The Elemental Sulfur-Responsive Protein (SipA) from the Hyperthermophilic Archaeon *Pyrococcus furiosus* Is Regulated by Sulfide in an Iron-Dependent Manner[∇]

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The gene (*sipA*) encoding the sulfur-induced protein A (PF2025) is highly upregulated during growth of *Pyrococcus furiosus* on elemental sulfur (S⁰). Expression of *sipA* is regulated by sulfide, the product of S⁰ reduction, but in an iron-dependent manner. SipA is proposed to play a role in intracellular iron sulfide detoxification.

Pyrococcus furiosus is a hyperthermophilic archaeon that grows optimally near 100°C. It utilizes carbohydrates as carbon and energy sources, producing acetate, carbon dioxide, and hydrogen (4). When elemental sulfur (S⁰) is added, hydrogen is replaced by hydrogen sulfide (1), hereafter referred to as sulfide, which is represented by comparable amounts of H₂S and HS[−] (pH ~7; 100°C) (2). Transcriptional analyses of cells grown with and without S⁰ revealed that PF2025, which encodes a 19-kDa protein of unknown function, was the most highly upregulated by S⁰ and was proposed to encode S⁰-induced protein A, or SipA (11). A subsequent study revealed a two-part response by *P. furiosus* when S⁰ is added at mid-log phase (12). The primary response occurred within 10 min after the addition of S⁰ but did not involve *sipA*; rather, it included upregulation of the gene (*nsr*) encoding a cytoplasmic coenzyme A (CoA)-dependent NAD(P)H sulfur oxidoreductase (NSR) proposed to reduce S⁰ and produce sulfide intracellularly. Expression of *sipA* was significantly upregulated as part of the secondary response, occurring within 30 min of the addition of S⁰. The goal of the present study was to provide insight into the function of SipA by determining what regulates its production.

To determine if SipA might be produced in response to the sulfide that would accumulate as a result of intracellular reduction of S⁰ by NSR, the effects of extracellular sulfide on the production of SipA were determined. Sodium sulfide was added to concentrations of 5, 10, and 20 mM at 25°C from a deaerated stock to uninoculated growth media in closed bottles (12). The pH was adjusted to 7, using deaerated 6 N HCl. The actual sulfide concentrations in aqueous solution measured colorimetrically (12) were 2.3 to 7.7 mM (Table 1). *P. furiosus*

was grown in the same media, and expression of *sipA*, measured by quantitative PCR (QPCR) (12), was upregulated 2-, 19- and 550-fold, respectively, compared with that for cells grown without added sulfide (Fig. 1a). In contrast, expression of *nsr*, which is part of the primary response to S⁰ (12), was unaffected by sulfide (Fig. 1b). Sulfane sulfur (S⁰) concentrations (10) were low and not statistically related to the added sulfide. Consequently, these data indicate that expression of *sipA* is regulated by sulfide and not by S⁰. As might be expected, the degree of upregulation when sulfide is added extracellularly is lower (by an order of magnitude) than that measured when sulfide is generated intracellularly by the reduction of S⁰ catalyzed by NSR (Fig. 1a).

The production of SipA protein was measured in cells grown in the presence of sulfide by immunoanalysis (3) following electrophoresis (8) of samples precipitated with 20% (wt/vol) trichloroacetic acid. Polyclonal antibodies to SipA, generated using recombinant protein with an N-terminal His₆ tag produced in *Escherichia coli* and purified in the presence of 6 M guanidinium chloride (Research Animal Resources Facility, Athens, GA), and to *P. furiosus* superoxide reductase ([SOR] a gift from Frank Jenney, Philadelphia College of Osteopathic Medicine, Suwanee, GA) were purified from serum using a protein A column (GenScript, Piscataway, NJ). As shown in Fig. 2a, the upregulation of *sipA* transcription in response to sulfide led to increased amounts of SipA protein compared to that of the constitutively expressed SOR.

In addition to the upregulation of *sipA* transcription, the secondary response of *P. furiosus* to S⁰ includes upregulation of genes predicted to be involved in iron transport and iron-sulfur cluster assembly, suggesting that cells experience intracellular iron limitation when reducing S⁰ (12). To determine if iron affected the regulation of *sipA* by sulfide, expression was measured in cells grown in the standard medium to which iron had not been added, which reduces the iron concentration from 7.4 μM to ~0.7 μM. Surprisingly, expression of *sipA* was not upregulated by increasing extracellular sulfide concentrations in this iron-limited medium, and even more surprising, *sipA* expression was also largely unaffected by S⁰ (Fig. 1a). These data were confirmed at the protein level using immunoanalysis

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TABLE 1. Sulfide and sulfane sulfur concentrations in iron-sufficient and iron-limited growth media^a

Amount of sulfide added (mM)	Conc of sulfide or sulfane in iron-sufficient media (7.4 μ M)		Conc of sulfide or sulfane in iron-limited media (0.7 μ M)	
	Sulfide (mM)	Sulfane (mM)	Sulfide (mM)	Sulfane (mM)
5	2.26 $\pm$ 0.30	0.116 $\pm$ 0.056	4.28 $\pm$ 0.75	0.082 $\pm$ 0.057
10	4.21 $\pm$ 0.66	0.139 $\pm$ 0.066	8.02 $\pm$ 1.14	0.100 $\pm$ 0.049
20	7.65 $\pm$ 1.74	0.237 $\pm$ 0.061	15.43 $\pm$ 2.08	0.164 $\pm$ 0.059

^a Sulfide and sulfane sulfur concentrations were measured colorimetrically in growth media prior to inoculation. Values are from at least two separate media preparations $\pm$ standard deviations.

(Fig. 2b). We therefore concluded that the production of SipA is upregulated by sulfide but that the reaction is iron dependent. Interestingly, the expression of *nsr* was downregulated 5-fold by sulfide in the absence of iron, suggesting that it may also be regulated by sulfide in a product-related manner (Fig. 1b).

To determine if expression of *sipA* was regulated simply by decreased iron availability, independently of sulfide, *P. furiosus* was grown in the standard medium to which the iron chelator bathophenanthroline disulfonic acid (BPS) had been added. At BPS concentrations above 65 μ M, no significant growth of *P. furiosus* was observed by planktonic cell counts using light microscopy and a Petroff-Hausser counting chamber (data not shown). However, BPS concentrations below 65 μ M did not significantly affect either cell growth rate or final cell density compared to those of cells grown in the absence of a chelator (data not shown). Cells were therefore grown in the presence

FIG. 1. RNA levels change with added sulfide and iron. QPCR results for *sipA* (a) and *nsr* (b) from *P. furiosus* grown with sulfide (5, 10, or 20 mM) or 1 g/liter S⁰, with or without 7.4 μ M iron present. Results are compared to base media with no sulfide or S⁰ added. Data are from three separate growths and are graphed as log₂-fold change values with error bars indicating standard deviations. Corresponding fold change values for significant regulation are labeled.

FIG. 2. SipA protein levels change with added sulfide and iron. (a) Western blot analysis of SipA in whole-cell extract from *P. furiosus* grown in at least two independent cultures with added sulfide or S⁰. The constitutively expressed protein SOR was used as a loading control. Lane 1, 1 g/liter of S⁰; lanes 2 to 4, no S⁰ or sulfide; lanes 5 to 6, 5 mM sulfide; lanes 7 to 8, 10 mM sulfide; lanes 9 to 10, 20 mM sulfide. (b) Western blot analysis of SipA in whole-cell extract of *P. furiosus* from cultures grown with and without 1 g/liter of S⁰ and with and without 7.4 μ M iron added.

of 22.5 and 45 μ M BPS, and the expression of *sipA* and *nsr* was analyzed by QPCR. However, their expression levels were not significantly different under these growth conditions (data not shown). Decreased iron availability is therefore not sufficient to regulate *sipA* expression. Taken together, these results demonstrate that SipA regulation is specifically due to sulfide rather than elemental sulfur and that this regulation is absolutely dependent on iron.

Two recent studies reported that production of *sipA* is also upregulated under oxidative stress conditions in *P. furiosus* (13, 14). One possible explanation for this is that reactive oxygen species, such as superoxide and hydrogen peroxide, degrade iron-sulfur clusters to release free ferrous iron into the cytoplasm (6), as well as leaving the remnant iron-sulfur cluster. Thus, oxidative stress produces both iron and sulfide in the form of an iron-sulfur cluster, leading to the upregulation of *sipA*. Ferrous iron further reacts with hydrogen peroxide to form the highly toxic hydroxyl radical (6). The iron storage protein Dps is also upregulated under oxidative stress conditions (13), which sequesters iron as an iron-oxyhydroxide core, using hydrogen peroxide as the oxidant, removing the threat of both free iron and hydrogen peroxide (9). Based on the results presented herein, the role of SipA may well be that of the sulfide equivalent of Dps, whereby it facilitates the reaction of iron in a controlled fashion, not with peroxide, but with some form of sulfide.

The evidence therefore suggests that production of SipA is upregulated in *P. furiosus* when the cell must limit iron availability intracellularly to prevent its reaction with sulfide. This sulfide can be generated either by intracellular reduction of S⁰ or by the destruction of iron-sulfur clusters through oxidative damage. When anaerobic solutions of sulfide and ferrous iron are mixed, they spontaneously react to generate insoluble iron sulfide. Consequently, if free sulfide accumulates in the cytoplasm it could react with available iron, resulting in an iron sulfide precipitate. In addition to the physical problems associated with uncontrolled precipitation, iron sulfide has been shown to bind DNA *in vitro* (5). In iron-deficient cells, however, free ferrous iron would not be available to react with sulfide, so it would not be necessary to upregulate SipA either

when intracellular sulfide is produced by the reduction of S^0 or when extracellular sulfide is added (Fig. 1 and 2).

While the definite function of SipA cannot yet be assigned, the results presented here, specifically that SipA may be involved in sequestration of iron and/or sulfide by an as-yet-unknown mechanism, suggest possibilities that can help direct future investigations. Interestingly, while the protein contains no recognizable metal-binding motif and only one cysteinyl residue, it does show sequence similarity to NifB (21% identity), which is involved in the biosynthesis of the iron-sulfur cofactor of nitrogenase (7). However, it remains to be seen if this is relevant to the proposed role of SipA in iron and sulfide metabolism in *P. furiosus*.

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