# A Phosphoprotein from the Archaeon *Sulfolobus solfataricus* with Protein-Serine/Threonine Kinase Activity

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Sulfolobus solfataricus contains a membrane-associated protein kinase activity that displays a strong preference for threonine as the phospho-acceptor amino acid residue. When a partially purified detergent extract of the membrane fraction from the archaeon *S. solfataricus* that had been enriched for this activity was incubated with  $[\gamma^{-3^2}P]$ ATP, radiolabeled phosphate was incorporated into roughly a dozen polypeptides, several of which contained phosphothreonine. One of the phosphothreonine-containing proteins was identified by mass peptide profiling as the product of open reading frame [ORF] *sso0469*. Inspection of the DNA-derived amino acid sequence of the predicted protein product of ORF *sso0469* revealed the presence of sequence characteristics faintly reminiscent of the "eukaryotic" protein kinase superfamily. ORF *sso0469* therefore was cloned, and its polypeptide product was expressed in *Escherichia coli*. The recombinant protein formed insoluble aggregates that could be dispersed using urea or detergents. The solubilized polypeptide phosphorylated several exogenous proteins in vitro, including casein, myelin basic protein, and bovine serum albumin. Mutagenic alteration of amino acids predicted to be essential for catalytic activity abolished or severely reduced catalytic activity. Phosphorylation of exogenous substrates took place on serine and, occasionally, threonine. This new archaeal protein kinase displayed no catalytic activity when GTP was substituted for ATP as the phospho-donor substrate, while Mn<sup>2+</sup> was the preferred cofactor.

The versatility of covalent phosphorylation-dephosphorylation as a mechanism for regulating protein function and transducing extracellular signals has been compellingly demonstrated in numerous studies encompassing a broad spectrum of eucarval and bacterial organisms (reviewed in references 5, 15, 19, 22, 23, and 28). However, while protein phosphorylation has been detected in several members of the third phylogenetic domain, the Archaea (24, 41, 50, 51, 53, 54, 56, 57), we know relatively little concerning the chemical nature, enzymatic catalysts, and physiological roles of archaeal protein phosphorylation-dephosphorylation events (reviewed in reference 27). Only a few archaeal proteins have been implicated as the targets of protein phosphorylation to date. They include a CheY homolog in Halobacterium salinarium (45), a methyltransferase-activating protein from Methanosarcina barkeri (11), an aminopeptidase from Sulfolobus solfataricus (9), and a glycogen synthase from Sulfolobus acidocaldarius (7). In addition, the N-terminal sequences of three phosphotyrosine-containing proteins from Thermococcus kodakaraensis KOD1 have been determined, although the full sequences of these phosphoproteins have yet to be identified (24). Homologs of the eukaryotic cell cycle protein Cdc6 from Methanobacterium thermoautotrophicum and Pyrobaculum aerophilum also have been reported to autophosphorylate on serine when incubated with  $[\gamma^{-32}P]ATP$  in vitro (18).

Homology searches have revealed the presence of open reading frames [ORFs] encoding potential protein kinases and protein phosphatases within nearly every archaeal genome published to date (26, 27, 32, 42, 49). However, in only a few instances have the inferences of these in silico analyses been translated into the direct, empirical identification and characterization of defined gene products displaying the predicted functional capabilities. Included among these are a CheA-like histidine kinase from *H. salinarium* (44, 45); a faintly "eukaryotic" protein-serine kinase from *S. solfataricus* P2 (35); a set of PPP-family protein-serine/threonine phosphatases from *S. solfataricus* P1 (31), *Methanosarcina thermophila* TM-1 (55), and *Pyrodictium abyssi* TAG11 (36); and a member of the conventional protein-tyrosine phosphatases from *T. kodakaraensis* that exhibited dual-specific capabilities in vitro (24).

Recently, our laboratory identified a glycosylated polypeptide in the membrane fraction of the extreme acidophilic archaeon *S. solfataricus* P1 that exhibited protein kinase activity toward itself as well as several exogenous proteins and peptides (33, 34). Following solubilization from the membrane pellet with detergent, the activity of this protein kinase, tentatively designated SsoPK1, could be enriched up to 10-fold by ionexchange chromatography. In an effort to identify endogenous phosphoprotein substrates from *S. solfataricus*, this enriched membrane extract was incubated with [ $\gamma$ -<sup>32</sup>P]ATP.

While in vitro labeling of potential phosphoproteins incurs the risk that nonphysiologic phosphorylation events may result from interactions between proteins whose normal spatial relationship inside the cell has been perturbed, this method has met with considerable success in manifesting physiologically relevant phosphorylation events in eukaryotic organisms. Moreover, it offers several important operational advantages over in vivo labeling in intact cells grown in the presence of  $[^{32}P]$ orthophosphate. Sensitivity of detection is greatly enhanced by the ability to directly introduce  $[\gamma-^{32}P]$ ATP of high specific activity into samples that have been depleted of organ-

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ophosphate-containing metabolites, as well as the ability to enrich for the protein kinase activity of interest and/or potential substrates. It is also possible to test alternative phospho donors such as  $[\gamma^{-32}P]$ GTP. In vitro labeling also greatly reduces the total quantity of radioactivity required and eliminates several potentially hazardous experimental manipulations of radioactive materials.

Intriguingly, one of the polypeptides that became phosphorylated during our incubations displayed many of the sequence features characteristic of the eukaryotic protein kinase family. Assays of the recombinant version of this protein, the product of ORF *sso0469*, confirmed that it possessed the catalytic capabilities implied by its amino acid sequence. Herein we describe the identification and initial characterization of a new archaeal protein kinase, SsoPK3.

### MATERIALS AND METHODS

**Materials.** Purchased materials included [ $\gamma^{-32}P$ ]ATP and [ $\gamma^{-32}P$ ]GTP (NEN Research Products, Boston, Mass.), [ $\beta^{-32}P$ ]GDP (ICN Biomedical Research Products, Irvine, Calif.), protein assay reagent and prestained standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Richmond, Calif.), chelating Sepharose (Pharmacia Biotech, Piscataway, N.J.), sequencing-grade modified trypsin (Promega, Madison, Wis.), genomic DNA from *S. solfataricus* P2 (American Type Culture Collection, Rockville, Md.), and tamoxifen and other inhibitors (Sigma-Aldrich, St. Louis, Mo.). General laboratory reagents and culture media were from Fisher (Pittsburgh, Pa.) or Sigma-Aldrich.

**Routine procedures.** Protein concentrations were determined as described by Bradford (6) using premixed reagent and a standardized solution of bovine serum albumin (BSA). SDS-PAGE was performed as described by Laemmli (29). Two-dimensional electrophoresis was performed essentially as described by Gorg et al. (17), with all equipment and materials purchased from Pharmacia (Uppsala, Sweden). Gels were stained with Coomassie brilliant blue as described by Fairbanks et al. (14). Electronic autoradiography was performed using a Packard (Meriden, Conn.) InstantImager.

**Preparation of DE-52 fraction.** The DE-52 fraction was prepared as described by Lower et al. (33). Briefly, *S. solfataricus* P1 (ATCC 35091) was grown in continuous culture with vigorous aeration at 75°C in ATCC medium 1304 with the level of yeast extract increased to 2 g/liter. Kanamycin sulfate, 20 mg/liter, was added daily. Cells were harvested at an optical density at 600 nm of 0.5 to 0.8 and stored at  $-20^{\circ}$ C until needed.

Frozen S. solfataricus, 20 g wet weight, was thawed and resuspended in 2 volumes of 20 mM MES, pH 6.5, containing 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 µg of DNase I/ml. The cells were lysed by two passages through a French pressure cell at 12,000 lb/in<sup>2</sup>. The lysate was centrifuged at 1,000 × g for 10 min at 4°C. Next, the supernatant liquid was centrifuged at 100,000 × g for 75 min at 4°C. The pellet was washed and resuspended in 40 ml of 20 mM sodium acetate, pH 5.0, containing 0.5 M NaCl, and particulate material was collected by centrifugation at 100,000 × g for 75 min at 4°C. The pellet was resuspended in 40 ml of 20 mM MES, pH 6.5, containing 125 mM NaCl and 25 mM octyl glucoside. The mixture was centrifuged at 100,000 × g for 75 min at 4°C, and the supernatant liquid was retained as the detergent extract.

The detergent extract was diluted fivefold by the addition of 4 volumes of 20 mM MES, pH 6.5, containing 0.5 mM EDTA and applied to a 1.5- by 18-cm column of DE-52 cellulose that had been equilibrated in 20 mM MES, pH 6.5, containing 25 mM NaCl and 12.5 mM octyl glucoside (equilibration buffer). The column was washed with three column volumes of equilibration buffer and developed with a linear gradient, 150 ml total, of 25 to 500 mM NaCl in equilibration buffer. Fractions, 3 ml, were collected and assayed for protein kinase activity. Fractions corresponding to the major peak of protein kinase activity, which eluted at an NaCl concentration of  $\approx$ 200 mM, were pooled and retained as the DE-52 fraction.

Radiolabeling and electrophoretic separation of phosphoproteins in the DE-52 fraction. The DE-52 fraction was desalted by adding 6.5 volumes of 20 mM MES, pH 6.5, containing 15 mM octyl glucoside and reducing it to its original volume by centrifugal ultrafiltration using a Centricon-10 concentrator. This process was repeated twice more. Next, the desalted DE-52 fraction, 60 to 75  $\mu$ g of protein, was incubated in 100  $\mu$ l of 20 mM MES, pH 6.5, containing 12.5 mM octyl glucoside, 2 mM dithiothreitol (DTT), 5 mM MnCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 50

 $\mu$ M ATP, and 1  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP/ $\mu$ l for either 5 or 60 min, as indicated, at a temperature of 65°C. Where indicated, equal quantities of GTP and [ $\gamma$ -<sup>32</sup>P]GTP or GDP and [ $\beta$ -<sup>32</sup>P]GDP were substituted for ATP and [ $\gamma$ -<sup>32</sup>P]ATP. The reaction was terminated by the addition of 3 volumes of ice-cold acetone, and precipitated proteins were collected by centrifugation. For SDS-PAGE, the protein pellet was resuspended in hot SDS-PAGE sample buffer. For two-dimensional electrophoresis, the pellet was incubated for 1 to 2 h at room temperature in 125  $\mu$ l of 9 M urea containing 4% (wt/vol) 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate, 100 mM DTT, and 2% (vol/vol) immobilized pH gradient buffer, pH 4 to 7 (Pharmacia).

**Phosphoamino acid analysis.** Phosphoamino acid analysis was performed essentially as described by Kamps and Sefton (25). Radiolabeled proteins were isolated by two-dimensional electrophoresis and transferred to an Immobilon P membrane. The portion of the membrane containing the radiolabeled phosphoprotein was incubated for 1 h in 6 N HCl at 95°C, and the supernatant fluid was concentrated in a Speed Vac. The hydrolysate was then applied to a 10- by 10-cm silica gel thin-layer chromatography plate, along with standards of phosphoserine, phosphothreonine, and phosphotyrosine. The plate was subjected to two-dimensional thin-layer electrophoresis. The pH of the buffer used for the first dimension was 1.9, and that for the second dimension was 3.5. Phosphoserine, phosphothreonine, and phosphotyrosine standards were visualized by ninhydrin staining, and the radiolabeled species were located by electronic autoradiography.

Mass peptide profiling. The section of a Coomassie-stained two-dimensional gel containing the protein of interest was excised using a clean razor blade and chopped into pieces approximately 1 mm<sup>3</sup> in size, and the pieces were placed in a 1.5-ml Eppendorf tube. A 100-µl aliquot of a 25 mM solution of ammonium bicarbonate, pH 8.0, containing 50% (vol/vol) acetonitrile was added, and the mixture was agitated for 10 min using a Vortex mixer. The supernatant liquid was removed, and the process was repeated three times more. Next, sufficient 25 mM ammonium bicarbonate, pH 8.0, containing 10 mM DTT was added to cover the gel fragments, and the mixture was incubated for 1 h at 56°C. The mixture was cooled to room temperature, and the supernatant liquid was removed and replaced by an equal volume of 25 mM ammonium bicarbonate, pH 8.0, containing 55 mM iodoacetamide. The mixture was kept at room temperature, protected from light, and occasionally agitated using a Vortex mixer. After 45 min, the supernatant liquid was removed and replaced with 100 µl of 25 mM ammonium bicarbonate, pH 8.0, and agitated for 10 min using a Vortex mixer. The supernatant liquid was removed and replaced with 100 µl of ammonium bicarbonate, pH 8.0, containing 50% (vol/vol) acetonitrile. Following continuous mixing for 10 min, the supernatant liquid was removed and replaced with 100 µl of 25 mM ammonium bicarbonate, pH 8.0, and the entire process was repeated.

The gel fragments were dried for 30 min in a Speed Vac, and then 1 volume of 25 mM ammonium bicarbonate, pH 8.0, containing 0.1 mg of sequencinggrade modified trypsin/ml was added. Following initial agitation for 5 min using a Vortex mixer, the mixture was incubated for 12 to 16 h at 37°C. Next, 2 volumes of distilled water was added, the mixture was agitated for 5 min using a Vortex mixer, and the supernatant liquid, which contained the tryptic peptides, was removed and transferred to a fresh Eppendorf tube. The gel slices were washed twice by adding 2 volumes of 50% (vol/vol) acetonitrile containing 5% (vol/vol) trifluoroacetic acid (TFA), agitating for 5 min on a Vortex mixer, and withdrawing the free liquid with a pipettor. The supernatant liquids were pooled, reduced in volume to  $\approx$ 10  $\mu$ l using a Speed Vac, and then brought up to a volume of  $\approx$ 25  $\mu$ l by the addition of 50% (vol/vol) acetonitrile containing 0.1% (vol/vol) TFA and stored at  $-20^{\circ}$ C.

For mass spectral analysis, 0.5- $\mu$ l portions of the tryptic peptide mixture were mixed with 0.5  $\mu$ l of a saturated solution of  $\alpha$ -hydroxycinnamic acid in 50% (vol/vol) acetonitrile containing 0.1% (vol/vol) TFA. Mass spectral determination of peptide masses was performed on a Kompact Seq matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer equipped with a nitrogen UV laser from Kratos Analytical (Chestnut Ridge, N.Y.). Gel slices from regions lacking visibly stained polypeptides were used as controls to identify mass peaks arising from autodigestion of trypsin and other nonspecific sources. Peptide mass profiles were matched to potential protein sources within an error tolerance of  $\pm 1$  atomic mass units using the web-based ProFound software package available from Rockefeller University (http:www .proteometrics.com [63]). All peptides were assumed to bear a charge of  $\pm 1$ .

**Cloning of and expression of** *sso0469*. ORF *sso0469* was cloned using the materials provided in the TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) following the manufacturer's protocols. Briefly, *sso0469* was amplified by PCR using genomic DNA (0.55  $\mu$ g) from *S. solfataricus* as template according to standard procedures (48). The sequences of the forward and reverse primers were, respectively, 5'ACAGAGAATTTAGAAGTTGGATTGAGA-3' and 5'-G



FIG. 1. Multiple proteins become phosphorylated when a partially purified detergent extract of *S. solfataricus* is incubated with  $[\gamma^{-32}P]ATP$ . The DE-52 fraction, containing 75 µg of protein, was incubated for 60 min at 65°C with  $[\gamma^{-32}P]ATP$  as described in Materials and Methods. Incubation was terminated by the addition of ice-cold acetone. The precipitated proteins were collected by centrifugation, washed, and resolved from one another by two-dimensional electrophoresis. Shown is the Coomassie-stained gel (left), with the positions and  $M_r$  of molecular weight standards to the left and the pH of the isoelectric focusing gel across the top, along with an autoradiogram thereof (right). The arrow indicates the polypeptide of apparent  $M_r \approx 67$  and pI  $\approx 5.7$  that was selected for further analysis by mass peptide profiling.

CTGATCAAAATTTGGAAGAAATAG-3'. The presence of a potential transcriptional stop codon was noted immediately upstream of the predicted initiation codon, i.e., -6 to -3. To preclude any interference with recombinant protein expression, a second round of PCR amplification was performed using 15 ng of the initial PCR product as template and a new forward primer, 5'-ATGA TGGAAAGTATTTTTGAAGTAG-3', to produce a shorter PCR product lacking the nucleotides of concern. This second PCR product was then ligated into vector PCR T7/NT TOPO, which adds oligonucleotides encoding an N-terminal extension containing a hexahistidine sequence and recognition epitope for the anti-Xpress antibody. The resulting plasmid was used to transform *Escherichia coli* strain TOPO 10 F'. The transformed cells were cultured overnight on Luria-Bertani medium containing 0.1 mg of ampicillin/ml, and the plasmid was isolated therefrom. DNA sequence analysis of the cloned DNA was performed to verify the fidelity of PCR amplification.

rSsoPK3, the recombinant protein product of *sso0469*, was expressed in *E. coli* cells by using standard procedures (48). Cells were harvested by centrifugation and stored at  $-20^{\circ}$ C until needed. The cell pellet from a 200-ml culture was thawed and resuspended in 4 ml of 50 mM morpholinepropanesulfonic acid (MOPS), pH 7.0, containing 0.1 mg of lysozyme/ml, 5 µg of RNase A/ml, 2 U of DNase I/ml, and 1 mM phenylmethylsulfonyl fluoride and incubated on ice for 10 min. The cells were then lysed by sonication using three bursts, each of 30-s duration, at 30% power of a Heat Systems-Ultrasonics model W140 sonifier cell disruptor equipped with a microprobe. The lysate was centrifuged for 15 min at 3,000 × g at a temperature of 4°C, and the supernatant liquid was discarded.

The pellet was resuspended in 50 mM MOPS, pH 7.0, containing 5 M urea by passing several times through a pipette and then centrifuged for 15 min at 3,000  $\times$  g at 4°C. The supernatant liquid was purified by metal chelate chromatography using established procedures (48), with the exception that Triton X-100, 0.3% (vol/vol), was included in all solutions.

**Site-directed mutagenesis.** Site-directed mutagenesis of *sso0469* was performed using a Gene Editor in vitro site-directed mutagenesis system (Promega) according to the manufacturer's instructions using plasmids containing the cloned gene as template. Primers used for mutagenesis included 5'-CCTACCC GACACTGCAGTTGTAATAATTTGAC-3' (Lys<sub>215</sub> to Ala), 5'-GATATCATAG CTGACTCTATTAATAATTTATTGGATGAAATAGAA-3' (Asp<sub>349</sub> to Ala), and 5'-GAAACTGAAGCTTATGATATAG-3' (Asp<sub>344</sub> to Ala).

Assay of protein kinase activity. Protein kinase activity was routinely assayed in solution by the filter paper method. Briefly, rSsoPK3 was incubated at 35°C in a volume of 100 µl of 20 mM MES, pH 6.5, containing 50 µM ATP (300 µCi of  $[\gamma^{-32}P]$ ATP/ml), 5 mM MnCl<sub>2</sub>, 2 mM DTT, 0.3% (vol/vol) Triton X-100, and a phospho-acceptor substrate such as casein, usually at a concentration of 1 mg/ml. Inhibitors such as tamoxifen that do not readily dissolve in water were delivered as 10-fold-concentrated solutions in ethanol (ethanol alone had no effect on enzyme activity.). Reactions were initiated by addition of ATP. Following incubation at 35°C for periods of up to 60 min, reactions were terminated by spotting a 30-µl portion of each reaction mixture onto a 2- by 2-cm square of Whatman 3MM paper that was immediately immersed in 10% (wt/vol) trichloroacetic acid containing 4% (wt/vol) sodium pyrophosphate (10). After gently stirring for 20 min, filter papers were transferred to a solution of 5% (wt/vol) trichloroacetic acid containing 2% (wt/vol) sodium pyrophosphate and gently stirred for 20 min. After repeating the last-named step three times more, the filter paper squares were dried and the quantity of [<sup>32</sup>P]phosphate immobilized on the filter paper was determined by liquid scintillation counting in 1 ml of ScintiSafe Plus 50% (Fisher).

Phosphorylation of rSsoPK3 by the DE-52 fraction in vitro. Portions (25  $\mu$ g) of rSsoPK3 were loaded onto an SDS–10% (wt/vol) polyacrylamide gel and resolved by electrophoresis under standard conditions. The protein was then transferred to an Immobilon P membrane using established procedures (35). The membrane was washed briefly in methanol and air dried. The membrane was divided into sections corresponding to the lanes of the SDS-polyacrylamide gel. Each section was incubated for 2 h at 65°C in 10 ml of 20 mM MES, pH 6.5, containing 0.1% (vol/vol) Triton X-100, 15 mM octyl glucoside, 2 mM DTT, 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 50  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (450  $\mu$ Ci) and, where indicated, 750  $\mu$ g of the DE-52 fraction.

## RESULTS

Labeling of potential archaeal phosphoproteins with [<sup>32</sup>P] phosphate. S. solfataricus P1 contains a membrane-associated protein kinase, SsoPK1, that can be solubilized with nonionic detergents and enriched ≈10-fold by ion-exchange chromatography (33). In an effort to identify potential protein substrates for SsoPK1, this partially purified detergent extract, referred to as the DE-52 fraction, was desalted and then incubated with  $[\gamma$ -<sup>32</sup>P]ATP, Mn<sup>2+</sup>, and Mg<sup>2+</sup>. The individual protein components of the mixture were subsequently resolved by SDS-PAGE and two-dimensional electrophoresis. As seen in Fig. 1, at least a dozen radioactive species were visible when a twodimensional gel was analyzed by autoradiography. In some areas of the gel the pattern of phosphoproteins observed, i.e., sets of closely spaced spots nearly identical in  $M_r$  that differed in pI by small regular intervals, was suggestive of the presence of differentially modified forms of a single polypeptide. While shortening the period of incubation from 60 to 5 min resulted in some decrease in the gross level of protein-bound <sup>32</sup>P ra-

TABLE 1. Phosphoamino acid content of  ${}^{32}P$ -labeled polypeptides from the membrane fraction of *S. solfataricus<sup>a</sup>* 

Polypeptide		Dhamhaamina aaid	
Mass (kDa)	pI	r nosphoamino acid	
105	6.1	P-Thr	
71	5.3	_	
70	5.4	P-Thr	
69	5.5	P-Thr	
68	5.6	P-Thr	
67	5.7	P-Thr	

<sup>*a*</sup> The DE-52 fraction, containing 75 µg of protein, was incubated for 60 min at 65°C with  $[\gamma^{-32}P]$ ATP as described in Materials and Methods and subjected to two-dimensional electrophoresis (Fig. 1). Selected sections of the gel containing the indicated <sup>32</sup>P-labeled polypeptides were then analyzed for phosphoamino acid content by acid hydrolysis and thin-layer electrophoresis as described by Kamps and Sefton (25). Abbreviation: P-Thr, phosphothreonine. For samples in which only inorganic phosphate was present in the acid hydrolysate, a dash (—) is used to indicate that no phosphoamino acid could be detected.

dioactivity, the population of proteins that were labeled was not perceptibly altered. Phosphoamino acid analysis of several of the more prominent phosphopolypeptides indicated that many contained phosphothreonine (Table 1), consistent with phosphorylation by SsoPK1, which displayed a strong preference for phosphorylating threonine residues in vitro (33).

Identification of one of the phosphoproteins as the product of ORF sso0469. One of the more prominent of the phosphothreonine-containing polypeptides on the gel (Fig. 1), with an apparent  $M_r$  of  $\approx 67$  kDa and a pI of  $\approx 5.7$ , was selected for further examination. The section of the gel containing this polypeptide was excised, washed, and then incubated with trypsin. The resulting peptides were extracted from the polyacrylamide matrix, and their masses were determined by MALDI-TOF mass spectrometry. Eleven peaks were detected in the mass spectrum. The estimated masses of five of these, assuming that each bore a net charge of  $\pm 1$ , corresponded within  $\pm 1$ atomic mass units to the calculated values for potential tryptic peptides derived from the deduced protein product of ORF sso0469 from S. solfataricus (Table 2). Although the predicted protein product of ORF sso0469, SsoPK3, could not account for every peak in the mass spectrum, its calculated  $M_r$  of 66,755 agreed closely with that estimated for the phosphothreoninecontaining polypeptide by SDS-PAGE. The empirically determined pI of the polypeptide was somewhat more acidic than that calculated for SsoPK3, 6.2. However, the observed deviation was consistent in both direction and magnitude with covalent modification by one or more phosphoryl groups (16).

In our experience, it is not uncommon that only a portion of the peaks in the mass spectrum derived from an in situ tryptic digestion of what appeared to be a single polypeptide spot or band from an electrophoretic gel can be traced to a single ORF (43). The extreme sensitivity of contemporary mass spectrometers renders them capable of detecting peptides from other, relatively minor species that might be present in the section of gel that was analyzed. Moreover, any chemical or other modification of an amino acid residue, e.g., oxidation, deamidation, and phosphorylation, will confound mass peptide profiling. Another potentially confounding factor is that the strain of *S. solfataricus* used for the initial proteomic work, *S. solfataricus* P1, differs from that whose genome was utilized, *S. solfataricus* 

TABLE 2. Mass peptide profiling of phosphothreonine-containing protein with a mass of  $\approx$ 67 kDa and pI of  $\approx$ 5.7 from *S. solfataricus*<sup>4</sup>

Peptide mass (AMU)	Peptide sequence	Calculated mass (AMU)	Difference (AMU)
537.8	$NA^b$		
538.1	NA		
559.8	NA		
568.1	NA		
581.0	NA		
581.7	DAHLK <sub>369</sub>	582.3	-0.6
633.0	NEIMK <sub>95</sub>	633.3	-0.3
662.1	QTSDGR <sub>27</sub>	662.3	-0.2
680.3	GTISFR <sub>33</sub>	679.4	+0.9
690.3	NA	NA	NA
877.8	LLRHGRK <sub>487</sub>	878.6	-0.8

<sup>*a*</sup> The portion of the two-dimensional gel shown in Fig. 1 containing the phosphoprotein indicated by the arrow was excised and incubated with trypsin as described in Materials and Methods. The resulting peptides were extracted from the gel matrix and analyzed by MALDI-TOF mass spectrometry. The spectrum obtained was used to search the archaeal protein library of the Profound program. Listed here are the masses of each peptide, as determined assuming each bears a net charge of +1, and the DNA-derived amino acid sequence of and calculated molecular masses of potential matching peptides from the predicted product of ORF *sso0469* from *S. solfataricus* P2 (47).

<sup>b</sup> NA, not applicable. The DNA-derived amino acid sequence of SsoPK3 does not predict that a monocationic peptide of this mass will be produced upon cleavage of the protein by trypsin.

P2. While these strains were originally isolated from adjacent volcanic hot springs (65), recent studies reveal that many populations of hyperthermophilic archaeons have been subject to an unexpectedly high degree of recent evolutionary divergence following separation by physical or geographic barriers (62). As even highly conservative amino acid changes confound mass peptide profiling, such divergence might also provide a source of extraneous mass species.

**DE-52 fraction phosphorylates the recombinant protein product of ORF** *sso0469* **in vitro.** In order to ascertain whether SsoPK3 was in fact the phosphoprotein observed on two-dimensional gels, ORF *sso0469* was amplified by PCR and cloned into an expression vector encoding an N-terminal fusion domain that contained a hexahistidine sequence. The recombinant protein (rSsoPK3) formed inclusion bodies in *E. coli* that could be dispersed using buffers containing 5 M urea. Following dispersal into urea, rSsoPK3 could be maintained in a soluble, active state using nonionic detergents such as Triton X-100 or octyl glucoside. Following solubilization, rSsoPK3 was purified to apparent electrophoretic homogeneity by metal-chelate chromatography.

rSsoPK3 tended to precipitate when heated to 65°C or more, so in order to test whether the DE-52 fraction was capable of phosphorylating this protein at near-physiologic temperatures, it was decided to immobilize the protein on a membrane. Therefore, samples of rSsoPK3 were resolved by SDS-PAGE and blotted onto an Immobilon P membrane, and sections of the membrane corresponding to the individual lanes of the gel were incubated with [ $\gamma$ -<sup>32</sup>P]ATP in the presence or absence of the DE-52 fraction. As can be seen in Fig. 2, membraneimmobilized rSsoPK3 did become phosphorylated in the presence of the DE-52 fraction. Phosphorylation took place predominantly on threonine. While these observations do not represent definitive proof, the DE-52 fraction-dependent phosphorylation of the immobilized protein on threonine was



FIG. 2. The DE-52 fraction phosphorylates rSsoPK3 in vitro. Portions (25 µg each) of rSsoPK3 were applied to an SDS-polyacrylamide gel, electrophoresed, and then blotted onto an Immobilon P membrane. Sections of the membrane corresponding to each lane of the gel were excised and incubated for 2 h at 65°C with  $[\gamma$ -<sup>32</sup>P]ATP in the absence (lane 1) or presence (lane 2) of the DE-52 fraction. Shown are the electronic autoradiograms of the two membrane segments containing rSsoPK3. At right are the molecular mass and positions of the protein standards as well as that of rSsoPK3. The additions present in each incubation are indicated below the autoradiograms, as well as the results of any phosphoamino acid analyses performed. Abbreviations: PAA, phosphoamino acid; n.a., not applicable; +++, >50 cpm of the indicated [32P]phosphoamino acid was detected upon phosphoamino acid analysis of an acid hydrolysate; +/-, <10 cpm of the indicated [32P]phosphoamino acid was detected upon phosphoamino acid analysis of an acid hydrolysate.

consistent with our initial identification of SsoPK3 as the phosphoprotein of  $M_{\rm r} \approx 67$  kDa, pI 5.7 on the two-dimensional gel shown in Fig. 1.

**rSsoPK3 has protein kinase activity.** Computer searches offered little insight into the possible function of SsoPK3. However, manual inspection of its DNA-derived amino acid sequence indicated the presence of weak, but plausible, candidates for the functionally essential amino acid residues that are conserved among the members of the so-called eukaryotic protein kinase paradigm (20, 60). These included the univer-

sally conserved glycine (subdomain I), lysine (subdomain II), and glutamate residues (subdomain III) of the ATP-binding domain, the universally conserved Asp-Xaa<sub>4</sub>-Asn sequence of the catalytic loop (subdomain VIb), the universally conserved aspartate (subdomain VII) that participates in binding the divalent metal ion cofactor, and the highly conserved aspartate (subdomain IX) and arginine (subdomain XI) that form a salt bridge stabilizing the conformation of the C-terminal catalytic peptide-substrate binding domain (Fig. 3). The presumed subdomain VIb region did not, however, contain either the lysine residue that is generally indicative of specificity for serine and threonine residues or the arginine that correlates with selectivity for tyrosine (21). It should be noted, however, that functionally competent eukaryotic protein kinases lacking either of these basic amino acids also have been characterized from both yeast (3, 12, 13, 58) and bacteria (64). These latter protein kinases belong to subfamilies whose line of descent and development diverged from that of the prototypical eukaryotic protein kinases that predominate in eucaryal organisms (32).

The putative catalytic domain of SsoPK3 occupied the entire central third and a substantial portion of the C-terminal third of the 582-residue polypeptide. The  $\approx 180$  N-terminal amino acids preceding the putative catalytic domain exhibited no obvious homology with other proteins, nor were any regions resembling well-characterized primary sequence motifs such as transmembrane helices apparent. The  $\approx 70$  amino acids at the C-terminal end of the protein did, however, contain a potential leucine zipper motif (Fig. 2). Leucine zippers are protein-protein interaction domains found in both eukaryotic (61) and bacterial (37) proteins that bind to complementary leucine zipper sequences on other polypeptides (2).

rSsoPK3 proved capable of catalyzing the transfer of a phosphoryl group from  $[\gamma^{-32}P]$ ATP to a number of exogenous proteins in vitro, including casein, BSA, myelin basic protein, and reduced carboxyamidomethylated and maleylated lysozyme (Table 3). It would not, however, phosphorylate the tyrosinerich copolymers poly(Glu-Tyr) or poly(Glu<sub>4</sub>-Tyr), a pair of commonly utilized substrates for the detection and assay of protein-tyrosine kinases. It should be noted, however, that these experiments were performed at 35°C rather than at the growth temperature for S. solfataricus, i.e., 65 to 80°C, because the majority of these eukaryotic proteins denature at temperatures above 45 to 55°C. Mutagenic alteration of three amino acid residues predicted to be essential for catalysis, the lysine in subdomain II of the ATP binding domain (Lys<sub>215</sub>), the conserved aspartate in the catalytic loop (Asp<sub>349</sub>), or the aspartate predicted to bind the metal ion cofactor  $(Asp_{394})$ , all resulted in either the complete abolition or a marked diminution of catalytic activity (Table 4).

Analysis of the exogenous proteins that were most efficiently phosphorylated by rSsoPK3 in vitro revealed that each had been phosphorylated on serine or, in the case of casein, on both serine and threonine residues (Table 3). No phosphotransferase activity could be detected when  $[\gamma^{-32}P]$ GTP was used as phospho-donor substrate in place of  $[\gamma^{-32}P]$ ATP (data not shown), or when Mg<sup>2+</sup>, Ni<sup>2+</sup>, or Zn<sup>2+</sup> was substituted for Mn<sup>2+</sup> as cofactor (Fig. 4). Low but detectable activity, approximately one-eighth that with Mn<sup>2+</sup>, was observed when Ca<sup>2+</sup> was tested as a cofactor, however. Repeated attempts to determine whether rSsoPK3 itself became phosphorylated when

MMESIFEVEE	GKLREAKIIT	RQTSDGRGTI	<u>SFR</u> NYIVEFP	FSLKDKLGIG	KLLAVNTIKE	60
NYYLILEVAD	IIPMHYGMIN	LDSTIPKEIR	<u>NEIMK</u> KVSES	WYSNDEKEIW	IDSITYPLGY	120
ILEINSNNIQ	FKKGYFPPLL	GSSVKILNKK	AYASFVCANS	NVSLGNILHE	QLSLDINLEK	180
AIKYHLGIFA	FTGS <b>G</b> KSNLA Ogx <b>G</b> xogxv Subdomain	SLIARKVLDN I	LPDT <b>K</b> VVIFD oao <b>K</b> xo Subdomain	VSMEYAILLL II	DKLLEVPSRV Exoo Subdomain II	240 []
ISLDRVPPNP	ADASRKFLRS	HVIPDDIIDI	RDKIKKSAEI	LHQNGKMKQL	YVPPEGFTYL oo Sub-	300 -
TVADLIDLVK 000*00 domain V	K <u>QIEDK</u> YTAI OX Si	SQKPLLYTFL x*oxx+oxoo ubdomain VIa	SKLDNFMRER h	KLTVDDII <b>D</b> D oohr <b>D</b> o Subdom	SIN <b>N</b> LLDEIE k+x <b>N</b> oo ain VIb	360
NLGK <u>DAHLK</u> E	NSSLFTFISG	IRAYISLGIR o Su	ETE <b>D</b> YDIENL ko+ <b>D</b> fgo+ bdomain VII	AIEILDSSRD	SPRLFILELP g+xxox Subdomain	420 1
NLEEGRQVVA +pE00 VIII	TVINQIYNRR	KRMYS <b>D</b> NPKV <b>D</b> 00+0 Subdor	LFIIDEAQEF gooooxo main IX	IPYDTKQKDK	SEASSTAIEK ooxx Subdo-	480 -
<u>LLRHGRK</u> YHL o main X	HSLISTQRLA	YLNTNALQQL OOX SI	HSYFISTLP <b>R</b> xooxxxxxx <b>R</b> ıbdomain XI	PYDRQLLAET x+	FGISDMLLDK Leu-	540
TLELEPGQWL cine Zipp	LVSFKSALPH er	DVPVFFAAEN	NLDLLKDRIN	KL 582		

FIG. 3. The predicted protein product of ORF *sso0469* contains sequence features characteristic of eukaryotic protein kinases. Shown is the DNA-derived amino acid sequence of SsoPK3, the predicted protein product of ORF *sso0469*. Sequence features identified by mass peptide profiling (Table 1) are underlined. Regions exhibiting potential homology, as determined by eye, to the most highly conserved subdomains of the eukaryotic protein kinase paradigm are indicated by the presence of the consensus sequences for each of these subdomains, as described in Hanks and Hunter (20), immediately below. Universal, functionally essential residues are highlighted in bold type. Other symbols used: lowercase letters, highly conserved amino acid residues; o, positions conserving nonpolar residues; #, positions conserving charged residues; \*, positions conserving small residues with near-neutral polarity; x, positions showing no clear pattern of conservation. The amino acid residues that form the potential leucine zipper domain are italicized.

incubated with  $[\gamma^{-32}P]$ ATP at temperatures ranging from 35 to 65°C both in solution and in gel following SDS-PAGE consistently yielded negative results. While this behavior suggests that the enzyme does not autophosphorylate, the tendency of

TABLE 3. Phosphorylation of exogenous proteins and peptides by rSsoPK3 in vitro<sup>*a*</sup>

Substrate	Activity (nmol of <sup>32</sup> P/min mg)	Phosphoamino acid(s)	
Casein	$2.214 \pm 0.135$	P-Ser, P-Thr	
BSA	$0.745 \pm 0.021$	P-Ser	
Myelin basic protein	$0.721 \pm 0.055$	P-Ser	
Histone H4	$0.056 \pm 0.008$	Not determined	
RCM-lysozyme	$0.678 \pm 0.081$	P-Ser	
Poly(Glu-Tyr)	< 0.005	Not determined	
Poly(Glu <sub>4</sub> -Tyr)	< 0.005	Not determined	

 $^a$  rSsoPK3 (0.15 µg) was assayed for protein kinase activity using the following potential phospho-acceptor substrates, each at a concentration of 1 mg/ml, at 35°C for 30 min. Shown are the averages of triplicate determinations  $\pm$  the standard error. Where indicated, a portion of each phosphorylated substrate was isolated by SDS-PAGE and then analyzed to determine the type of amino acid residue(s) that had become phosphorylated. For further details, see Materials and Methods.

the recombinant protein to precipitate at or near 65°C may have masked potential autophosphorylation events.

A variety of compounds that act as active site-directed inhibitors of eucaryal representatives of the eukaryotic protein kinase superfamily proved ineffective at slowing the rate at which rSsoPK3 phosphorylated casein (Table 5). These included PKI, a highly specific inhibitor of the cyclic AMP (cAMP)-dependent protein kinase that targets its peptide substrate binding site (8, 46), as well as several compounds that target the ATP-substrate binding sites of eukaryotic protein kinases with various degrees of selectivity: staurosporine (38), genistein (1), M9 (40), and H7 (40). While the aforementioned compounds produced no detectable inhibition at even the highest concentrations that we tested, tamoxifen, a compound that inhibits protein kinase C (39, 59) and a variety of calmodulin-dependent enzymes such as cardiac myosin light chain kinase (59) and a cAMP-phosphodiesterase (30), did inhibit the protein kinase activity of rSsoPK3 with an apparent 50% inhibitory concentration (IC<sub>50</sub>) of 0.5 mM. Intriguingly, tamoxifen also inhibited the activity of SsoPK2, another faintly eukaryotic protein kinase encoded by the genome of S. solfataricus (35).

TABLE 4. Catalytic activity of mutagenically altered forms of rSsoPK3<sup>a</sup>

Substitution	Corresponding residue in cAPK	Catalytic activity (% rSsoPK3)
K <sub>215</sub> to A	Lys <sub>72</sub> of subdomain II (contacts $\alpha$ - and $\beta$ -phosphates of ATP)	<1
$D_{349}$ to A	Asp <sub>166</sub> of subdomain VIb (catalytic loop)	<1
D <sub>394</sub> to A	Asp <sub>184</sub> of subdomain VII (ligand to the Mg <sup>2+</sup> that coordinates $\alpha$ - and $\beta$ -phosphates of ATP)	8

<sup>*a*</sup> Listed below are specific amino acid substitutions introduced into rSsoPK3 by site-directed mutagenesis and the catalytic activity of the mutagenically altered proteins, as measured under standard conditions using casein as phospho-acceptor substrate, relative to that of the unaltered enzyme. The numbering and assignment of functional properties to residues in the catalytic subunit of the cAMP-dependent protein kinase (cAPK) are according to Taylor et al. (60).

SsoPK3 became phosphorylated when the DE-52 fraction was incubated with  $[\gamma^{-3^2}P]$ GTP or  $[\beta^{-3^2}P]$ GDP. Since rSsoPK3 apparently was incapable of catalyzing its own phosphorylation, the phosphorylation event that occurred when the DE-52 fraction was incubated with  $[\gamma^{-3^2}P]$ ATP presumably was catalyzed by a second, exogenous protein kinase. A probable candidate for this second protein kinase would appear to be SsoPK1, inasmuch as (i) the DE-52 fraction was specifically enriched for the activity of this protein kinase and (ii) SsoPK3 was phosphorylated on threonine, consistent with the strong threonine preference of SsoPK1 observed previously (33).

In addition to its selectivity for threonine as the phosphoacceptor amino acid, SsoPK1 displayed another very unusual property relative to the vast majority of previously characterized protein kinases—the ability to use  $[\gamma^{-32}P]$ GTP or  $[\beta^{-32}P]$ GDP as alternative phospho-donor substrates in vitro (4, 33). We attempted to exploit this attribute in order to evaluate the possibility that SsoPK3 had been phosphorylated by SsoPK1 in vitro. When the DE-52 fraction was incubated with  $[\gamma^{-32}P]$ GTP, Mn<sup>2+</sup>, and Mg<sup>2+</sup> for periods of either 5 or 60 min, the pattern of phosphorylated species observed on a twodimensional gel was nearly identical to that elicited with  $[\gamma^{-32}P]$ ATP (data not shown). Next,  $[\beta^{-32}P]$ GDP was tested as a phospho-donor substrate. Unfortunately, as we had utilized all of the DE-52 fraction used for the experiment described in



FIG. 4. Metal ion preference of rSsoPK3. The protein kinase activity of rSsoPK3 (50 ng) was assayed as described in Materials and Methods using casein as phospho-acceptor substrate, with the exception that the indicated compounds, each at a concentration of 5 mM, were substituted for the normal divalent metal ion cofactor,  $Mn^{2+}$ . Shown are the results of duplicate determinations  $\pm$  the standard error.

Fig. 1, a second batch of the DE-52 fraction prepared from a fresh culture of S. solfataricus was needed. As can be seen in Fig. 5, the polypeptide composition of the new preparation of the DE-52 fraction was somewhat simpler than that used in the experiment portrayed in Fig. 1. Also, the degree of radiolabeling was much lower than that obtained in Fig. 1, consistent with the markedly lower efficiency of  $[\beta^{-32}P]GDP$  as a phosphodonor substrate for SsoPK1 relative to purine nucleotide triphosphates (33). The longer exposure time required to visualize the phosphopeptides present therefore lent greater apparent prominence to phosphorylated species not visibly associated with Coomassie blue-stained polypeptides, most notably the boot-shaped area on the left side of the autoradiogram. However, a similar "boot" also became visible when the gel from Fig. 1 was exposed for longer periods; in fact, the "foot" of the boot could already be seen. However, the experiment in Fig. 5 also revealed that several radiolabeled polypeptides, including one whose apparent  $M_r$  and pI matched those of SsoPK3, became phosphorylated when the partially purified membrane extract was incubated with  $[\beta^{-32}P]GDP$ .

## DISCUSSION

Incubation of the DE-52 fraction, a detergent extract of the membrane fraction of *S. solfataricus* P1 that had been enriched for the activity of a threonine-preferring protein kinase by ion-exchange chromatography, with  $[\gamma^{-32}P]$ ATP led to the appearance of about a dozen phosphorylated polypeptides on polyacrylamide gels. Mass peptide profiling of one of the radiolabeled polypeptides, that with an  $M_r$  of  $\approx 67$  kDa and a pI of  $\approx 5.7$ , indicated that it was the product of ORF *sso0469*. Tests with the recombinant protein product of ORF *sso0469*, rSsoPK3, indicated that the DE-52 fraction could catalyze the former's phosphorylation in vitro. Moreover, it did so on predominantly threonine residues, consistent with our initial identification of SsoPK3 as a phosphoprotein.

Computer analysis of the DNA-derived amino acid sequence of the predicted protein product of ORF *sso0469*, SsoPK3, revealed the presence of a potential leucine zipper near its C terminus. However, evidence of global homology with proteins of known function was not forthcoming. Visual inspection subsequently revealed the presence of sequence features faintly reminiscent of the eukaryotic protein kinase paradigm. While several ORFs potentially encoding eukaryote-like protein kinases have been identified in members of *Archaea* (32, 49, 52), to date only one of these has been demonstrated to possess the catalytic potential implied from their sequence (35). We therefore asked whether the recombinant protein product of ORF *sso0469*, rSsoPK3, behaved as a protein kinase.

TABLE 5. General properties of	the known inhibitors of eukar	votic protein kinases that we	re tested against rSsoPK3 <sup>a</sup>
1 1		2 1	0

Inhibitor (reference)	Known target(s)	Mode of action	IC <sub>50</sub> (µM) range	Max. concn (µM)
Staurosporine (38)	Broad specificity	ATP binding site	0.001–3 µm	20
Genistein (1)	PTKs	ATP binding site	1-30	2,000
ML-9 (40)	cAPK, PKC, MLCK	ATP binding site	4–54	80
H-7 (40)	Broad specificity	ATP binding site	3-780	2,000
PKI-peptide (7, 46)	cAPK specific	Peptide binding site	0.0023	0.9
Tamoxifen (30, 39)	PKC, CaM kinases	Antagonizes activators	2–100 μM	2,500

<sup>*a*</sup> Listed are below are the names of several known inhibitors of eukaryotic protein kinases, the enzymes they are known to target, their mode of action, the range of  $IC_{50}$  values against established targets, and the highest concentration used when testing their efficacy versus rSsoPK3. Abbreviations: cAPK, cAMP-dependent protein kinase; PKC, protein kinase C; PTK, protein-tyrosine kinase; CaM kinase, calmodulin-dependent protein kinases; MLCK, myosin light-chain kinase.

Our analyses revealed that rSsoPK3 was capable of phosphorylating several exogenous, albeit nonphysiological, proteins in vitro. The phosphorylation of these proteins took place almost exclusively on serine. rSsoPK3 appeared to be ATP specific, as no phosphotransferase activity was detected when  $[\gamma^{-3^2}P]$ GTP was tested as a potential phospho-donor substrate. Mn<sup>2+</sup> was the preferred cofactor, although weak activity also was observed in the presence of Ca<sup>2+</sup>. rSsoPK3 displayed no capacity to autophosphorylate. Taken together, these behaviors suggest that SsoPK3 was not the source of the membraneassociated protein kinase activity previously characterized in *S. solfataricus*, as the latter enzyme displayed a strong preference for threonine over serine as its phospho-acceptor amino acid, broad nucleotide specificity, and the ability to autophosphorylate in vitro. However, at this point in time, we cannot rule out the possibility that the differences in behavior between rSsoPK3 and SsoPK1 might represent some unidentified consequence of the recombinant expression of the former, as the  $M_r$  and pI of the two proteins appear to be quite similar.

Site-directed mutagenesis was used to individually alter



FIG. 5. SsoPK3 becomes phosphorylated when a partially purified detergent extract of *S. solfataricus* is incubated with  $[\beta^{-32}P]$ GDP. The DE-52 fraction, containing 60 µg of protein, was incubated for 5 min at 65°C with either  $[\gamma^{-32}P]$ ATP (left) or  $[\beta^{-32}P]$ GDP (right) as described in Materials and Methods. Incubation was terminated by the addition of ice-cold acetone. The precipitated proteins were collected by centrifugation, washed, and resolved from one another by two-dimensional electrophoresis. Shown are the Coomassie-stained gels (top), with the positions and masses of molecular weight standards to the left and the pH of the isoelectric focusing gel across the top, along with electronic autoradiograms thereof (bottom). The arrow indicates the position of SsoPK3.

three residues predicted to correspond to functionally critical amino acids that are universally conserved among established members of this enzyme superfamily. As predicted, substitution of the putative conserved lysine of subdomain II, Lys<sub>215</sub>, with Ala abolished catalytic activity, as did replacement of the putative conserved aspartate of subdomain VIb, Asp<sub>349</sub>, by Ala. Substitution of the putative conserved aspartate of subdomain VII, Asp<sub>394</sub>, by Ala produced an enzyme of greatly reduced catalytic efficiency, ≈8% of wild type. In eukaryotic protein kinases, the latter substitution would be expected to not just reduce but completely eliminate catalytic activity (60). However, the presence of an adjacent glutamate residue, Glu<sub>393</sub>, which is not present in any of the well-studied eucaryal members of this enzyme family (20), may account for the retention of weak residual catalytic activity by this mutationally altered form of rSsoPK3.

Since rSsoPK3 appears to be incapable of phosphorylating itself, it is logical to assume that it was phosphorylated by another protein kinase activity present in the DE-52 fraction. Two lines of evidence suggest that the protein kinase responsible was SsoPK1, the membrane-associated protein kinase activity characterized previously. First, SsoPK3 was phosphorylated on threonine. Second, SsoPK3 was phosphorylated when either  $[\gamma^{-32}P]$ GTP or, more importantly,  $[\gamma^{-32}P]$ GDP was substituted for  $[\gamma^{-32}P]$ ATP as phospho-donor substrate. However, a definitive determination of whether SsoPK1 was responsible for phosphorylating SsoPK3 in vitro, and whether this phosphorylation event forms part of a signal transduction cascade in the intact organism, must await the identification of the gene that encodes the former.

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