MTH1745, a protein disulfide isomerase-like protein from thermophilic archaea, *Methanothermobacter thermoautotrophicum* involving in stress response

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Abstract MTH1745 is a putative protein disulfide isomerase characterized with 151 amino acid residues and a CPAC active-site from the anaerobic archaea *Methanothermobacter thermoautotrophicum*. The potential functions of MTH1745 are not clear. In the present study, we show a crucial role of MTH1745 in protecting cells against stress which may be related to its functions as a disulfide isomerase and its chaperone properties. Using real-time polymerase chain reaction analyses, the level of MTH1745 messenger RNA (mRNA) in the thermophilic archaea *M. thermoautotrophicum* was found to be stress-induced in that it was significantly higher under low (50°C) and high (70°C) growth temperatures than under the optimal growth temperature for the organism (65°C). Additionally, the expression of MTH1745 mRNA was up-regulated by cold shock (4°C). Furthermore, the survival of MTH1745 expressing *Escherichia coli* cells was markedly higher than that of control cells in response to heat shock (51.0°C). These results indicated that MTH1745 plays an important role in the resistance of stress. By assay of

enzyme activities in vitro, MTH1745 also exhibited a chaperone function by promoting the functional folding of citrate synthase after thermodenaturation. On the other hand, MTH1745 was also shown to function as a disulfide isomerase on the refolding of denatured and reduced ribonuclease A. On the basis of its single thioredoxin domain, function as a disulfide isomerase, and its chaperone activity, we suggest that MTH1745 may be an ancient protein disulfide isomerase. These studies may provide clues to the understanding of the function of protein disulfide isomerase in archaea.

Keywords Archaea · *Methanothermobacter thermoautotrophicum* · Stress · Chaperone activity · Protein disulfide isomerase

Introduction

Archaea, one of the three major groups of life forms, is a highly diverse and abundant group including lots of “extremophiles” that thrive in harsh environments such as hot springs, salt lakes, and submarine volcanic habitats (Madigan et al. 2000; Eckburg et al. 2003). The study of stress genes in archaea began very recently compared with earlier studies in their counterparts, bacteria, and eukaryotes. Since the 1980s, the stress response in archaea has been studied, but it was only in 1991 that an archaeal stress gene (*hsp70*) was cloned and sequenced for the first time (Macario et al. 1991; Klumpp and Baumeister 1998; Macario et al. 1999). Since then, a few studies have been conducted to elucidate how and when these genes are expressed and how they respond to stress. However, many aspects of the mechanism of the capacity of such microbes to withstand stress still remain for further study.

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To understand protein folding under environmental stress is now a key challenge in biology (Gilbert 1997). Studies have shown that disulfide bonds usually contribute to the stabilization of a folded protein conformation (Creighton 1986). Many secreted proteins have disulfide bonds that are crucial for their structure or function. In Gram-negative bacteria, disulfide bond formation is located within the periplasmic space and is catalyzed by a group of enzymes belonging to the Dsb (disulfide bond formation) family. These proteins have a single thioredoxin-like active site consisting of a pair of cysteines in a CXXC motif (Missiakas and Raina 1997). In eukaryotic cells, the disulfide bond formation is located within the endoplasmic reticulum (ER) which has a high concentration of the enzymes and molecular chaperones involved in the folding and assembly of proteins. The ER-resident enzyme, protein disulfide isomerase (PDI), catalyzes thiol–disulfide exchange reactions. PDI has two thioredoxin-like active sites (CGHC) that are involved in disulfide bond formation and rearrangement reactions (Freedman et al. 1994; Gilbert 1997; Huppa and Ploegh 1998). Unexpectedly, there are large numbers of proteins with disulfide bonds in the cytoplasm of certain thermophilic and hyperthermophilic archaea (Mallick et al. 2002). These include *Pyrococcus ferodoxin* (Gorst et al. 1995), DNA polymerases from *Thermococcus gorgonarius* (Hopfner et al. 1999), and *Thermococcus* sp. (Rodriguez et al. 2000). Disulfide bonds are also present in archaeal secreted or membrane proteins (Eichler and Adams 2005). These raise the questions concerning the redox state of the archaeal cytoplasm and the nature of the proteins that are involved in disulfide bond formation in archaea (Mallick et al. 2002).

To date, little structural or functional information is available on these disulfide bond formation proteins isolated from archaea, and considering the limited information available, it is too early to assign any of them a particular physiological role. In fact, other archaeal protein disulfide oxidoreductases (PDOs) have been isolated from *Sulfolobus solfataricus* (SsPDO; Guagliardi et al. 1994), *Pyrococcus horikoshii* (PhPDO; Kashima and Ishikawa 2003), *Methanococcus jannaschii* (Mj0307; Bult et al. 1996), and *Methanobacterium thermoautotrophicum* (Mt0807, Mt0895; McFarlan et al. 1992).

Methanothermobacter thermolithotrophicus Δ H, isolated in 1971 from sewage in Urbana, Ill., is a lithoautotrophic, strictly anaerobic, thermophilic archaea that grows at the optimal temperature of 65°C (Smith et al. 1997). Recently, a whole genome analysis of *M. thermolithotrophicus* Δ H has revealed classes of small redox protein which have true thioredoxin-like activities. MTH1745, a putative protein disulfide isomerase, is a 151-residue protein, which is characterized by a single thioredoxin-like domain. However, very few studies have been directed at the understanding of

isomerase activity and its anti-stress role in the archaea. To clarify the role of MTH1745, we undertook the messenger RNA (mRNA) expression profile after different culture temperatures and after cold shock treatment. We then analyzed its reductive/isomerase activities, molecular chaperone activity, and acquired thermotolerance in *Escherichia coli*. Our data indicated that MTH1745 plays certain crucial roles in protecting against stress and exhibited both disulfide reductase and molecular chaperone functions.

Materials and methods

Culturing *M. thermoautotrophicum* Δ H at different temperature and cold shock treatment

For the cultivation of *M. thermoautotrophicum* Δ H, a modification medium was used. It was composed of (per liter deionized water): $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g; NaCl 0.6 g; K_2HPO_4 , 1.5 g; NH_4Cl , 1.5 g; sodium cysteine 0.5 g; NaHCO_3 , 1.0 g; sodium resazurin 0.001 g; trace element solutions 10 ml ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 3 g; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.5 g; NaCl 10 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.1 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g; CuSO_4 0.01 g; $\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ 0.01 g; H_3BO_3 0.01 g; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.01 g; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 0.025 g; $\text{NaSeO}_3 \cdot 6\text{H}_2\text{O}$ 0.0003 g). After adjustment to pH 7.0, 60 ml medium was prepared per 600-ml anaerobic bottle and sterilized under a strictly anaerobic H_2 and CO_2 atmosphere (80:20). *M. thermoautotrophicum* Δ H was cultured in this medium at different temperatures (50, 60, 65, 70°C) using Hungate technology. The cells were harvested until $\text{OD}_{600}=0.2$, which were in log growth phases. For cold shock treatment, *M. thermoautotrophicum* Δ H was exposed to 4°C for 2 h after culturing at 65°C.

RNA extraction

All samples were homogenized in Trizol Reagent (Invitrogen, CA, USA), and total RNA was prepared according to the manufacturer's instructions. Total RNA was quantified on a Genova UV/visible spectrophotometer at 260 nm.

Quantification of MTH1745 expressions by real-time PCR

The complimentary DNA was synthesized from 2 μg of total RNA from each sample using random decamer primer and murine moloney leukemia virus reverse transcriptase (Promega, Madison, WI, USA) in a 25 μl reaction. The expression pattern of MTH1745 was analyzed in various samples by gene-specific primers 1745F, 1745R (Table 1) using MiniOpticon™ System (Bio-Rad, USA). Polymerase chain reaction (PCR) products were detected with SYBR Green I using the iQ SYBR Green Supermix kit (Bio-Rad).

Table 1 Primers used in this work

Primer	Sequence (5'-3') ^a
1745F	AGGCTTCGAGGACAGGTAAA
1745R	GACTGCGATGAAATCCTCTG
16SF	AAACCAGGTCGTAGTTCGGAT
16SR	AAGGAGCAGGGACGTATTCA
E1745F	GGAATTCcatatgATGGAGCATGGTAAATTGGA
E1745R	CCCaagcttTACCCCAGATAGGATATGA
MTH1745-1F	CGGggtaccACACGGAGACACAGATTACCTCTCATGGAGCATGGTAAATTGGA
MTH1745-1R	CCCaagcttCTACCCCAGATAGGATATGA
ApssF	GGggtaccACACGGAGACACAGATTACCTCTCGTGAAACAAAGCACTATTG
ApssR	CATCCAATTTACCATGCTCGAATTCCGGGATCTCGGC
MTH1745-2F	GCCGAGATCCCGGAATTTCGAGCATGGTAAATTGGATG

^a Lowercase indicate restriction endonuclease recognition sequences

Each PCR reaction mix contained 12.5 µl of Supremix, 200 nM primer 1745F, 200 nM primer 1745R, and 1 µl RT-PCR products. Standard curve of MTH1745 was constructed using purified plasmids subcloned MTH1745 by tenfold serial dilutions as 1:10⁴, 1:10⁵, 1:10⁶, 1:10⁷, and 1:10⁸ along with a non-template control. Standard curve of 16S ribosomal RNA (rRNA) was constructed using purified plasmids subcloned 16S fragment amplified with the primers 16SF and 16SR (Table 1). Then, this plasmid was diluted by tenfold series as 1:10⁵, 1:10⁶, 1:10⁷, 1:10⁸, and 1:10⁹. All samples were analyzed three times. The MTH1745 gene expression profiles obtained from real-time PCR were normalized by the amount of 16S rRNA transcript to correct the differences in RNA amounts and reverse transcription reactions. Melting curve analysis and agarose gel electrophoresis was performed after real-time PCR reaction to monitor the PCR specificity.

Cloning, expression, and purification of MTH1745

A DNA fragment of MTH1745 (1595827..1596279) was PCR-amplified from the chromosomal DNA of *M. thermoautotrophicum* ΔH. The forward primer (E1745F) contained a 5'-*Nde*I site (indicated in lowercase) and reverse primer (E1745R) contained a unique *Hind*III (indicated in lowercase) with stop codon. The PCR product was digested with *Nde*I and *Hind*III and ligated with *Nde*I-plus-*Hind*III digested pET-28a, generating pET-28a-MTH1745 which encodes MTH1745 with N-terminal His6 extension.

MTH1745 transcripts contain seven AGA/G codons, and therefore, pSJS1240 (obtained from Steven J. Sandler, University of Massachusetts Amherst, Amherst), which encodes transfer RNA (tRNA)_{AUA} and tRNA_{AGG/A}, was transformed into *E. coli* BL21(DE3) cells, together with pET-28a-MTH1745, to increase the availability of these otherwise rare tRNAs in *E. coli* (Kim et al. 1998; Ng and

Jarrell 2003). Synthesis of His6-MTH1745 was induced by addition of isopropyl-β-D-thiogalactoside (IPTG; 1 mM final concentration) to exponentially growing cultures of *E. coli* (pSJS1240, pET-28a-MTH1745) in Lauria-Bertani (LB) medium containing 100 µg of kanamycin and 100 µg of spectinomycin per milliliter. After exposing the cells to 1 mM IPTG at a cell density of *A*=0.6 for 4 h at 37°C, the cells were lysed and the His6-tagged protein was purified by Ni affinity chromatography. Based on Coomassie blue staining after sodium dodecyl sulfate–polyacrylamide gel electrophoresis, MTH1745 was purified to 90% homogeneity.

Thermotolerance experiments with transformed *E. coli* cells

For thermotolerance experiments, cell cultures were grown as described above. IPTG (1 mM) was added to mid-log phase cultures (OD₆₀₀=0.6), and cell was incubated at 30°C for 30 min. After exposing to 51.0°C for 0, 15, 30, 45, and 60 min, the cultures were diluted to 5.0×10⁶ cells per milliliter. Then, samples (50 µl) were plated in triplicate on LB plus appropriate antibiotic plates. The plates were incubated overnight at 37°C before scoring colony formation to determine the percentage of survivors. The survival was calculated by dividing the number of viable cells per plates in the treated samples versus untreated samples (exposing to 51.0°C for 0 min).

Assay of insulin reductase activity

Reductase activity was assayed by Holmgren's turbidimetric method (Holmgren 1979; Pedone et al. 2004) with a few modifications. The catalytic reduction of insulin disulfide bonds was measured at 30°C. The analysis was performed in the presence of increasing concentrations of the purified proteins (0.8, 1.6, and 2.4 µM), as well as in their absence (the spontaneous precipitation reaction). The purified protein was added in 1 ml 100 mM sodium phosphate buffer

(pH 7.0), containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 1 mg bovine insulin (Sigma). The control cuvette contained only buffer and insulin. The reaction was started by the addition of 2 mM dithiothreitol to both cuvettes. Increasing turbidity from precipitation of the insulin B chain was recorded at 650 nm.

Assay of disulfide isomerase activity

Isomerase activity was assayed according to the reactivation of reduced denatured RNase A (RNase A from bovine pancreas, Sigma) by monitoring the absorbance increase at 296 nm due to the hydrolysis of cytidine 2':3'-cyclic monophosphate (cCMP) at pH 8.0 (0.1 M Tris-acetate buffer, 50 mM NaCl, 1 mM EDTA), 25°C in the absence or presence of PDI proteins (Lyles and Gilbert 1991). All samples contained a glutathione redox buffer (0.2 mM GSSG, 1.0 mM GSH). The concentration of reduced RNase A was 8 µM. The relative activity was calculated as $(A - A_0)/(A_1 - A_0) \times 100\%$, where A is the activity of MTH1745, A_1 is the activity of human PDI (hPDI), and A_0 is the blank in the absence of protein.

Assay of chaperone activity

The stock solution of citrate synthase (from porcine heart, Sigma) was diluted with 40 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)/KOH buffer at pH 7.5 to a final concentration of 150 nM in 1.0-ml cuvettes and heated at 43°C with 150 nM of purified MTH1745. The

molarity of MTH1745 in reaction mixtures was based on monomeric molecular masses of MTH1745 variants. Citrate synthase aggregation was monitored by measuring solution turbidity at 360 nm with a SPECTRAMax PLUS spectrophotometer every 4 min for 1 h. Bovine serum albumin (BSA) was used at 150 nM to evaluate nonspecific protection of citrate synthase (Chen et al. 2007).

Complementation of *dsbA* phenotypes by MTH1745

The plasmid pFK198 was constructed by amplifying a DNA fragment of MTH1745 using primers MTH1745-1F and MTH1745-1R (Table 1), which was cloned in between the *KpnI* and *HindIII* sites of pFK093 (obtained from Jon Beckwith, Harvard Medical School, Boston, Massachusetts). To export MTH1745 to the periplasmic space, a fragment encoding the alkaline phosphatase signal sequence (APss) was amplified from the plasmid pFK017 using the primers APssF and APssR, and another fragment encoding MTH1745 was amplified using the primers MTH1745-2F and MTH1745-1R. APss was fused at its N terminus, and the reconstituted gene APss-MTH1745 was created using the two fragments as template with the primers APssF and MTH1745-1R, which was cloned in between the *KpnI* and *HindIII* sites of pFK093 to produce the plasmid pFK200 (Table 2). The plasmids pFK198 and pFK200 were transformed into JCB571 (obtained from Hiroshi Kadokura, Department of Microbiology and Molecular Genetics Harvard Medical School, Boston) to construct strains JCB573 and JCB580, respectively. To get better

Table 2 Strains and plasmids used in this work

Strain or plasmid	Relevant genotype or features	Reference
Strains		
<i>M. thermoautotrophicum</i>	<i>M. thermoautotrophicum</i> DSMZ1053	DSMZ
<i>E. coli</i> BL21 (DE3)	<i>E. coli</i> B (DE3) [F^- dcm ompT hsdS _B (r_B^- m _B ⁻)]	Studier et al. 1990
JCB570	MC 1000 <i>phoR</i> zih12::Tn10	Jonda et al. 1999
JCB571	JCB 570 <i>dsbA</i> ::kan1	Jonda et al. 1999
JCB573	JCB571 with pFK198	This work
JCB579	JCB571 with pFK198 and pSJS1240	This work
JCB580	JCB571 with pFK200	This work
JCB581	JCB571 with pFK200 and pSJS1240	This work
MTCON	<i>E. coli</i> BL21(DE3) with pET-28a	This work
MTDX01	<i>E. coli</i> BL21(DE3) with pET-28a-MTH1745	This work
MTDX02	<i>E. coli</i> BL21(DE3) with pSJS1240, pET-28a-MTH1745	This work
Plasmids		
pFK017	pBAD43 with APss-γ	Katzen and Beckwith 2000
pFK093	pBAD18 with DsbD	Katzen and Beckwith 2000
pFK198	pBAD18 with MTH1745	This work
pFK200	pBAD18 with APss-MTH1745	This work
pSJS 1240	Derivative of pRI952 [derivative of pACYC 184 containing ileX gene (encoding Trn ^{ile} _{AUA}) and argU gene (encoding tRNA ^{arg} _{AGA/AGG}), Sp ^r]	Kim et al. 1998
pET-28a-MTH1745	pET28a(+) with MTH1745	This work

MTH1745 expression, pSJS1240 was co-transformed into JCB573 and JCB580 to construct strains JCB579 and JCB581, respectively. The strains JCB573, JCB579, JCB580, and JCB581 carried out the complementation assay, respectively (Katzen and Beckwith 2000; Sinha et al. 2004). Alkaline phosphatase (AP) activities were measured as described by Brickman and Beckwith (1975). The AP activities was calculated as $1,000 \times (\text{OD}_{420} - (1.75 \times \text{OD}_{550})) / (T \times V \times \text{OD}_{660})$, where T is the reaction time (min), and V is the volume (ml).

Results

MTH1745 mRNA expression in *M. thermoautotrophicum* at different growth temperatures and under cold shock conditions

MTH1745 mRNA expression of *M. thermoautotrophicum* at different culture temperatures can be seen in Fig. 1a and under cold shock treatment in Fig. 1b. The MTH1745 mRNA expression can be seen to be highly increased at culture temperatures far from the optimal growth temperature of 65°C (Fig. 1a) and in the case of cold shock treatment (Fig. 1b). The result suggests that the level of MTH1745 mRNA was stress-induced, hence exhibiting an uptrend at culture temperatures far from the optimal or by cold shock (4°C).

MTH1745 confers thermotolerance on *E. coli* cells

E. coli expressing His-MTH1745 was more resistant to heat stress than cells containing only the pET-28a vector. As shown in Fig. 2, after exposing to 51.0°C, the survival of MTCON cells (with pET-28a) dropped to about 3.7% at 1 h, whereas that of the MTDX01 cells (with pET-28a-MTH1745) was 26.6%. These results show that expression of MTH1745 elevates the cells thermotolerance ability.

MTH1745 exhibits reductase activity in vitro

All concentration of MTH1745 was active in the insulin reductase assay, and the activity was dose-dependent. The optimal activity was presented at 2.4 μM. This showed that MTH1745 has the reductase activity in vitro.

MTH1745 exhibits disulfide isomerase activity in vitro

The ability to refold reduced denatured RNase A is used to measure the protein disulfide isomerase activity in vitro (Lyles and Gilbert 1991). The isomerase activity of MTH1745 was about 28% of hPDI. These results suggest that MTH1745 exhibited disulfide isomerase activity in vivo.

MTH1745 exhibits chaperone activity in vitro

Purified MTH1745 effectively protected citrate synthase against heat-induced denaturation, while BSA provided almost no protection. At a dose of 150 nM, heat-induced turbidity was reduced by 38.5% after 60 min at 43°C (Fig. 3). This result indicated that MTH1745 reduced heat-induced denaturation of citrate synthase, and thus exhibited a chaperone activity in vitro.

MTH1745 complements DsbA deficiency partly

DsbA is an extremely efficient catalyst of disulfide bond formation in the bacterial periplasm, but has poor isomerase activity. *E. coli dsbA*[−] mutants have many functional defects in many proteins that require disulfide bond formation in the periplasm for their activity (e.g., AP, β-lactamase, and outer membrane protein A; Kim and Wyckoff 1991; Knodler et al. 1999). The strains JCB573, JCB579, JCB580, and JCB581 recovered AP folding partly (Fig. 4). The results showed that MTH1745 could be, in part, a functional substitute for DsbA.

Fig. 1 MTH1745 mRNA expression in *M. thermoautotrophicum* analyzed by real-time PCR. The level of MTH1745 mRNA was normalized by 16S rRNA levels and indicated in the respective growth

temperature (a) or cold shock at 4°C for 2 h (b). The value of 65°C was set to 1 and the rest of the value normalized. The results were expressed as means±SEM

Fig. 2 Survival versus time at 51.0°C. Two transformed *E. coli*, MTCO (with pET-28a) and MTDX01 (with pET-28a-MTH1745), were cultured as described and subjected to 51.0°C treatment for up to 60 min. After heat treatment, samples were taken at the time indicated, then diluted, and immediately plated on LB plus kanamycin plates. Survival (%) of MTDX01 cells (open triangle) and MTCO cells (open square) are shown. Mean survival is expressed as a percentage of the survival obtained in cultures

Discussion

M. thermolithotrophicus is a strictly anaerobic thermophilic archaea which grows at the optimal temperature of 65°C and often faces stress conditions, such as temperature fluctuations, decrease in pH, hypersalinity, and the presence of heavy metals. In the present study, the synthesis of MTH1745 mRNA has been observed in *M. thermoautotrophicus* under stress conditions. All organisms have evolved a variety of strategies for coping with stress, and *M. thermoautotrophicus* was not an exception. One of its strategies may be to increase the synthesis of MTH1745. This study about the different expression patterns at various temperature and acquired thermotolerance in *E. coli* suggests that MTH1745 has an important role for enhancing the survival potential of the extremophile archaea upon exposure to such stress.

Fig. 3 MTH1745 prevents heat-induced denaturation of citrate synthase. Purified MTH1745 was incubated at 43°C with 150 nM citrate synthase, and the solution turbidity was measured at 360 nm. MTH1745 concentrations were: 0.0 nM (open square); 150 nM (ex mark). BSA concentrations were 150 nM (open triangle)

In vitro MTH1745 reduced heat-induced denaturation of citrate synthase in *E. coli* (transformed with pET-28a-MTH1745), which showed that MTH1745 also possesses a chaperoning activity to recognize and bind with unfolded proteins to prevent protein aggregation in vivo. The recent studies demonstrate that PDI, DsbA, DsbC, thioredoxin, and thioredox reductase have chaperone activities in vitro and may function as a chaperone in vivo (Lyles and Gilbert 1994; Tang et al. 1994; Darby and Creighton 1995; Holst et al. 1997; Wang 1998; Klappa et al. 1998; van den Berg et al. 1999; Chen et al. 1999; Ferrari and Söling 1999; Vinci et al. 2000; Kern et al. 2003; Pandhare and Deshpande 2004). These examples suggest that proteins with a thioredoxin domain may play an important role in recognizing and binding to unfolded proteins to prevent protein aggregation. Our results suggest that this is also the same case with MTH1745. After deviation from the optimal growth temperature, higher or lower, MTH1745 mRNA accumulated abundantly in cells to protect *M. thermoautotrophicus* from stress without adversely affecting the normal metabolism (Fig. 1).

An important property of disulfide isomerase proteins is the presence of multiple thioredoxin catalytic domains. PDI is a eukaryotic disulfide isomerase, having four thioredoxin domains (Kersteen and Raines 2003). DsbC is a periplasmic disulfide isomerase of Gram-negative bacteria and displays about 30% of the activities of eukaryotic PDI as isomerase and as thiol-protein oxidoreductase (Chen et al. 1999). Structurally, DsbC is a 2×23-kDa homodimer (McCarthy et al. 2000). A previous study indicates that proteins which consist of a single thioredoxin domain, such as thioredoxin, DsbA, and the N-terminal active-site domain a and the non-active-site domain b of PDI, exhibit low or no isomerase and chaperone activity (Zhao et al. 2003). The primitive

Fig. 4 In vivo restoration of DsbA-dependent alkaline phosphatase activity with periplasmic expressed MTH1745 in *E. coli*. Alkaline phosphatase activity is expressed as $\Delta A_{420\text{nm}} \text{ min}^{-1} \Delta A_{660\text{nm}}^{-1}$. *E. coli* strains, JCB570, JCB571, JCB573 (with MTH1745), JCB579 (with MTH1745, pSJS1240), JCB580 (with APss-MTH1745), JCB581 (with APss-MTH1745, pSJS1240) were used

eukaryote *Giardia lamblia* PDIs (gPDI1, gPDI2, gPDI3) which have only one active site exhibit lower activity compared with chick PDI (Knodler et al. 1999). Furthermore, dimerization by domain hybridization (DsbCn-Trx, DsbCn-DsbA, DsbCn-PDIa, and DsbCn-PDIb) creates chaperone and isomerase activities for monomeric thiol-protein oxidases or reductases (Zhao et al. 2003). For all the proteins containing the thioredoxin domain, PDI is the most efficient, catalyzing maximum re-activation of rRNAase, while thioredoxin is the least efficient (Hawkins et al. 1991). Based on the above description, it is not surprising that MTH1745, which is an 18-kDa molecule, characterized by single thioredoxin domain, displays about 28% of the isomerase activity of hPDI. It is indicated that PDI need either multiple thioredoxin domains or single thioredoxin domains, and multiple domains bestow upon PDI more isomerase activity. In addition, PDI activity can be demonstrated by complementation of the phenotype associated with a *dsbA* mutation, such as the increasing yield of heterologously expressed disulfide-containing protein AP (Humphreys et al. 1995; Ostermeier et al. 1996; Ferrari and Söling 1999). The result showed that MTH1745 could be functionally a partial substitute for DsbA (Fig. 4). It was also to be expected that some single active-site PDIs (such as *Giardia* PDI-1, PDI-3) failed to complement an *E. coli dsbA* mutant (Knodler et al. 1999; McArthur et al. 2001).

A question rises: what was the common ancestry of all eukaryotic PDIs? Phylogenetic analyses supports the suggestion that eukaryotic PDIs came from a thioredoxin ancestor and independent duplications of thioredoxin-like domains of which the yeast single-domain PDIs and gPDI appear to be direct descendants (Knodler et al. 1999; McArthur et al. 2001). However, little structural or functional information is available on these proteins isolated from archaea. It has been reported that protein disulfide oxidoreductase (PfPDO) from *P. furiosus*, a PDI-like archaeal protein, displays oxidative, reductive, and disulfide isomerase activities, which is characterized by two redox sites (CXXC). In fact, other archaeal PDOs have been isolated from *S. solfataricus* (SsPDO; Guagliardi et al. 1994), *P. horikoshii* (PhPDO; Kashima and Ishikawa 2003), *M. jannaschii* (Bult et al. 1996), and *M. thermoautotrophicum* (McFarlan et al. 1992). Here, we show that MTH1745 has the disulfide isomerase and chaperone activities with a single thioredoxin domain. Given the “archaic” nature of archaea and their hypothesized role in eubacterial and eukaryotic evolution (Amegbey et al. 2003), it is tempting to speculate that MTH1745 is a molecular “fossil” representing an early branch point in the evolution of protein disulfide isomerase.

There were also two thioredoxin proteins (Mt0807 and Mt0895) identified in the microbe which exhibited disulfide oxidize/reductive activity (Amegbey et al. 2003). It was possible that there existed a series of precise integrity

system (included proteins MTH1745, Mt0807 and Mt0895) to sustain the protein disulfide bonds formation and bind to unfolded proteins to prevent protein aggregation under stress condition in *M. thermoautotrophicum*. Numerous problems related to protein disulfide folding in archaea remain unsolved to date, but genetic and in vivo approaches hold out the best options to continue such a study area. The protein Mt1715 from *M. thermoautotrophicum* described here may provide some contribution to our understanding of PDI in archaea and the capacity of thermophilic archae to withstand stress.

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