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Hydrogenase of the hyperthermophile *Pyrococcus furiosus* is an elemental sulfur reductase or sulfhydrogenase: Evidence for a sulfur-reducing hydrogenase ancestor

(hydrogen activation/polysulfide reduction/geothermal biology/evolution)

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ABSTRACT **Microorganisms growing near and above 100°C have recently been discovered near shallow and deep sea hydrothermal vents. Most are obligately dependent upon the reduction of elemental sulfur (S^0) to hydrogen sulfide (H_2S) for optimal growth, even though S^0 reduction readily occurs abiotically at their growth temperatures. The sulfur reductase activity of the anaerobic archaeon *Pyrococcus furiosus*, which grows optimally at 100°C by a metabolism that produces H_2S if S^0 is present, was found in the cytoplasm. It was purified anaerobically and was shown to be identical to the hydrogenase that had been previously purified from this organism. Both S^0 and polysulfide served as substrates for H_2S production, and the S^0 reduction activity but not the H_2-oxidation activity was enhanced by the redox protein rubredoxin. The H_2-oxidizing and S^0-reduction activities of the enzyme also showed different responses to pH, temperature, and inhibitors. This bifunctional "sulfhydrogenase" enzyme can, therefore, dispose of the excess reductant generated during fermentation using either protons or polysulfides as the electron acceptor. In addition, purified hydrogenases from both hyperthermophilic and mesophilic representatives of the archaeal and bacterial domains were shown to reduce S^0 to H_2S. It is suggested that the function of some form of ancestral hydrogenase was S^0 reduction rather than, or in addition to, the reduction of protons.**

The ability of microorganisms to reduce elemental sulfur (S^0) is a very recent discovery (1) and is still limited in the microbial world (2). The notable exceptions are the hyperthermophilic archaea (formerly archaebacteria) that thrive at temperatures near and even above 100°C in shallow and deep sea hydrothermal vents (3–5). All of these organisms have been shown to be dependent, to a greater or lesser extent, upon S^0 reduction for optimal growth. The majority are obligately dependent upon the reduction of S^0 to H_2S and use either H_2 or organic compounds as electron donors. On the other hand, some of these organisms can grow by fermentative-type metabolisms and produce H_2. In these cases addition of S^0 to the growth medium leads to the production of H_2S and usually stimulates growth. It has been suggested that sulfur reduction is a "detoxification" mechanism to lower the amount of H_2 produced because H_2 inhibits growth (6). However, we have recently shown that in at least one fermentative hyperthermophile, *Pyrococcus furiosus* (6), S^0 reduction appears to be an energy-conserving reaction (7).

Little is known about the mechanism of S^0 reduction in hyperthermophilic organisms. Pihl *et al.* (8) reported that the activity was membrane-bound in the autotrophic, S^0-respiring *Pyrodictium brockii*, which grows optimally at 105°C. Moreover, there have been few reports on S^0 reduction in mesophilic organisms. The activity was also shown to be membrane-associated in *Wollinella succinogenes* (9) and *Spirillum 5175* (10, 11), whereas it was associated with a periplasmic protein in some *Desulfovibrio* sp. (12) and with a soluble protein in *Chlorobium thiosulfatophilum* (13). Only one sulfur reductase has been partially purified: that from *W. succinogenes*, an organism that can obtain energy by S^0 respiration (10). This membrane-bound enzyme contained an Fe–S cluster but no heme iron and appeared to use either S^0 or polysulfide as the electron acceptor (14).

We report here on the S^0-reducing activity of the hyperthermophile *P. furiosus*, which grows optimally at 100°C (6). This organism reduces either S^0 or polysulfide to H_2S during growth and can do so remote from the insoluble sulfur particles present in the medium (15). However, the abiotic reduction of S^0 to H_2S also occurs at the growth temperature of *P. furiosus* (15), and so it was far from clear whether any enzyme is actually involved in catalyzing S^0 reduction. In addition, assuming the reaction is enzymatic, two mechanisms have been suggested by which the excess reductant produced during fermentation might be coupled to S^0 reduction rather than H^+ reduction (H_2 production) (4). These did not involve membrane electron transport, which might be anticipated if S^0 reduction is an energy-conserving reaction (7). However, we show here that *P. furiosus* does have sulfur reductase activity, defined as the release of H_2S from S^0, but it is located in the cytoplasmic fraction. Moreover, it is associated with and seems to be identical to the hydrogenase previously purified from this organism (16).

MATERIALS AND METHODS

Growth of Bacterium. *P. furiosus* (DSM 3638) was routinely grown at 85°C in a 500-liter fermenter, as described (16).

Enzyme Assays. Sulfur reductase was assayed at 80°C in 8-ml sealed vials with H_2 as the gas phase. The 2-ml assay mixture routinely contained 100 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-3-propanesulfonic acid (EPPS)/NaOH, pH 8.4, 0.1 g of sublimed sulfur (Baker), and 0.8 mM sodium dithionite. The vials were shaken at 160 rpm in a water bath, and cell-free extract or purified fractions were added to initiate the reaction. Where indicated, *P. furiosus* hydrogenase (5 μg) was added immediately before the addition of the extract. At intervals, aliquots of the assay mixture were removed and assayed for hydrogen sulfide (H_2S) by methylene blue formation (17). Because the H_2S is distributed between the gas and liquid phases, the total amount of H_2S produced in this assay was calculated from a standard curve prepared by using known amounts of sodium sulfide (Na_2S) under the same conditions. One unit of sulfur reductase activity is defined as the production of 1 μmol of H_2S per min, calculated from the amount of H_2S produced over a 3-hr

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period. Hydrogenase activity was determined by H_2 evolution of H_2 oxidation as described (15). One unit of activity is defined as 1 μmol of H_2 evolved or consumed per min.

Polysulfide Preparation and Determination. A 0.5 M polysulfide solution was prepared by reaction of 12 g of Na_2S with 1.6 g of elemental sulfur in 100 ml of anoxic water (18). Polysulfide was measured by cold cyanolysis (19).

Purification of Sulfur Reductase. All steps were performed under anaerobic conditions at room temperature. All buffers were repeatedly degassed and flushed with Ar and were maintained under a positive pressure of Ar. They all contained dithiothreitol (1 mM) to protect against trace O_2 contamination. All columns were controlled by a fast protein liquid chromatography system (Pharmacia LKB). *P. furiosus* cells (12 g, wet weight) were suspended in 50 mM Tris·HCl, pH 8.1 (1 g of cells per 4-ml buffer) containing lysozyme (1 mg/ml) and DNase (0.1 mg/ml). The cells were lysed during incubation of the cell suspension at 35°C for 2 hr with stirring. A cell-free extract was obtained by centrifugation at 50,000 × *g* for 1 hr. The cell-free extract was applied to a column (2.8 × 13 cm) of Q Sepharose Fast Flow (Pharmacia LKB) that was equilibrated with 50 mM Tris·HCl buffer, pH 8.1, at 2.5 ml per min. The absorbed proteins were eluted with a 500-ml linear gradient from 0 to 0.7 M NaCl in the same buffer. Sulfur reductase activity was located in the fractions eluting at 0.35–0.5 M NaCl. These were diluted 2-fold with the same buffer and were applied to a column (1 × 10 cm) of Q Sepharose HP (Pharmacia LKB) preequilibrated with 50 mM Tris·HCl buffer, pH 8.1. The column was washed with 20 ml of the same buffer and then with a 120-ml linear gradient from 0 to 0.5 M NaCl. Sulfur reductase activity was detected in the fractions eluting at 0.28 M NaCl. These fractions were applied directly to a column (1 × 10 cm) of hydroxyapatite HR (Behring Diagnostics) previously equilibrated with 50 mM Tris·HCl buffer, pH 8.1, at 1.0 ml/min. The column was washed with 20 ml of the same buffer. The protein was eluted with a 100-ml linear gradient from 0 to 0.15 M potassium phosphate in the same buffer (pH 8.1). The sulfur reductase activity was found in the fractions eluting at 0.03 M phosphate. These fractions were applied to a Mono Q HR column (0.5 × 5 cm) equilibrated with 50 mM Tris·HCl, pH 8.1 at 0.5 ml/min. The column was washed with 10 ml of the same buffer, and the protein was eluted with a 40-ml linear gradient from 0 to 0.4 M NaCl. The sulfur reductase activity was found in the fractions eluting at 0.24 M NaCl. Those judged pure by native and SDS/gel electrophoresis were combined and stored as pellets in liquid N_2.

Other Methods. Protein concentrations were estimated by the Lowry method with bovine serum albumin as the standard (20). The hydrogenase (16), ferredoxin (21), and rubredoxin (22) of *P. furiosus*; the hydrogenase of the hyperthermophilic bacterium *Thermotoga maritima* (23) and the hyperthermophilic archaeon *Thermococcus litoralis* (24); and the hydrogenase I of the mesophilic anaerobe *Clostridium pasteurianum* (25) were purified as described in the references. Partially purified cofactor F_{420}-reducing hydrogenase from *Methanobacterium thermoautotrophicum* strain ΔH (26) was from Robert Scott of the Chemistry Department, University of Georgia, Athens. Purification of hydrogenase from the hyperthermophilic archaeon ES-4 will be described elsewhere (J. M. Blamey and M.W.W.A., unpublished data).

RESULTS AND DISCUSSION

Cell-free extracts of *P. furiosus* were found to catalyze the reduction of S^0 to H_2S at 80°C using H_2 as the electron donor. The specific activity ranged from 0.15 to 0.20 units/mg. There was no significant difference in the specific activity of cell-free extracts from cells grown either with or without S^0 (1%, wt/vol). In both cases, most activity (>90%) was found associated with the cytoplasmic fractions. It should be noted that in the sulfur reductase assay used, the production of H_2S from S^0 was measured by using H_2 as the electron donor. Under all conditions tested and with either cell-free extracts or purified fractions, H_2S could not be detected unless a protein-containing fraction was added to the assay mixture.

In the initial attempts to purify the sulfur reductase activity from cell-free extracts, purified *P. furiosus* hydrogenase was added to the assay medium to provide a means of activating H_2 as a source of reductant. However, control experiments showed that addition of hydrogenase, although stimulatory, was not obligatory for H_2S production, and it was therefore omitted for routine assays. The sulfur reductase activity was purified ≈30-fold compared with the cell-free extract with an overall yield of ≈10% (Table 1). Analysis of the purified enzyme by SDS/gel electrophoresis revealed why the addition of hydrogenase was not required for H_2 activation: the purified sulfur reductase showed the same pattern of subunits as that exhibited by the hydrogenase previously purified from this organism (16). Furthermore, that the enzymes responsible for S^0 reduction and H_2 evolution in *P. furiosus* were one and the same was confirmed by the co-elution of these two activities throughout the purification procedure. In addition, pure samples of *P. furiosus* hydrogenase previously purified in our laboratory all exhibited sulfur reductase activity. The enzymatic nature of the H_2S-producing activity of what we shall refer to as the "sulfhydrogenase" enzyme was shown by the proportional increase in the rate of S^0 reduction with increased amounts of the purified protein (between 0 and 50 μg/ml), and the catalytic rate constant (k_{cat}) value for S^0 reduction (593 s^{-1}). Moreover, the rate of H_2S production remained constant over at least 10 hr, at which time >100 μmol of H_2S can be produced by the 2-ml reaction mixture.

Sulfhydrogenase exhibited maximal S^0 reduction activity at pH 8.4 at 80°C (Fig. 1). The activity was barely measurable at 30°C but increased linearly with temperature with maximum (100%) activity at 80°C (decreasing to 15% at 90°C). In contrast, the H_2-oxidizing activity of the enzyme has a slightly lower pH optimum (8.0), and the optimum temperature is >95°C (5, 16). It is not clear whether this difference arises because of the instability of some catalytic domain of the protein >80°C that is required for S^0 reduction but not H_2 oxidation or to the fact that S^0 undergoes a structural transition near 94°C (27). The rate of the S^0 reduction to H_2S by sulfhydrogenase depended on the S^0 concentration with an optimum of 5% (wt/vol; Fig. 2). H_2S was not detected when S^0 was replaced by other sulfur-containing compounds such as Na_2SO_4, Na_2SO_3, or $Na_2S_2O_3$ (each up to 30 mM) or cystine (2.2 mM).

During the measurement of S^0-reducing activity of sulfhydrogenase, it was observed that the final assay mixture became bright yellow. The visible absorption of the solution was identical to that of polysulfide (14). This can be considered an alternative form of elemental sulfur (27) and appears to be generated during S^0 reduction by sulfhydrogenase (Fig. 3). That polysulfide is an intermediate in S^0 reduction was confirmed by the observation that polysulfide is reduced to H_2S by sulfhydrogenase in the absence of S^0 (Fig. 1). These data also support the hypothesis that polysulfide is an intermediate

Table 1. Purification of sulfur reductase from *P. furiosus*

Step	Protein, mg	Activity, units	Specific activity, units/mg	Yield, %
Cell-free extract	560	112	0.2	100
Q Sepharose	222	78	0.3	69
Q Sepharose HP	33	46	1.4	41
HAP	5	23	4.5	21
Mono Q	2	11	5.7	10

HAP, hydroxyapatite.

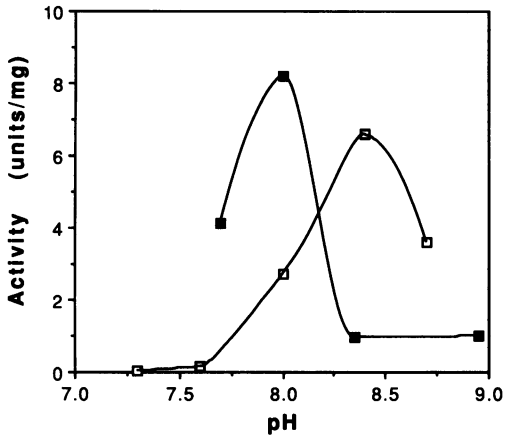

FIG. 1. pH dependence of S^0 reduction activity. Activity was measured at 80°C with either S^0 (0.05 g/ml, □) or polysulfide (25 mM, ■) as substrate. The 2-ml reaction mixture also contained sulfhydrogenase (56 μg), rubredoxin (5.3 μM), and 100 mM EPPS buffer at the indicated pH value (measured at 23°C after addition of S^0 or polysulfide).

in S^0 reduction *in vivo* (15). Moreover, because S^0 is insoluble in aqueous solutions and polysulfide is generated abiotically by the reaction of H_2S and S^0 (27), polysulfide is presumably the true substrate for sulfhydrogenase. If so, the apparent lag phase seen in production of both H_2S and polysulfide from S^0 (Fig. 3) suggests that quite high concentrations of sulfide (1–3 mM) are required for efficient polysulfide production (and thus H_2S production) from S^0. Interestingly, the pH dependence of polysulfide reduction by sulfhydrogenase was not the same as for S^0 reduction (Fig. 1), although this may reflect the effect of pH on the abiotic conversion of S^0 to polysulfide by sulfide (27). At present we do not know whether sulfhydrogenase actually uses S^0 as a substrate, as trace amounts of sulfide could be responsible for the initial production of polysulfide from S^0. It should be noted, however, that under the assay conditions there was no H_2S generated from either S^0 or polysulfide, and no polysufide was produced from S^0, unless sulfhydrogenase was added.

As depicted in Fig. 4, the rate of S^0 reduction by sulfhydrogenase at pH 7.6 was increased ≈3-fold by the presence of the redox protein rubredoxin, which we had previously isolated from *P. furiosus* (22). However, rubredoxin had no effect if the assay was conducted at pH 8.4, and it did not stimulate either the H_2 oxidation or H_2 evolution activity of sulfhydrogenase over the pH range 7.0–8.4. On the other hand, *P. furiosus* ferredoxin (21), which is thought to be the *in vivo* electron donor to the hydrogenase for H_2 production (16, 28), had no effect on the S^0 reduction activity of sulfhydrogenase at pH 7.6 or 8.4. Neither ferredoxin nor rubredoxin catalyzed S^0 reduction in the absence of sulfhydrogenase. The function of rubredoxin in *P. furiosus* has yet to be established (22, 28); hence, a role in S^0 reduction is an intriguing possibility.

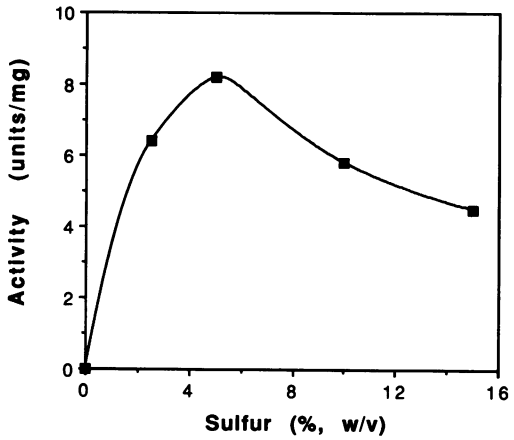

FIG. 2. Effect of S^0 concentration on S^0 reduction activity. H_2S production from S^0 was determined as described in text, except that the pH was 8.4 and the amount of S^0 added was varied as indicated. w/v, Wt/vol.

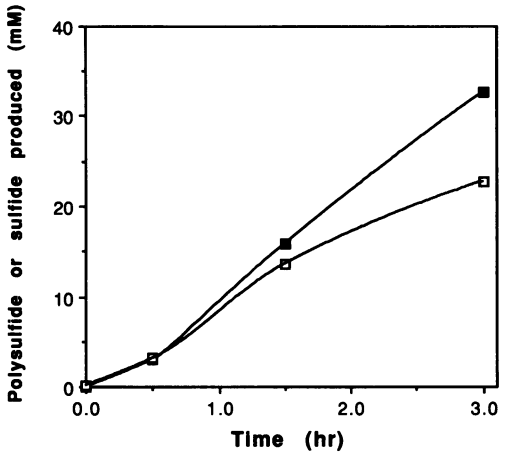

FIG. 3. Polysulfide and H_2S production from S^0. H_2S production from S^0 (■) and polysulfide production from S^0 (□) were measured as described in text, except that the reaction mixture contained sulfhydrogenase (35 μg/ml) and S^0 (0.05 g/ml) in 100 mM EPPS buffer, pH 8.4.

The results presented so far demonstrate that the moiety responsible for catalyzing S^0 reduction in at least one hyperthermophile is a cytoplasmic hydrogenase. To determine if this was a unique property of the *P. furiosus* enzyme, we examined the S^0 reduction activity of the cytoplasmic hydrogenases that we have recently purified from other fermentative, S^0-reducing hyperthermophiles, which include the archaeon *Ts. litoralis* (ref. 29; optimum *T*, 90°C) and the (eu)bacterium *Ta. maritima* (ref. 30; optimum *T*, 80°C). We also assayed the hydrogenase of the hyperthermophilic archaeon ES-4, which reportedly grows only by S^0 respiration (ref. 31; optimum *T*, 100°C). Table 2 shows that these hydrogenases also reduced S^0 to H_2S at 80°C. The hydrogenases of ES-4 and *P. furiosus* showed the highest specific activities in S^0 reduction and also the highest ratios of S^0-reducing activity to H_2-oxidizing activity.

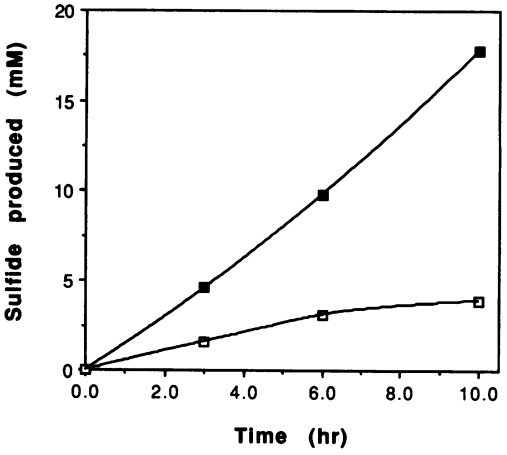

FIG. 4. Effect of rubredoxin on S^0 reduction activity of sulfhydrogenase. The 2-ml reaction mixture contained 100 mM EPPS, pH 7.6, 0.1 g of sublimed sulfur, 0.8 mM sodium dithionite, and 70 μg of sulfhydrogenase with (■) or without (□) *P. furiosus* rubredoxin (7 μM).

Table 2. The sulfur reductase activity associated with the hydrogenases from different organisms

Organism	Sulfur reductase activity,* units/mg		Ratio
	Sulfur reductase	Hydrogenase	
P. furiosus (80°C)	5.7	44	0.130
ES-4 (80°C)	7.2	132	0.050
Ts. litoralis (80°C)	0.02	1.4	0.014
Ta. maritima (80°C)	0.06	2.4	0.025
M. thermoautotrophicum (55°C)	0.05	5.8	0.009
C. pasteurianum (30°C)	4.4	510	0.009

The assay temperature is given in parentheses.
*Hydrogenase was measured by the H_2-dependent reduction of methyl viologen at pH 8.0, and S^0 reduction was measured at pH 8.4.

To investigate whether S^0 reduction might be a general property of all hydrogenases, the enzymes from the mesophilic bacterium *C. pasteurianum* and from the moderately thermophilic methanogen *M. thermoautotrophicum* were also assayed for S^0 reducing activity. They, too, catalyzed H_2S production (Table 2); in fact, the rate of S^0 reduction by the clostridial enzyme was linear with time for >6 hr, at which time >50 mM sulfide was produced. These data with the mesophilic enzymes are consistent with the ability of some methanogens to reduce S^0 to H_2S *in vivo* (32) and suggest that H_2-producing saccharolytic clostridia such as *C. pasteurianum* might dispose of the excess reductant generated from fermentation as H_2S. As far as we are aware, the growth of such organisms in the presence of S^0 has not been reported.

S^0 reduction by hydrogenases, therefore, appears to be a general phenomenon. Indeed, because the hyperthermophiles are considered the most ancient organisms known at present (33), one could speculate that the primary or even sole function of a prototypical "hydrogenase" in ancestral microorganisms growing in sulfur-rich, geothermal environments was to reduce sulfur species (S^0 or polysulfide) rather than protons. Subsequently, in most cases, the main role of such an enzyme then evolved into the metabolism of H_2. The exception would be the hyperthermophiles, in which "sulfhydrogenase" maintained (or acquired) a dual physiological role—the reduction of both protons and sulfur species.

Finally, we have recently shown that the production of H_2S from S^0 by *P. furiosus* does, in fact, represent an energy-conserving reaction, although it does not appear to occur by a conventional respiratory mechanism (7). On the other hand, our data show that this organism contains a cytoplasmic S^0 reduction activity that is stimulated by a cytoplasmic redox protein (rubredoxin) at physiological pH. This result also argues against a membrane-bound respiratory chain. Although the role of rubredoxin is unknown, it might serve to stabilize polysulfide species near neutrality. We note, however, that in contrast to most other hyperthermophilic organisms, *P. furiosus* grows well without S^0 (6). Thus, it remains to be determined whether hyperthermophiles that obligately depend upon S^0 reduction for growth—e.g., ES-4—contain an enzyme (in addition to a cytoplasmic hydrogenase) that can reduce S^0. In any event, apparently the production of intracellular sulfide from polysulfide and S^0 is readily catalyzed by probably all hydrogenases, and this has some unknown bioenergetic benefit, at least to some organisms that grow near and above 100°C.

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