interval; $n = 18$). The rate of phenylacetate production in the peptides-plus-maltose-plus-S^0 medium [0.98 ± 0.06 nmol $(h \cdot 10^6 \text{ cells})^{-1}$; $n = 11$] was very similar to that measured in the same medium without maltose when the values were normalized by dividing the production rates by their corresponding specific growth rates. Thus, the production rates in the peptides-plus-S^0 medium were not affected by the presence of maltose. In contrast, when cells were grown with maltose as the only carbon source, very little phenylacetate was produced [0.03 ± 0.01 nmol $(h \cdot 10^6 \text{ cells})^{-1}$; $n = 4$]. Hence, phenylacetate production appears to be a very good measure of peptide utilization. A low level of phenylacetate was produced when cultures were grown on maltose plus S^0 [0.19 ± 0.25 nmol $(h \cdot 10^6 \text{ cells})^{-1}$; $n = 9$] or on peptides plus maltose without S^0 [0.23 ± 0.02 nmol $(h \cdot 10^6 \text{ cells})^{-1}$; $n = 13$]. However, these rates were not significantly different from one another, but they were higher than the rate measured for the maltose medium and lower than the rates measured in medium containing peptides plus S^0 (with or without maltose). Thus, the production rates in the peptides-plus-S^0 medium were not affected by the presence of maltose, while the production rates in the maltose, maltose-plus-S^0, and maltose-plus-peptides media were all much lower than those seen during growth on peptides plus S^0 both with and without maltose. Furthermore, the phenylacetate production data correlated well with the growth data and confirmed that growth on peptides was to a large extent dependent upon S^0 availability.

Enzyme activities. In view of the differential utilization of peptides depending on the presence of S^0, the activities of a variety of enzymes in the fermentative pathways were measured in cells grown under the five growth conditions (Table 1). In order to assess the activities of both cytoplasmic and membrane-bound enzymes, the proteins from WCEs of cells were fractionated into defined cytoplasmic (CYT), membrane-associated (MA), and membrane-bound (MB) samples. Protein assays showed that of the total protein present in the WCE, 75% ± 8% ($n = 15$), was recovered in the combined CYT and 50 mM Tris-HCl-wash fractions. Only a very small percentage of the total protein (3% ± 1%; $n = 14$) was recovered in the MA fraction obtained by the high-salt wash, while the MB fraction contained 12% ± 5% ($n = 15$) of the total protein. The remaining protein (~10%) was presumably lost during

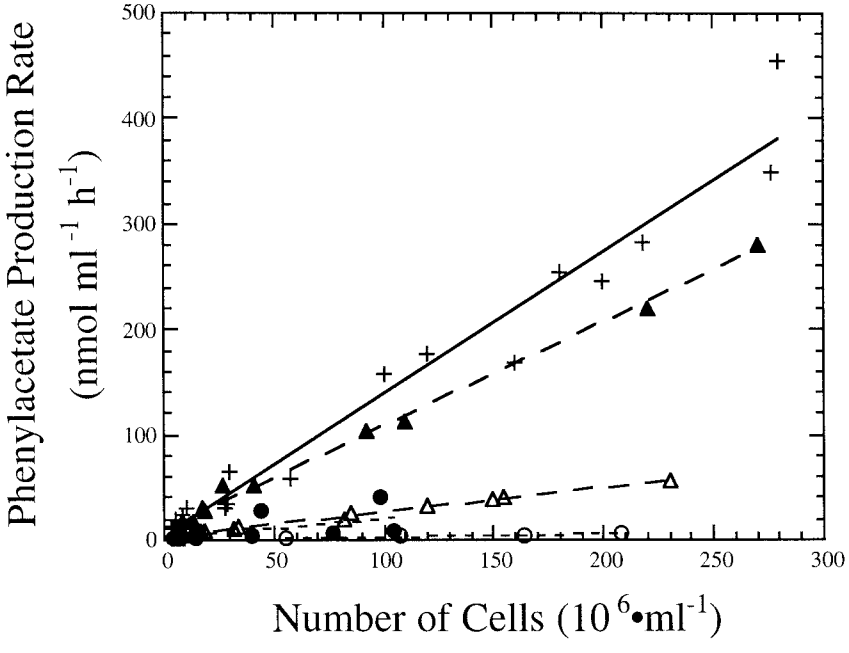

FIG. 3. Phenylacetate production rates for cultures grown on media containing peptides plus S^0 (plus signs), maltose (open circles), maltose plus S^0 (solid circles), maltose plus peptides (open triangles), and maltose plus peptides plus S^0 (solid triangles).

TABLE 2. Specific activities of selected glycolytic, peptidolytic, and related enzymes of cells grown with various substrates

Enzyme	Sp act (U mg^{-1})[a] on the following growth medium:				
	Maltose + peptides	Maltose	Maltose + peptides + S^0	Maltose + S^0	Peptides + S^0
Glycolysis, GAPOR	1.63 ± 0.71	4.35 ± 3.21	1.22 ± 0.83	2.25 ± 0.85	0.42 ± 0.38
Peptidolysis					
Prolidase	8.35 ± 3.16	9.20 ± 0.82	9.64 ± 0.94	11.43 ± 5.20	6.86 ± 3.57
Aminoacylase	1.53 ± 0.71	1.81 ± 0.16	0.80 ± 0.16	1.03 ± 0.78	1.03 ± 0.55
GDH	2.81 ± 1.08	1.73 ± 0.77	1.73 ± 0.20	0.73 ± 0.20	**5.70 ± 0.67**
KGOR	0.20 ± 0.06	0.13 ± 0.05	0.26 ± 0.06	0.20 ± 0.03	**0.44 ± 0.02**
IOR[b]	0.17 ± 0.04	0.06 ± 0.03	0.10 ± 0.01	0.02 ± 0.01	0.25 ± 0.09
VOR	2.12 ± 0.45	2.16 ± 0.23	1.88 ± 0.37	**0.79 ± 0.08**	1.80 ± 0.61
FOR	2.36 ± 0.18	2.37 ± 0.49	3.58 ± 0.93	**0.83 ± 0.22**	3.99 ± 0.56
Both pathways					
POR[c]	7.90 ± 1.27	9.70 ± 1.12	6.81 ± 0.15	4.89 ± 0.87	4.95 ± 1.60
AOR	2.30 ± 0.47	2.48 ± 0.37	2.42 ± 0.75	**0.99 ± 0.22**	2.19 ± 0.42
ACS I	**0.57 ± 0.08**	0.24 ± 0.07	0.21 ± 0.05	0.15 ± 0.01	0.35 ± 0.12
ACS II	0.14 ± 0.11	0.06 ± 0.00	0.07 ± 0.01	0.08 ± 0.04	0.08 ± 0.02
H_2ase I + II	**4.17 ± 1.72**	**2.57 ± 0.87**	0.45 ± 0.12	0.16 ± 0.10	0.39 ± 0.06
MB H_2ase	**18.06 ± 8.15**	**14.52 ± 7.37**	2.64 ± 0.53	0.51 ± 0.09	1.09 ± 0.46
Other					
NROR	0.019 ± 0.015	**0.033 ± 0.013**	0.004 ± 0.002	**0.053 ± 0.007**	0.012 ± 0.002
NROR + FNOR	1.55 ± 0.49	1.35 ± 0.07	0.99 ± 0.01	0.96 ± 0.20	0.81 ± 0.09
SOR	32.33 ± 9.88	31.53 ± 23.51	30.22 ± 5.79	25.98 ± 5.66	25.98 ± 6.49
FDH	0.01 ± 0.01	0.02 ± 0.02	0	0	0
PpsA[d]	1.13 ± 0.12	2.27 ± 0.29	1.49 ± 0.21	1.02 ± 0.55	1.59 ± 0.09
AK	0.27 ± 0.10	0.37 ± 0.01	0.32 ± 0.03	0.22 ± 0.06	0.31 ± 0.05
GK	0.06 ± 0.04	0.06 ± 0.01	0.06 ± 0.00	0.03 ± 0.02	0.07 ± 0.02

[a] All enzyme activities were measured using the cytoplasmic protein fraction except for the MB hydrogenase (H_2ase). Values are means ± SDs. Boldfaced values are significantly different ($P < 0.05$) from all values that are not boldfaced.
[b] Statistical trend: peptides plus S^0 > maltose = maltose plus S^0 = peptides plus maltose plus S^0.
[c] Statistical trend: maltose > maltose plus S^0 = peptides plus S^0.
[d] Statistical trend: maltose > maltose plus S^0 = peptides plus maltose.

sample transfer and manipulations. The washing procedure was effective at removing CYT protein from the MA and MB fractions, as judged by the amount of GDH activity in the various fractions. GDH is a soluble protein (40) and was used as a marker to establish that the wash protocol provided complete separation of CYT proteins from the membrane. Of the total GDH activity, 77% ± 15% ($n = 15$) was recovered after the first centrifugation step (100,000 × *g*) and only a trace amount could be measured in the MA and MB protein fractions (0.5% ± 0.5% and 0.1% ± 0.1%, respectively; $n = 15$).

The specific activities and standard deviations for each of the 21 enzyme assays that were carried out using cytoplasmic and membrane fractions from cells derived from each of the five growth conditions are summarized in Table 2. Notably, 14 of the 21 enzymes showed significant differences in activity with change in the growth condition. Those that remained essentially unchanged under the five growth conditions were FNOR, ACS II, AK, GK, prolidase, aminoacylase, and SOR.

On the other hand, the specific activities of both the cytoplasmic and membrane-bound hydrogenases increased approximately 10-fold when S^0 was omitted from the media. This was independent of whether cells were grown on maltose or maltose plus peptides. To determine whether these differences were due to enzyme inhibition by residual S^0 (or its metabolites) in the protein sample, aliquots of the cytoplasmic protein fraction from cells grown on maltose in the absence of S^0 were combined separately with aliquots of cytoplasmic, membrane, and WCE fractions from cells grown with maltose plus S^0. In each case, hydrogenase activities were additive, showing that the extracts of S^0-grown cells do not contain inhibitors of these enzymes.

The activities of the peptidolytic pathway-related enzymes, AOR, FOR, and VOR, were each unchanged during growth on maltose or peptides-plus-S^0 (with and without maltose) media but decreased significantly when the organism was grown on maltose plus S^0 (Table 2). In contrast, the specific activity of NROR was highest under this growth condition, slightly lower on the maltose-only medium, and lower still in the three peptide-based media. When *P. furiosus* was grown on peptides-plus-S^0 medium, the specific activities of the peptidolytic pathway-related enzymes, KGOR and GDH, were significantly higher than in cells grown under any other condition, which were all similar to each other. In addition, IOR activity was higher in cells from the peptides-plus-S^0 medium than it was in cells grown with maltose with and without S^0 or peptides, which were all similar to each other. The specific activity of the glycolytic enzyme, GAPOR, was lowest in the peptides-plus-S^0 medium, although the values obtained varied considerably with the different growth conditions probably because of the inhibitory effect of sodium dithionite (which was present in all buffers) on the enzyme (35). Both POR and PpsA showed a significant trend toward higher specific activity when cultures

were grown on the maltose medium, while the activity of ACS I was significantly higher in the maltose-plus-peptides medium. The specific activity of FDH was very low in cells grown without S^0, and the enzyme could not be detected in cells grown in the presence of S^0.

DISCUSSION

The results presented here show for the first time that *P. furiosus* efficiently utilizes peptides for growth only if S^0 is present, and peptides (plus S^0) appear to be more favorable for growth than maltose as the carbon source. Yet S^0 appears to have little effect on the metabolism of maltose by *P. furiosus*. Similarly, peptides appear to have little effect on cell growth with maltose in the absence of S^0. These conclusions are supported by results showing that phenylacetate production increased sixfold when S^0 was added to the peptides-plus-maltose medium. Also, growth rates were highest when both peptides and S^0 were present in the medium. The presence of maltose in the peptides-plus-sulfur medium had no effect on the rate of phenylacetate production, suggesting that peptidolysis coexists with glycolysis. This is the first study to demonstrate growth substrate preference by *P. furiosus* and a link between peptide utilization and S^0 availability. Peptides are the favored carbon source, presumably because they eliminate the need for the likely energy-requiring, de novo synthesis of some amino acids necessary during growth on maltose. Moreover, a link between peptide utilization and S^0 availability enables rationalization of some previously reported data. For example, Raven and Sharp (37) have shown that *P. furiosus* does not grow after 20 h of incubation with 0.5% (wt/vol) peptone and 0.1% (wt/vol) yeast extract in the absence of S^0, whereas growth occurred when the peptide source was replaced with 20 mM maltose. However, this apparent preference for maltose over peptides was determined without considering S^0. The results presented here show that peptides could be utilized only in the presence of S^0.

There have been several reports on the nutritional characteristics of *P. furiosus*, but typically maltose is the carbon source and S^0 is omitted from growth media. For example, using a defined minimal medium for the continuous culture of *P. furiosus* on maltose (no S^0), it was shown that biotin, proline, and cysteine are required (37), while other researchers have reported lower growth rates on the same medium (23) and that Ile and Val are also essential amino acids (16). In the present study, the low concentration of yeast extract satisfied the essential amino acid and vitamin requirements of the organism, and we show here that these were independent of S^0 during growth with maltose as the carbon source. Like *P. furiosus*, *P. woesei* and *P. glycovorans* are reported to grow on maltose as the main carbon source (2, 4), but in contrast to the situation with these strains, the growth rates and cell yields of other *Pyrococcus* spp., including *P. abyssi*, *P. horikoshii*, *Pyrococcus* sp. strain ES-4, and *Pyrococcus* sp. strain GB-D, are low or zero in the absence of S^0 and peptides (9, 12, 19, 36). It seems that these organisms cannot grow without S^0 because they can only utilize peptides, and this appears to be a S^0-dependent process.

P. furiosus is therefore unusual among known *Pyrococcus* spp. in that it will grow to high densities using maltose as the carbon source in the absence of peptides and S^0. Maltose utilization has been studied in the hyperthermophile *Thermococcus litoralis* (17, 50), which, like *P. furiosus*, grows on maltose without S^0 (39). Maltose binds to the membrane protein MalE and crosses the membrane via the MalFG ATP-binding cassette (ABC) membrane transporter complex, where, in *P. furiosus*, it is converted to glucose by α-glucosidase (50). As might be expected, homologs of *malEFG* are present in the genome sequence of *P. furiosus* (30), but they are absent in the genomes of *P. horikoshii* (30) and *P. abyssi* (www.genoscope.cns.fr/cgi-bin/Pab.cgi). This could explain the inability of these organisms to utilize maltose, and presumably these genes would not be present in the other known *Pyrococcus* spp. that can grow only on peptides plus S^0.

It has been established that in media lacking S^0, the rate of H_2 production by *P. furiosus* is similar to the combined rates of H_2 and H_2S (~40:60 ratio) production in the same media containing S^0 (10, 45). These results suggest that S^0 reduction simply "replaces" H_2 evolution as a means of disposing of excess reductant (10) and that, as previously suggested, the cytoplasmic hydrogenases reduce S^0 as well as produce H_2 (28, 29). However, the results presented herein show that S^0 reduction may not occur by this simplistic mechanism, since the activities of the cytoplasmic hydrogenases are dramatically reduced by the presence of S^0 (Table 2). In fact, it is reasonable that the activities of the cytoplasmic hydrogenases, as well as that of the membrane-bound enzyme, increase in the absence of S^0, as this would allow increased rates of H_2 production to compensate for the loss of S^0 reduction activity. Therefore, it does not appear that the hydrogenases are responsible for significant S^0 reduction, at least under the conditions used to grow *P. furiosus* described above. The question is, what does catalyze this reaction? FNOR reduces S^0 (24), but its activity did not vary with S^0 availability (Table 2), suggesting that this is probably a fortuitous reaction. A hyperthermophilic membrane-bound sulfur reductase has been purified and characterized from the autotroph *Pyrodictium abyssi* (8). We have been unable to detect any S^0-reducing activity in the membranes of *P. furiosus* using H_2, reduced ferredoxin, or NAD(P)H as the electron donor (J. F. Holden, R. Sapra, and M. W. W. Adams, unpublished data), and the genome sequence of *P. furiosus* does not contain any obvious homologs of the three genes that encode the membrane-bound sulfur reductase complex of mesophilic organisms (14). The S^0-reducing entity of *P. furiosus* is therefore unknown at this time. The membrane protein composition of *P. furiosus* changed with sulfur availability (21; Holden et al., unpublished), and characterization of these S^0-responsive proteins may lead to an understanding of the role of sulfur in metabolism. Our results correlate well with those reported for the growth of *Thermococcus* sp. strain ES1 in a peptide-based medium where hydrogenase (and FDH) activity decreased with increasing amounts of S^0 (27).

In prior studies of *P. furiosus*, the only enzymes of the glycolytic and peptidolytic pathways that were shown to be regulated were GAPOR and GAPDH (48). The activities of these enzymes increased fivefold and decreased sevenfold, respectively, when *P. furiosus* was grown on cellobiose relative to growth on pyruvate (48). Expression of the GAPOR gene (*gor*) is regulated at the transcriptional level, while the activity of GAPDH appears to be regulated posttranslationally. Accordingly, from our analyses, the highest GAPOR activity was mea-

sured in cells grown on maltose, and this decreased when peptides were the sole carbon source (Table 2). Of the other enzymes tested that are involved in carbohydrate metabolism, both POR (Fig. 1) and the gluconeogenic enzyme PpsA (Fig. 1) showed higher activity in a maltose-only medium, although the differences were not large. Expression of *ppsA* is reported to be higher in cells grown on maltose and tryptone cells than in cells grown on tryptone only (41). PpsA comprises about 5% of the cellular protein in maltose-grown *P. furiosus*, and it has been suggested that it might function under high carbohydrate concentrations to rid the cell of excess energy, which can be harmful to the cell (18, 43).

We show here that several of the enzymes involved in the metabolism of peptides by *P. furiosus* are also regulated. KGOR, IOR, and GDH (Fig. 2) show higher activities in cells grown on the peptides-plus-S^0 medium than in cells using maltose as the sole carbon source (Table 2). On the other hand, the activities of FOR and VOR (Fig. 2) are largely unaffected by the growth conditions, except in cells grown on maltose plus S^0, when both decrease significantly. The same is true for AOR, an enzyme that is postulated to be involved in removing aldehydes generated in both the peptidolytic (via IOR, VOR, and POR) and saccharolytic (via POR) pathways. The specific effector that is generated only by the metabolism of maltose plus S^0 (and not by maltose only) is not known. The other enzyme that appears to undergo regulation is NROR, which catalyzes the NADPH-dependent reduction of rubredoxin, possibly as part of a defense mechanism against oxygen toxicity (20, 25). Its activity increases when cultures are grown in the absence of peptides (with or without sulfur), but the reason for this is unclear.

Aside from GAPOR (48), it is not known if the regulation of the various enzymes listed in Table 2 occurs at the transcriptional, translational, or posttranslational level. Protein and mRNA analyses using two-dimensional gel electrophoresis and DNA microarrays are under way to address this issue. What is clear is that the metabolism of sugars, peptides, and S^0 by *P. furiosus* is not as straightforward as previously thought (Fig. 1 and 2), as key enzymes are tightly regulated, particularly those involved in the disposal of excess reductant.

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