PspE (phage-shock protein E) of *Escherichia coli* is a rhodanese

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Abstract The *psp* (phage-shock protein) operon of *Escherichia coli* is induced when the bacteria are infected by filamentous phage and under several other stress conditions. The physiological role of the individual Psp proteins is still not known. We demonstrate here that the last gene of the operon, *pspE*, encodes a thiosulfate:cyanide sulfurtransferase (EC 2.8.1.1; rhodanese). Kinetic analysis revealed that catalysis occurs via a double displacement mechanism as described for other rhodaneses. The K_ms for SSO_3^{2-} and CN^- were 4.6 and 27 mM, respectively. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: PspE; Phage-shock response; Rhodanese; *Escherichia coli*

1. Introduction

Induction of the *psp* (phage-shock protein) operon was first described as a response of *Escherichia coli* upon infection with filamentous phage [1]. Genetic analysis revealed that the operon consists of four or five genes [2]. The first gene, *pspA*, is abundantly expressed upon induction. It encodes a 26 kDa protein, which is peripherally associated with the cytoplasmic membrane, although a proportion is localized in the cytoplasm. It has a negative regulatory role in expression of the operon [3]. PspB and PspC are both small integral inner membrane proteins with one membrane-spanning segment each. Both act as positive regulators of the operon. It is not clear whether the fourth open reading frame, *pspD*, indeed encodes a functional protein since its expression has never been demonstrated in vivo [4]. The *pspE* gene encodes a periplasmic protein [5] and is transcribed both as part of the operon and from its own promoter [2].

It has been demonstrated that expression of gene IV of filamentous phage leads to the induction of the *psp* operon [1]. The gene IV product is a member of the secretin family and forms large multimeric channels in the outer membrane, through which the phage is extruded [6]. Also, expression of homologs of the gene IV product, involved in type II or type III secretion in various Gram-negative bacteria, leads to induction of the expression of the *psp* operon in *E. coli* [7–9]. Furthermore, various stress conditions, including severe heat shock, osmotic shock, exposure to ethanol [1,4] and obstruction of the Sec translocon [10], which is involved in protein translocation across the cytoplasmic membrane, have been reported to induce expression of the *psp* operon. These stress conditions might all lead to the dissipation of the protonmotive force (pmf), and it has been demonstrated that expression of the *psp* operon, and more specifically of *pspA*, helps the cells to maintain the pmf under such stress conditions [5]. However, how the *psp* gene products exert this function is not clear. In this study, we focused on the possible role of the *pspE* gene product. We demonstrate that PspE is a rhodanese and discuss its possible role in the function of the *psp* operon.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *E. coli* strains used in this study are SG13009 (Qiagen), harboring plasmid pREP4, containing *lacI* and a Kan^r marker (Qiagen), and CE1224 [11].

For cloning of *pspE* behind a *tac* promoter, the *Afl*II–*Eco*RI fragment of plasmid pJP378 [5] containing *pspE* (*Afl*II-overhanging ends were blunted with Klenow fragment) was subcloned in pJF119HE [12], digested with *Sma*I and *Eco*RI. For expression of *pspE*, strain CE1224 was transformed with the resulting plasmid, designated ptacE.

To facilitate purification of PspE, a recombinant gene encoding a His-tagged PspE was constructed. The *pspE* gene without its signal sequence-encoding DNA was amplified using pJP380 [5] as the template and the primers 5′-CACTGCCCGTGGATCCCGCTGAACA-3′ (HA-032) and 5′-CTCATGGTGACCCGGGTTAACCTTTGAC-3′ (HA-033). These primers contained a *Bam*HI and a *Sma*I site, respectively (underlined). The PCR was performed with an annealing temperature of 50°C for 60 s and extension at 68°C for 60 s in 30 cycles. The resulting PCR product was cloned in PCR II TOPO (Invitrogen) according to the manufacturer's instructions, resulting in TOPO-PspE. Subsequently, TOPO-PspE was digested with *Bam*HI and *Sma*I, and the fragment containing *pspE* was ligated into the corresponding sites of pQE31 (Qiagen), behind a T5 promoter with a *lac* operator, followed by transformation of SG13009[pREP4]. The resulting plasmid was designated pQE31-PspE.

2.2. Growth media and growth conditions

Bacteria were grown at 37°C in L-broth [11], supplemented when necessary, with the antibiotics kanamycin (25 μg/ml) or ampicillin (100 μg/ml). The expression of genes behind the *tac* or T5 promoter was induced in mid-log phase cells by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Cultures were grown for 4 h after addition of IPTG and then harvested by centrifugation as described below. To evaluate the cyanide resistance of a PspE overproducer strain, CE1224 carrying plasmid ptacE or pJF119HE was grown in LB in the presence of increasing concentrations of potassium cyanide.

2.3. Protein purification

Cells from an 800 ml culture of strain SG13009[pREP4] carrying pQE31-PspE, induced with IPTG, were harvested by centrifugation (5000×*g*, 4°C, 10 min). The cells were resuspended in MTPBS buffer [13] and disintegrated by passage through a French press twice at 8000 psi. The intact cells and cell envelopes were removed by centrifugation at 5000×*g* at 4°C for 10 min and at 260 000×*g* at 4°C for 90 min, respectively, and the supernatant was loaded onto a Ni^{2+}-NTA col-

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umn equilibrated with MTPBS buffer at 4°C. The flow rate was maintained at 2 ml/min. After washing with MTPBS buffer, His-tagged PspE was eluted with MTPBS buffer containing 250 mM imidazole. After dialysis overnight against MTPBS buffer, fractions containing His-tagged PspE were stored in aliquots at −20°C. A polyclonal rabbit antiserum was raised against the purified His-tagged PspE at Eurogentec. Protein concentrations were determined by the method of Bradford [14] with bovine serum albumin as the standard.

2.4. Assay of sulfurtransferase activity

Sulfurtransferase activity of PspE was measured at 25°C essentially as described [15]. Briefly, reaction mixtures contained 100 mM Tris-acetate (pH 8.6), 6 mM cysteine, 50 mM ammonium thiosulfate, 30 mM KCN, and enzyme in a final volume of 0.5 ml. The reactions were initiated by the addition of KCN and terminated after 1 and 2 min by the addition of 0.25 ml of 15% formaldehyde. Color was developed by the addition of 0.75 ml of ferric nitrate reagent [100 g of $Fe(NO_3)_3 \cdot 9H_2O$ and 200 ml of 65% HNO_3 per 1500 ml]. Assays were clarified by centrifugation, and the absorbance at 460 nm was determined. One unit is defined as the amount of enzyme that catalyzes the production of 1 μmol of thiocyanate per minute and corresponds to an absorbance change at 460 nm of 2.8 in this system. Total cellular enzyme activities were determined by using 100 ml of whole cells that were pelleted, resuspended in 10 mM Tris–HCl (pH 8), and disintegrated by passage through a French press as described above.

2.5. Western immunoblot analysis

Cells were harvested by centrifugation and solubilized in sample buffer at 100°C for 10 min. Total cellular proteins were separated by SDS–PAGE [16] on 15% polyacrylamide gels. After blotting onto nitrocellulose filters (0.45 μm, Schleicher and Schuell) using a Mini Trans-Blot Cell (Bio-Rad Laboratories), immunoincubations were performed essentially as described [5], using polyclonal antibodies directed against PspE.

3. Results and discussion

3.1. PspE is a rhodanese

In order to identify a possible role for PspE, a PSI-BLAST search [17] was performed using PspE as the query, hoping to identify related proteins with known function. In addition to numerous bacterial proteins with unknown function, several rhodanese-related sulfurtransferases were revealed by this search, including the recently identified rhodanese GlpE of *E. coli* [15]. Consistently, based on sequence similarity, PspE was included recently in a superfamily of transfer proteins, which includes sulfurtransferases as well as tyrosine and dual-specificity phosphatases [18]. Furthermore, a search in the database Clusters of Orthologous Groups of Proteins [19] revealed that PspE belongs to the group of rhodanese-related sulfurtransferases (COG0607). Fig. 1 shows the predicted secondary structure and an alignment of PspE with the conserved regions of GlpE and two other representative members of the group of rhodaneses. A comprehensive discussion of the superfamily, along with alignments for 37 members, has been published recently [18]. These data suggest that PspE might act as a thiosulfate:cyanide sulfurtransferase (EC 2.8.1.1), an enzyme traditionally given the name rhodanese. Rhodaneses catalyze the transfer of the sulfane sulfur from thiosulfate to cyanide forming thiocyanate and sulfite. To investigate whether *pspE* indeed encodes a protein with rhodanese activity, the gene was cloned behind the *tac* promoter, and the resulting plasmid, ptacE, was introduced in strain CE1224. Enzyme assays subsequently revealed a 10-fold increase of the thiosulfate:cyanide sulfurtransferase activity as compared to the cells containing vector pJF119HE (Table 1). Western blot analysis with antibodies directed against PspE showed that the increased rhodanese activity in the ptacE-containing cells corresponded to an increase in PspE production (data not shown).

3.2. Enzymatic properties of PspE

Previously, two distinct rhodanese activities have been described for *E. coli*. One was released from cells only after sonication [20], whereas the other was accessible to substrates when intact cells are added to assay mixtures suggesting a

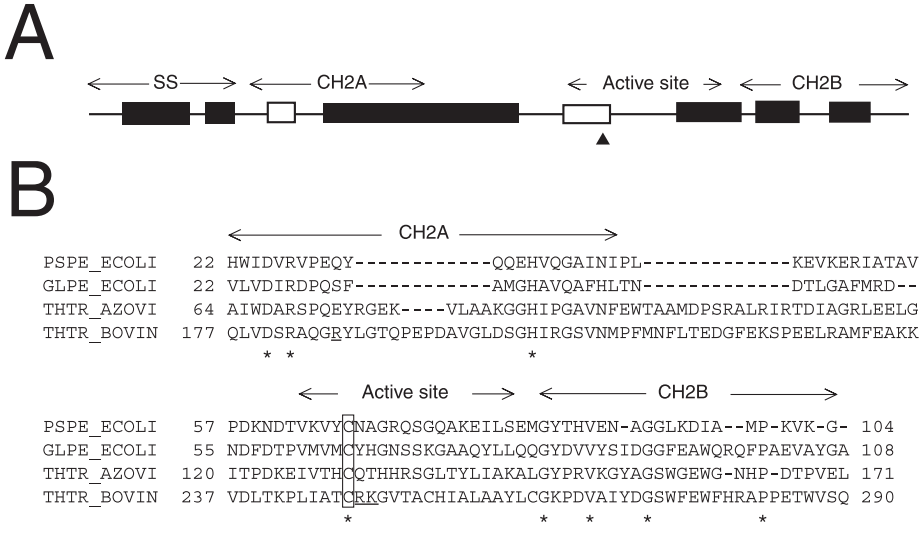

Fig. 1. Secondary structure prediction of PspE and comparison of the conserved region of PspE with other representative thiosulfate:cyanide sulfurtransferases. A: PspE is schematically outlined. Predictions of α-helical regions (black boxes) and β-sheet (white boxes) are shown. The program SSP was used for the prediction [37]. The active site cysteine (▲), signal sequence (ss) and two conserved structural motifs, designated CH2A and CH2B, are indicated. B: Alignment of the conserved regions of PspE with those of representative sulfurtransferases. The active site and the two conserved motifs, CH2A and CH2B, are indicated. The active-site cysteine is boxed, and asterisks highlight residues conserved in all four sequences. GLPE_ECOLI, *glpE*-encoded rhodanese of *E. coli* [15]; THTR_AZOVI, thiosulfate sulfurtransferase of *Azotobacter vinelandii* [38]; THTR_BOVIN, bovine rhodanese [39]. Accession numbers: PSPE_ECOLI, P23857; GLPE_ECOLI, P09390; THTR_AZOVI, P52197; and THTR_BOVIN, P00586.

Table 1
Overproduction of PspE and corresponding increase in rhodanese activity

Strain (plasmid)	Rhodanese s.a.[a] (U/mg)
CE1224 (pJF119HE)	0.06
CE1224 (ptacE)	0.6

[a]Specific activity (s.a.) determined by using crude extracts of cells disintegrated by a French press.

periplasmic localization [20,21]. The absence of a signal sequence and the enzymatic properties of GlpE [15] suggested that this enzyme does not correspond to the accessible rhodanese. Since PspE is synthesized with a signal sequence and its apparent molecular weight (~11 kDa from SDS–PAGE) is similar to the reported apparent molecular weight of accessible rhodanese (12–14 kDa), these two proteins might be identical. To compare the catalytic properties of PspE with those published for the accessible rhodanese, a His-tagged version of PspE was genetically constructed. The His-tagged PspE was expressed and a cell lysate was prepared from cells disrupted by a French press. After successive removal of unbroken cells and cell envelopes by centrifugation, the supernatant was subjected to immobilized metal ion affinity chromatography. Fractions containing His-tagged PspE were dialyzed, and the final protein preparation was more than 95% homogeneous as evaluated by Coomassie brilliant blue staining of a 15% SDS–PAGE gel (Fig. 2, lane 4). The purified His-tagged PspE had a specific activity of 800 U/mg which is two orders of magnitude higher than the value observed previously for the GlpE protein [15].

The well-characterized bovine liver rhodanese utilizes a double-displacement (ping-pong) mechanism for catalysis [22,23]. Data from activity measurements of purified His-tagged PspE with various concentrations of thiosulfate at fixed concentrations of cyanide were fitted to the equation describing this type of mechanism. Fig. 3 (top panel) shows the data and the fit obtained. Substrate inhibition by thiosulfate was observed above 100 mM, particularly at low cyanide concentrations (< 5 mM; data not shown). This type of inhibition was previously described for the GlpE [15] and the accessible rhodanese of *E. coli* [21]. The lower panel of Fig. 3 shows a secondary double reciprocal plot of the apparent maximum velocities in the top panel versus the cyanide concentration. The regression of the data to the equation describing a double-displacement mechanism yielded K_m values for thiosulfate and cyanide of 4.6 (±0.8) mM and 27 (±4) mM, respectively, in agreement with the reported K_m values of accessible rhodanese of 5 and 24 mM, respectively [21]. Therefore, it appears that the *pspE* gene encodes the previously identified accessible rhodanese, whereas GlpE or any of the other five putative *E. coli* rhodaneses that are identified in the COG0607 cluster, none of which contains a (putative) signal sequence, represents the inaccessible rhodanese.

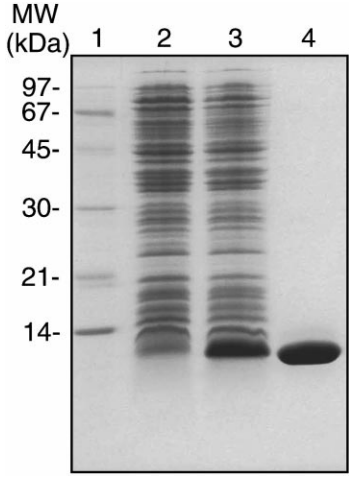

Fig. 2. SDS–PAGE gel showing overproduction and purification of His-PspE. Lane 1, molecular weight markers. Lane 2, total cellular proteins of strain SG13009 harboring pQE-31. Lane 3, total cellular proteins of SG13009 harboring pQE31-PspE. Lane 4, His-tagged PspE after purification by Ni^{2+}-NTA chromatography.

3.3. Physiological role of PspE

Although sulfurtransferases are present in many types of organisms from all three domains of life [19,24], their physiological role is still unclear. Proposed roles include cyanide detoxification [25], sulfur metabolism [26,27], and mobiliza-

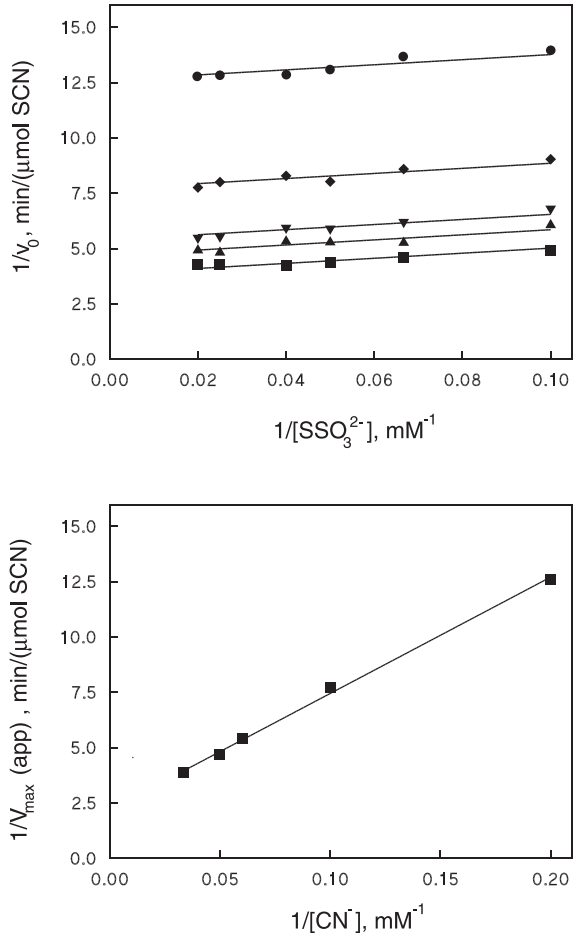

Fig. 3. Characterization of the kinetics of the thiosulfate:cyanide sulfurtransferase reaction catalyzed by PspE. Each assay, performed as described in Section 2, contained 10 µl (0.3 µg) of purified His-PspE. Upper panel: double-reciprocal plot of the rate of thiocyanate formation versus thiosulfate concentration at various fixed concentrations of cyanide: 5 mM (●), 10 mM (♦), 16.7 mM (▼), 20 mM (▲) and 30 mM (■). Lower panel: secondary double-reciprocal plot of apparent V_{max} from the data in the upper panel versus cyanide concentration. Data were fit to the equation describing a double-displacement mechanism.

tion of sulfur for the formation or restoration of iron–sulfur clusters [28–31]. The role of cyanide detoxification could be interesting in view of the function of the *psp* response. The *psp* operon is probably involved in the maintenance of the membrane potential under stress conditions [5], and the presence of cyanide can lead to de-energization of the cytoplasmic membrane by inhibition of the respiratory chain [32]. Under such conditions, PspE could be involved in the detoxification of cyanide. However, given the low affinity for cyanide ($K_m = 27$ mM), this seems unlikely to be the primary role of PspE in vivo. Indeed, overproduction of PspE did not lead to an increased resistance to cyanide when tested by growing cells in L-broth with various concentrations of cyanide (data not shown). It should be noted, however, that the *pspE* gene is present in *E. coli* and in its close relative, *Salmonella typhimurium*, but not in *Yersinia enterocolitica*, which does contain the other *psp* genes [33]. On the other hand, in the *Neisseria meningitidis* genome [34], a *pspE* homolog is present, but this organism apparently lacks homologs of the *pspA–D* genes, as we observed from a BLAST search (unpublished observation). Therefore, it could be that the function of PspE is not directly related to those of the other Psp proteins.

Alternatively, PspE could catalyze the formation or the repair of iron–sulfur centers, as was suggested previously for the accessible rhodanese [21]. The iron–sulfur clusters of succinate dehydrogenase [28] and mitochondrial NADH dehydrogenase [30] could indeed be reconstituted by bovine liver rhodanese. The products of the *hyd1* and *hyd2* genes are periplasmic [NiFe] hydrogenases [35,36], which probably have a role in hydrogen cycling during fermentative growth. PspE might be involved in the formation of the iron–sulfur clusters of these proteins and thereby in energy metabolism, consistent with the proposed role of the *psp* operon.

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