Amino Acids (2008) 34: 437–448
DOI 10.1007/s00726-007-0549-5
Printed in The Netherlands

Amino Acids

A DING phosphatase in *Thermus thermophilus*

A. A. Pantazaki[1], **G. P. Tsolkas**[1], and **D. A. Kyriakidis**[1,2]

[1] Laboratory of Biochemistry, Department of Chemistry, Aristotle University of Thessaloniki, Greece
[2] The National Hellenic Research Foundation, Athens, Greece

Received February 10, 2007
Accepted March 21, 2007
Published online May 14, 2007;

Summary. Phosphate transport in bacteria occurs via a phosphate specific transporter system (PSTS) that belongs to the ABC family of transporters, a multisubunit system, containing an alkaline phosphatase. DING proteins were characterized due to the N-terminal amino acid sequence DINGG GATL, which is highly conserved in animal and plant isolates, but more variable in microbes. Most prokaryotic homologues of the DING proteins often have some structural homology to phosphatases or periplasmic phosphate-binding proteins. In *E. coli*, the product of the inducible gene *Din*G, possesses ATP hydrolyzing helicase enzymic activity. An alkaline phosphorolytic enzyme of the PSTS system was purified to homogeneity from the thermophilic bacterium *Thermus thermophilus*. N-terminal sequence analysis of this protein revealed the same high degree of similarity to DING proteins especially to the human synovial stimulatory protein P205, the steroidogenesis-inducing protein and to the phosphate ABC transporter, periplasmic phosphate-binding protein, putative (*P. fluorescens* Pf-5). The enzyme had a molecular mass of 40 kDa on SDS/PAGE, exhibiting optimal phosphatase activity at pH 12.3 and 70 °C. The enzyme possessed characteristics of a DING protein, such as ATPase, ds endonuclease and 3′ phosphodiesterase (3′-exonuclease) activities and binding to linear dsDNA, displaying helicase activity on supercoiled DNA. Purification and biochemical characterization of a *T. thermophilus* DING protein was achieved. The biochemical properties, N-terminal sequence similarities of this protein implied that the enzyme belongs to the PSTS family and might be involved in the DNA repair mechanism of this microorganism.

Keywords: *Thermus thermophilus* – DING protein – Alkaline phosphatase – Phosphate specific transporter system (PSTS) – ATPase – Type II phosphodiesterase (3′-exonuclease)

Abbreviations: ABC, ATP binding cassette; DING, DINGGG-N-terminal sequence; ds, double stranded; PDE, phosphodiesterase; pDNA, plasmid DNA; PSTS, phosphate specific transporter system; SBP, substrate binding protein; ss, single stranded; T_{opt}, temperature optimum

Introduction

The name ''DING proteins'' was recently given to the ubiquitous members of a protein family of molecular mass of approximately 40 kDa that possess a characteristic DINGGGATL N-terminal sequence or a closely related sequence (Adams et al., 2002; Berna et al., 2002; Belenky et al., 2003; Scott and Wu, 2005). Human DING was first reported as a fragment of a larger protein of human synovial fluid possibly associated with rheumatoid arthritis (RA) (Adams et al., 2002). Other closely related proteins with properties of receptor or signalling proteins have been reported in various human and animal cells (Berna et al., 2002). DING proteins are not restricted to the animal kingdom, since they have been purified from several higher plant species. Plant DING proteins and their microbial relatives may elicit allergic responses leading to arthritic disease (Berna et al., 2002). Despite the high frequency of discovery of these proteins, complete gene sequences encoding for eukaryotic DING proteins have not yet been identified.

A variety of prokaryotic proteins related to phosphate transport or metabolism share significant homology with the first 14 amino acids of the typical N-terminus of eukaryotic DING proteins (Berna et al., 2002). These proteins are periplasmic alkaline phosphatases or periplasmic phosphate-binding proteins, where periplasmic binding proteins act to concentrate ions and other metabolites from the microenvironment, prior to uptake by cytoplasmic membrane transporters (Scott and Wu, 2005). Several microbial proteins related to DING proteins have phosphatase activity, which may relate to biomineralisation in eukaryotic systems (Berna et al., 2002). Bacterial adaptation to the environment often involves modification of enzymic activation and translocation (Huang et al., 1998). The activation of bacterial alkaline phosphatase biosynthesis upon phosphate starvation is a classical example of

induced enzyme biosynthesis in bacteria (Huang et al., 1998), which involves transmembrane signaling regulated by the level of phosphate in the environment. Control of this signal transduction pathway requires a phosphate specific transporter system (PSTS), a multisubunit system belonging to the ATP binding cassette (ABC) family of transporters, which bind ATP and couple ATP hydrolysis to the transport process across biological membranes (Kerr, 2002). PSTS of *E. coli* and *P. aeruginosa* have many similarities, including the presence of a porin, a periplasmic binding protein, an alkaline phosphatase and regulatory components (Tan and Worobec, 1993). The alkaline phosphatase (L precursor) of the PSTS of *P. aeruginosa* possesses phosphomonoesterase and phosphodiesterase activities (Tan and Worobec, 1993). Enzymes, possessing 3′-phosphodiesterase activities play an important role in DNA repair processes and contribute to genome stability, by removing potentially mutagenic DNA lesions that cannot be removed by polymerase-associated 3′-exonucleases (Sander, 1997). *E. coli din*G is a DNA damage-inducible gene which encodes a putative DNA helicase related to a group of eykaryotic helicases including Rad3 protein, a component of the DNA excision repair system in eukaryotes (Koonin, 1993). *E. coli Din*G gene product possesses ATP hydrolyzing enzyme activity, stimulated by ssDNA (Voloshin et al., 2003).

Thermophiles exhibit a preference for ABC-type transporters that are important for their survival in their natural habitat. ABC-type transporters of these organisms that thrive in nutrient-poor environments, such as in hot springs, have the advantage that they can scavenge solutes at very low concentrations due to the high binding affinities of their binding proteins (Albers et al., 2001). *Thermus thermophilus* that is classified as one of the oldest branches in bacterial evolution has been used as a model for structural and functional studies of biotechnologically important enzymes (Pantazaki et al., 2002; Lioliou et al., 2004).

We report here the isolation and characterization of a DING protein from *T. thermophilus*, which has the characteristics of the PSTS family of proteins. The biochemical properties and the N-terminal similarities of this protein with other of similar function proteins indicated the possible involvement of this enzyme in the DNA repair mechanisms.

Materials and methods

Bacterial source, media, nucleic acids and chemicals

T. thermophilus HB_8 (Oshima and Imahori, 1974), was grown at 75°C as previously described (Pantazaki et al., 1998). Calf thymus DNA was purchased from Sigma and plasmid pUC 119 was isolated from *E. coli* XL1 by the alkaline SDS lysis method (Stratagene, CA). Tryptone and yeast extract were purchased from Oxoid (Unipath LTD, Hampshire, UK). Chromatographic materials were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden) and chemicals from Sigma-Aldrich Chemie (GmbH, Steinheim, Germany), unless otherwise stated. Restriction endonucleases were purchased from New England BioLabs (Hertfordshire, UK). Molecular weight DNA and protein markers were obtained from Gibco-BRL (Karlsruhe, Germany).

Buffers

The buffer solutions used for the different pH values, in the range between 4 and 13, were previously described (Pantazaki et al., 1998). The pH values for all buffers were measured at 25 °C and corrected to alkaline phosphorolytic activity assay temperature of 70 °C by the -dpH/dt (unit/degree) coefficient, specific for each buffer.

Purification of an alkaline phosphorolytic activity

T. thermophilus cells (25 g wet weight) were suspended in 100 ml of buffer A (50 mM Tris–HCl pH 8.5) and disrupted by 30 sec cycle sonication (Branson) for 15 min in ice-bath. Cells debris was removed by centrifugation at $10{,}000 \times g$ for 20 min. The supernate was further centrifuged at $100{,}000 \times g$ for 1 h. The resulting supernate, designated as the crude extract, received 75 µl/ml extract of freshly prepared 10% (w/v) streptomycin sulphate, under constant stirring for 45 min at 4 °C. The mixture was centrifuged at $15{,}000 \times g$ for 20 min at 4 °C and 100 ml of the supernatant were applied on a DEAE-cellulose column, (21×2.6 cm) equilibrated in 50 mM Tris–HCl buffer, pH 8.5. The column was washed with 400 ml of the same buffer. A total 300 ml linear gradient of 0–0.5 M NaCl in buffer A was then applied. The fractions exhibiting phosphorolytic activity eluted at approximately 0.2 M NaCl, were pooled and applied on a Phenyl-Sepharose column, (7×2.6 cm), previously equilibrated with buffer A. The column was eluted with buffer A, to remove bulk proteins, followed by a 240 ml linear gradient of 0–70% (v/v) ethylene glycol in buffer A. Phosphorolytic activity was eluted at approximately 20% (v/v) ethylene glycol. Active fractions were combined and loaded onto a Cibacron blue-Sepharose column (7×2.6 cm) equilibrated with buffer A. The column was washed with buffer A and subsequently eluted by a 300 ml linear gradient of 0–0.7 M NaCl. Phosphorolytic activity was eluted at approximately 0.25 M NaCl. Active fractions were pooled and dialyzed against buffer A. The enzyme preparation from this step was applied on a Heparin-Sepharose column (13×1.5 cm) equilibrated with buffer A. The column was washed with the same buffer, to remove inactive protein, and enzyme activity was eluted with a total of 300 ml linear gradient of 0–0.2 M NaCl in buffer A. The active fraction was pooled, concentrated to 1 ml by Amicon ultrafiltration (PM 10) and applied to a Sephadex G-200 column. The active concentrated fraction was applied on a Sephadex G-200 column (100 ml volume) equilibrated with buffer A plus 0.2 M NaCl. The active fractions were pooled, dialyzed against buffer A and loaded on a Lysine-Sepharose column (8×2.6 cm) equilibrated with buffer A. The column was washed with buffer A and then eluted with a total of 200 ml linear gradient of 0–0.6 M NaCl in buffer A. The enzyme activity was eluted at the end of the gradient with 0.5 M NaCl. Active fractions were concentrated and dialyzed. Glycerol 5% (v/v) was then added, and the solution was stored at −20 °C.

Alkaline phosphatase assay

Alkaline phosphatase activity was assayed by following the release of p-nitrophenol from the hydrolysis of p-nitrophenyl phosphate. The reaction mixture consisted of 1 mM of p-nitrophenyl phosphate and 50 mM KCl–NaOH buffer pH 12.3 (measured at 25 °C) in a final

volume of 0.5 ml. The reaction was initiated by adding 50 µl of enzyme preparation and incubated at 70 °C during the period of time indicated in each experiment. Adding an equal volume of 0.25 M NaOH stopped the reaction and the absorbance of the mixture was measured at 410 nm, hereafter these being referred to as standard conditions. One enzyme unit represents the hydrolysis of 1 µmol of substrate per min under the aforementioned conditions. When phosphate esters other than p-nitrophenyl phosphate were used as substrates (such as phosphorylated nucleotides, sugars or phospho-amino acids), phosphatase activity was determined by measuring the amount of phosphate liberated during incubation at 70 °C, by using the malachite green procedure (Baykov et al., 1988). Controls without either the enzyme or substrate were used.

Protein determination

The protein concentration was determined by Bradford's method using bovine serum albumin as standard (Bradford, 1976).

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gel was performed according to Laemmli (1970). Protein bands were visualized after staining with silver nitrate.

Protein N-terminal sequence analysis

Protein sample was subjected to SDS-PAGE, electroblotted onto PVDF Immobilon-P membranes (Millipore Corp., Bedford, MA) and the protein band corresponding to alkaline phosphatase was visualized with Coomassie Brillant Blue. The 40 kDa band corresponding to the purified protein were excised from the membrane and subjected to Edman degradation. Automated N-terminal sequence analysis was performed on the Applied Biosystems protein sequencer model 492 A applying pulsed liquid blot mode chemistry (Wittman Institute of Technology and Analysis of Biomolecules (WITA GmbH), Teltow, Berlin, Germany). The deduced N-terminal sequence was compared with that of other proteins of databases (Bairoch and Boeckmann, 1992).

Phosphodiesterase type II (3′-exonuclease) activity assay

Phosphodiesterase activity was measured using p-nitro-phenyl thymidine-3′-monophosphate ester as substrate (Razzell and Khorana, 1959). The reaction mixture consisted of 1 mM of p-nitro-phenyl thymidine-3′-monophosphate ester (ammonium salt) and 50 mM KCl–NaOH buffer pH 12.3 (measured at 25 °C) in a final volume of 0.5 ml. The reaction was initiated by the addition of 50 µl of enzyme preparation and incubation at 70 °C for 1 h. Adding an equal volume of 0.25 M NaOH stopped the reaction and the absorbance of the mixture was measured at 410 nm. One unit represents the amount of enzyme that hydrolyzes 1 µmol of substrate per min under the aforementioned conditions.

Nucleolytic activity assay

Deoxyribonucleolytic activity was determined by monitoring the conversion of DNA to an acid-soluble form. Native or denatured calf thymus DNA was used as substrate. Denatured DNA was prepared by heating an aqueous solution of 2 mg/ml DNA in a boiling-water bath for 10 min followed by immediate cooling in an ice bath. Deoxyribonucleolytic activity was assayed at 70 °C in a total volume of 0.5 ml in the presence of enzyme, 0.6 mg/ml DNA and 50 mM of KCl–NaOH buffer pH 12.3 (measured at 25 °C). Reactions were terminated, by adding an equal volume of chilled 12.5% (v/v) perchloric acid. The reaction mixtures were placed in an ice bath for 10 min and the absorbance of the acid soluble products was measured at 260 nm. One unit of deoxyribonucleolytic activity is defined as the amount of enzyme that produces acid-soluble products giving an increase in absorbance of 0.1 at 260 nm after 1 h of incubation.

Agarose gel electrophoresis of DNA

Aliquots of assay mixtures containing 3 µg of each form of DNA were incubated in the presence of *T. thermophilus* alkaline phosphatase in a final volume of 20 µl at 70 °C for various time periods. The reaction was terminated by the addition of 5 µl loading buffer containing 0.25% (w/v) bromophenol blue and 30% (v/v) glycerol and the resulting products were separated by electrophoresis on agarose gels 1% (w/v), containing 1µg/ml ethidium bromide in 90 mM Tris–borate, and 2 mM Na_2EDTA. Agarose gel electrophoresis was performed with a horizontal gel apparatus (Mini-Sub™ DNA Cell, Biorad) for 2 h at 5V/cm. The gels were visualized under UV light DNA quantification was performed using Gelpro Analyzer V.3 computer program.

Results

Purification of an alkaline phosphorolytic activity

The purification of an alkaline phosphorolytic activity is described in Table 1, using p-nitrophelyl phosphate as substrate. The final purification was approximately 7,000-fold with 6.3% recovery. The purified to homogeneity alkaline enzyme displayed a single band on SDS/PAGE gel elec-

Table 1. Purification of a *T. thermophilus* phosphorolytic activity

Steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
1. Initial extract	4220	55000	13	1.0	100.0
2. Streptomycin sulfate precipitation	926.7	50600	54	4.2	92.0
3. DEAE-cellulose	157.7	45100	286	22	82.0
4. Phenyl-Sepharose	7.5	41250	5500	423	75.0
5. Cibacron Blue Sepharose CL-4B	1.2	34265	26700	2053.8	62.3
6. Heparin-Sepharose	0.62	24750	39800	3061.5	45.0
7. Sephadex-G200	0.205	11825	57600	4430.7	21.5
8. Lysine-Sepharose	0.038	3465	91184	7014.1	6.3

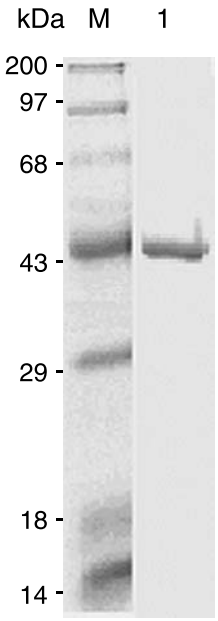

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified phosphorolytic enzyme. Purified enzyme (3 μg) was subjected to SDS-polyacrylamide gel electrophoresis in 10% (w/v) polyacrylamide. The gel was stained with silver nitrate. *1* Purified enzyme. *M* Molecular weight markers of the indicated masses

trophoresis (10% w/v in acrylamide) at molecular mass of 40 kDa (Fig. 1). The streptomycin sulphate step was included since the enzyme preparation contained large amounts of nucleic acids at all purification steps with ratio $A_{260\,nm}/A_{280\,nm}$ higher than 1.2.

Protein characterization: N-terminal sequence determination

The sequence of the first 14 amino acid residues, determined for the 40 kDa excised band of *T. thermophilus*, was as follows: ^{1}D/(G)VNG(G)(G)ATLPQPLY^{14}. The first amino acid is not absolutely clear whether it was aspartic acid (D) or glycine (G). Comparison of this N-terminal sequence with other sequences available in data bases (Altschul et al., 1997) showed that the purified alkaline phosphatase presented strong similarities with proteins belonging to the family of DING proteins (Table 2). It is surprising that the purified protein presented the same great similarity with the steroidogenesis-inducing protein, the human synovial stimulatory protein P205 (Hain et al., 1996) and the phosphate ABC transporter, periplasmic phosphate-binding protein, putative (*P. fluorescens* Pf-5). Furthermore, it displayed also significant similarities with other phosphate ABC transporters, alkaline phosphatases with the same molecular mass or other periplasmic phosphate-binding proteins. The signal peptide is missing from the *T. thermophilus* enzyme, indicating that the protein may be localized to the periplasmic space from where migrate towards the

Table 2. N-terminal amino acid sequences of DING proteins

Amino acid sequence	Source	Similarities (identities, positives) (%)	Reference
DVNGGGATLPQPLY	*T. thermophilus* alkaline phosphatase		This study
DVNGGGATLPQPLY	[Segment 2 of 3] steroidogenesis-inducing protein	100, 100	gi|62287179|sp||P83897_2
D INGGGATLPQPLY	phosphate ABC transporter, periplasmic phosphate-binding protein, putative [*P. fluorescens* Pf-5]	92, 100	gi|70730127|ref|YP_259866.1| gi|68344426|gb|AAY92032.1|
D INGGGATLPQPLY	human synovial stimulatory protein	92, 100	gi|41019457|sp||P80697_3
D INGGGATLPQQLY	ABC phosphate transporter *P. aeuginosa* UCBPP-PA14	92, 100	ZP_00138283
D INGGGATLPQQLY	hypothetical protein PA14_55410 [*P. aeruginosa* UCBPP-PA14]	85, 92	gi|116048595|ref|YP_792606.1| gi|115583816|gb|ABJ09831.1|
-VNGGGATLPQQLY	hypothetical protein PaerP_01000347 [*P. aeruginosa* PA7]	92, 92	gi|94418193|ref|ZP_01298009.1|
D INXGGATLPQPLY	human breast cancer	85, 92	TIGR TC105368
- - -GGGATLPEKLY	urinary tract stone matrix protein, 40 K – unidentified organism (fragment)	81, 90	gi|1082909|pir||A56049
AvTGGGA s LPAELY	*P. aeuginosa* alkaline phosphatase	72, 60	PIR: E83559
TvTGGGA smPAKLY	*P. aeuginosa* alkaline phosphatase		PIR: E83559
I NGAGATFPAPLY	phosphate-binding periplasmic protein [*Aquifex aeolicus* VF5]	69, 76	gi|15607002|ref|NP_214384.1|
S lTGAGAT f PAP -Y	*E. coli* periplasmic phosphate-binding protein	60, 57	PIR: H91211
- INGAGATFPAPLY	phosphate ABC transporter, phosphate-binding protein [*Synechococcus*]	69, 76	gi|86606216|ref|YP_474979.1|

Consensus DINGGGATLP Q-LY-T-VL-AGFAPY. Alignment of the N-terminal sequences of DING proteins from various species including the microbial relatives of these and the corresponding DING protein purified from *T. thermophilus* as well. Microbial sequences were deduced from DNA sequences and are located at or very near the predicted N-terminus of the mature protein

cytoplasm. The preponderant conserved motifs in these proteins are GGGA(T/S), GAGA (T/S) or GAGS(T/S). It is quite clear that the proteins of the PSTS family strictly conserve the glycine (G) of the consensus sequence. This sub-motif shows similarity with the identified ATP-binding site A (known as Walker A motif I (Walker et al., 1982), possessing the sequence A/G-X-X-G-X-G-K(T/S), characteristic of NTP-binding/hydrolyzing enzymes.

Biochemical properties

Optimal temperature and pH of DING alkaline phosphorolytic enzyme

The optimum temperature for the DING alkaline phosphorolytic activity, under the assay conditions, was 70 °C (Fig. 2A). When the assay was performed at 70 °C, at different pH values, a broad peak of activity between 11.5 and

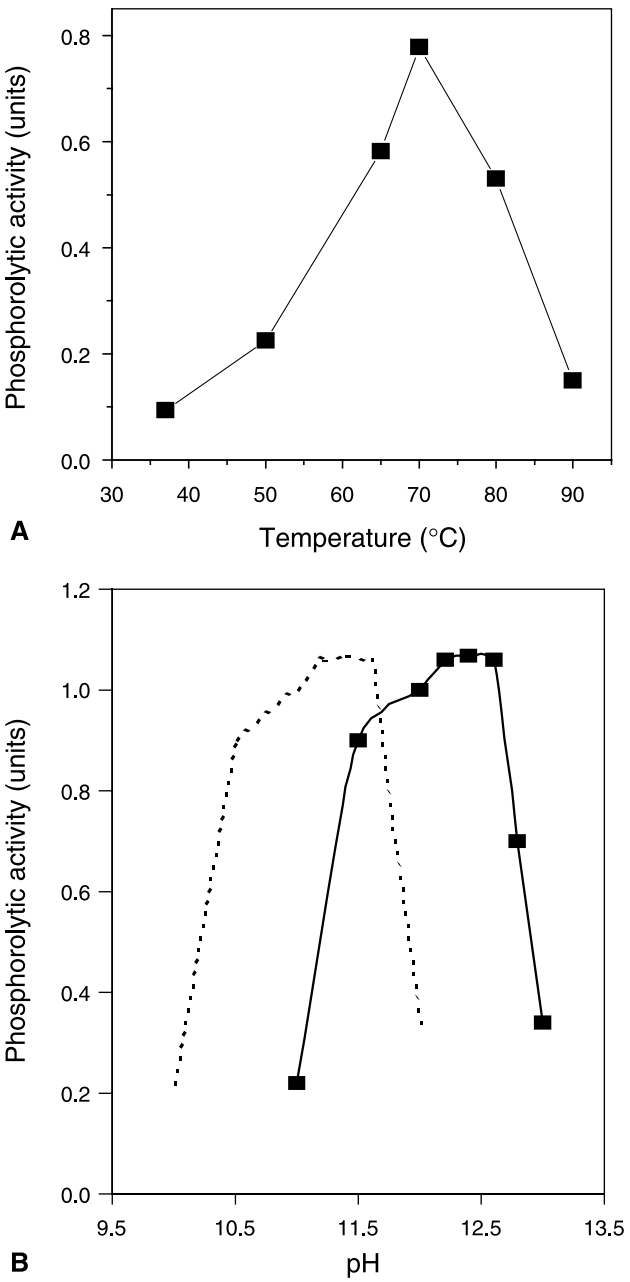

Fig. 2. Temperature (**A**) and pH optima (**B**) of DING phosphorolytic enzyme. Enzyme activity was measured at 50 mM of KCl–NaOH buffer pH 12.3 at different temperatures or at 70 °C at different pH values. Dotted line represents the curve corresponding to the pH values corrected for 25 °C

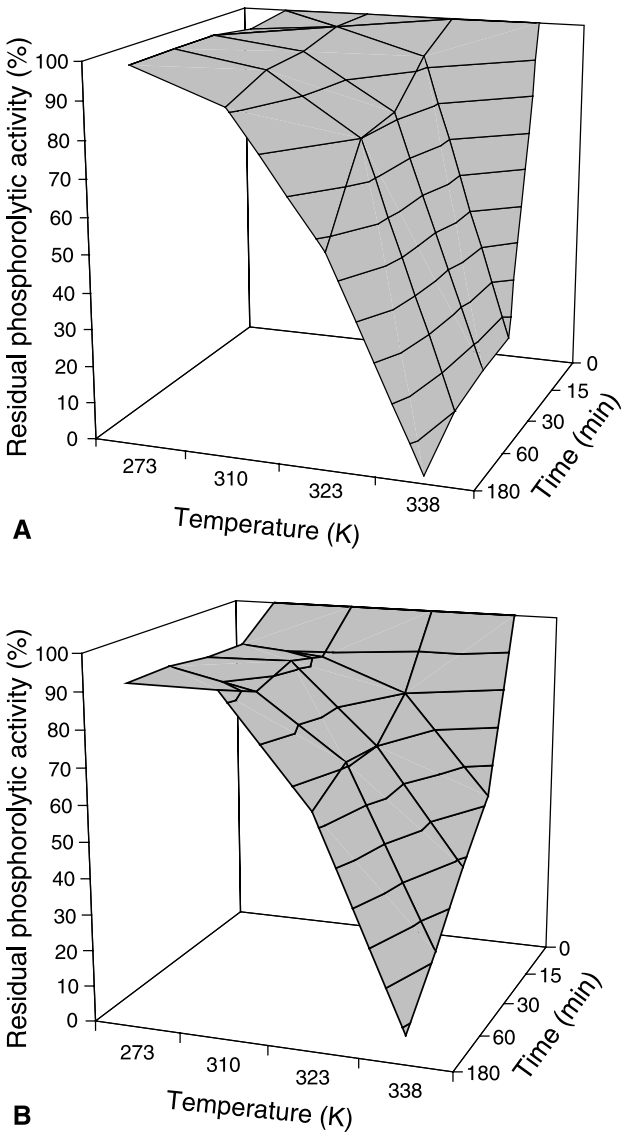

Fig. 3. Thermostability of DING phosphorolytic activity. The enzyme solutions were heat-treated at temperatures 37, 50, 65 and 80 °C for various periods (0–3 h) in 50 mM of KCl–NaOH buffer pH 12.3. Incubations were performed without addition of metal ion (**A**) or in the presence of 1 mM of Ca^{2+} (**B**). The residual activities were then measured at 70 °C as described in Materials and methods. The data are plotted as the residual enzyme activity (%) versus temperature (K) versus time during heat-treatment

12.6 was obtained (Fig. 2B). It should be noted that the actual pH values were 10.5–11.5 taking into account the correction needed for the enzyme assay at 70 °C (Fig. 2B, dotted line) as described under Materials and methods.

Thermal inactivation studies

The thermal stability of the purified enzyme (Lysine-sepharose step) was examined by measuring the residual activity after heat treatment at various temperatures at different times. Samples containing 1.2 μg of purified enzyme were incubated in the absence or presence of 1 mM Ca^{2+} or Mg^{2+} at different temperatures at the indicated periods of time. As shown in Fig. 3A, the enzyme maintained almost all its activity when it was pre-incubated at 37 or 50 °C. When the experiment was performed at 65 °C, the enzyme activity was gradually reduced, whereas at 80 °C most of its activity was lost after 15 min of incubation. Similarly, the enzyme stability was barely affected when the enzyme was pre-incubated at 37 or 50 °C in the presence of 1 mM Ca^{2+} or Mg^{2+} (Fig. 3B). When pre-incubation was performed at 65 or 80 °C, in the presence of metal ions, the enzyme activity was gradually reduced during the assay but it remained higher compared to the absence of metal ions. This result indicated that metal ions might have a protective role. The correlation of catalytic activity and thermostability of enzyme reactions have been extensively discussed previously (Peterson et al., 2004).

Table 3. Phosphohydrolytic activity of *T. thermophilus* on different phosphorylated substrates

Substrates	Enzyme activity (%)
Nucleotides	
p-Nitrophenyl phosphate (control)	100
ATP	157
2′-Deoxyadenosine 5′-triphosphate	100
ADP	133
AMP	41
NADP	27
Sugars	
Glucose-1-phosphate	37
Glucose-1,6-di-phosphate	39
D-fructose-6-phosphate	26
Phospho-amino acids	
Phospho-tyrosine	80
Phospho-serine	78
Phospho-threonine	75
Other metabolite	
Phospho(enol)pyruvate	168

All substrates used in the assay mixture were in a final concentration of 1 mM and the phosphate determination was performed by the malachite method as described under Materials and methods

Substrate specificity

The purified DING enzyme hydrolyzed a wide variety of phosphoryl-containing compounds but it displayed different specificities (Table 3). The enzyme showed a preference towards the phospho-nucleotides assayed, in the following order: nucleotides tri-phosphates > di-phosphates > mono-phosphates > nicotinamido-adenino-dinucleotide phosphate (ATP > ADP > AMP > NADP). Towards sugar derivatives, the activity was very low and increased in the following order: glucose-1-p > glucose-1,6-di-phosphate > fructose-6-p. Phospho(enol)pyruvate was hydrolyzed by the enzyme approximately to the same extent as ATP. The purified DING enzyme hydrolyzed phospho-amino acids in the order: p-nitrophenyl phosphate > phospho-tyrosine > phospho-serine > phospho-threonine (Table 3). This preference of the DING enzyme for ATP hydrolysis prompted us to elucidate its potential ATPase activity.

Activity of phosphodiesterase type II (3′-exonuclease)

Most of the ATPases have been reported to possess nucleolytic activities such as single-stranded (ss) DNA specific endonuclease activity or ATP-dependent double-strand (ds)-specific exonuclease activity. Furthermore, it is notable that the similar alkaline phosphatase (L precur-

Table 4. Determination of K_m and k_{cat} of purified DING phosphorolytic enzyme against various substrates

Substrates	K_m (mM)	k_{cat} (s^{-1})
p-Nitrophenyl phosphate	0.100	8.33
ATP	0.006	20.80
Phopshoenol-pyruvate	0.005	20.00
Thymidine 3′-monophosphate-p-nitrophenyl ester (ammonium salt)	0.080	13.33
Phospho-tyrosine	0.250	4.16

Double reciprocal plots of alkaline phosphorolytic enzyme versus concentration of substrates were performed against p-nitrophenyl phosphate, ATP, phosphotyrosine and thymidine 3′-monophosphate-p-nitrophenyl ester (ammonium salt). Enzyme activity was determined according to malachite green procedure as described in Materials and methods. The K_m was determined by the linear fitting procedure of plots. The maximum velocities of the reactions were determined for each substate and the k_{cat} values were calculated by dividing the V_{max} by the enzyme concentration used in the experiments. The enzyme concentration used was 0.12 μM

sor) (L-AP) from *P. aeruginosa*, exhibits phosphodiesterase activity, in addition to the phosphomonoesterase activity (Tan and Worobec, 1993). Thus, the capability of the purified DING enzyme to hydrolyze the synthetic substrate p-nitro-phenyl thymidine 3′-monophosphate, specific for phosphodiesterase activity determination, and especially for 3′-exonucleolytic activity, was examined. It was found that the enzyme hydrolyzed this substrate and therefore possessed exonucleolytic activity on DNA to the $3' \rightarrow 5'$ direction. The corresponding K_m and k_{cat} values are shown in Table 4.

Enzyme kinetics

The kinetic properties of *T. thermophilus* DING enzyme were examined at optimal temperature and pH, using various concentrations of p-nitrophenyl phosphate, ATP, phosphoenol-pyruvate, thymidine 3′-monophosphate-p-nitrophenyl ester (ammonium salt) and phospho-tyrosine, by measuring the release of phosphates monitored by the malachite green procedure. The DING enzyme was shown to hydrolyze all these substrates following typical Michaelis-Menten kinetics (Table 4), exhibiting the lowest K_m values for ATP and phopshoenolpyruvate. Recent analysis of raw wall preparations of *T. thermophilus* revealed the presence of significant amounts of covalently bound pyruvic acid (Cava et al., 2004). The high specificity for ATP revealed that DING enzyme might act as an ATPase consistent with the existence of the characteristic of NTP-binding/hydrolyzing enzymes Walker A motif I (Walker et al., 1982).

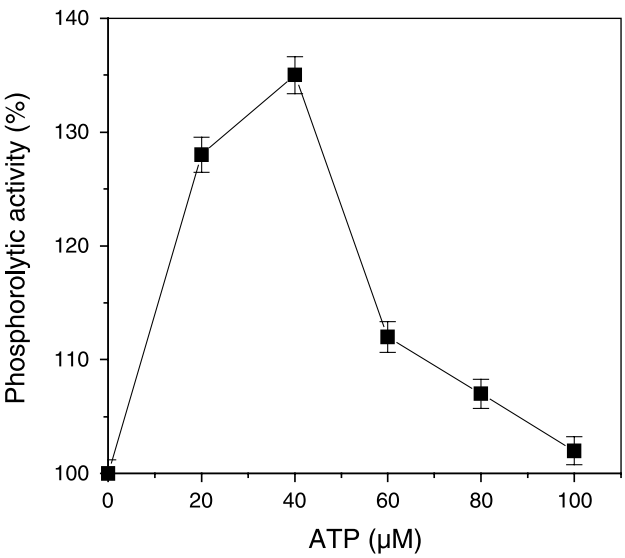

Fig. 4. Effect of ATP on DING phosphorolytic activity

Effect of ATP

The effect of ATP on the DING phosphorolytic and nucleolytic activities was tested, using p-nitrophenyl phosphate and DNA as substrates, respectively. As shown in Fig. 4, ATP enhanced the phosphorolytic activity by approximately 28 and 35% at concentrations 20 and 40 μM, respectively, while this activation was gradually reduced at higher concentrations. These results showed that ATP is an activator of both activities at low concentrations, indicating that this DING enzyme is ATP dependent.

DNA binding and nucleolytic activity

The interaction of *T. thermophilus* DING enzyme with various forms of DNA was assessed by agarose gel electrophoresis. The enzyme was able to interact efficiently with a random-sequence dsDNA. The time course of this interaction revealed that a small band shifting of the DNA and small amount of DNA approximately 8.2% enabled to enter into the gel after 30 min of incubation with the enzyme (Fig. 5A, lane 2, band 1). Furthermore, it revealed the concomitant appearance of a low molecular weight band of short oligonucleotides, or possibly mononucleotides, resulting from an endo- or exo-nuclease activity (Fig. 5A, lanes 2 and 3, band 3). This band represents the 16% (Fig. 5A, lane 2, band 3) and 15% (Fig. 5A, lane 3, band 3) of the total dsDNA existing in the corresponding lanes, respectively. In addition, a broad smearing of oligonucleotide products representing 81.5% of the total dsDNA was observed after 1h of incubation, indicating dsDNA endonuclease activity of the DING protein (Fig. 5A, lane 3). No degradation products were observed in the corresponding controls. The enzyme may act initially as a dsDNA endonuclease and subsequently as exonuclease since both oligonucleotides and mononucleotides were produced. This suggestion is in agreement with the data obtained with the synthetic substrate p-nitro-phenyl thymidine-3′-monophosphate (Table 4) indicating the 3′-exonucleolytic activity of the enzyme.

The DING protein interacts with a supercoiled substrate such as pDNA. Figure 5B demonstrates the distribution of topoisomers, which was created in the presence of *T. thermophilus* enzyme. A band-shifting of the pDNA and appearance of high molecular weight bands could be attributed to the formation of structures like concatamers (or catenanes) (Fig. 5B, lanes 2 and 3, bands 1 and 2), with reduced electrophoretic mobility compared to supercoiled and relaxed pDNA (Fig. 5B, lanes 2 and 3). The bands constituted the 10.6 and 21.3% of the total pDNA amount in the lanes 2 and 3 (band 1), and 4.3 and 4.4% in the same lanes

A

Lanes / Rows	Lane 1 Amount (%)	Lane 2 Amount (%)	Lane 3 Amount (%)
1	-	8.20	3.5
2	100	75.46	81.5
3	-	16.34	15

B

Lanes / Rows	Lane 1 Amount (%)	Lane 2 Amount (%)	Lane 3 Amount (%)
1	-	10.6	21.3
2	-	4.3	4.4
3	36.6	22.7	34.3
4	63.4	38.7	19.1
5	-	23.7	20.9

C

Lanes / Rows	Lane 1 Amount (%)	Lane 2 Amount (%)
1	-	5
2	100	72
3	-	23

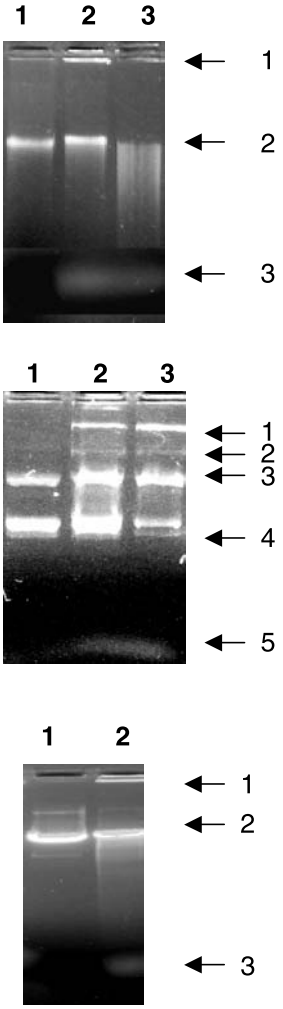

Fig. 5. Binding of the purified DING protein to various DNA forms. The protein was incubated with dsDNA and pDNA (10 μg) and the type of activity was assessed by gel electrophoresis. The reactions were carried out in the assay buffer in the absence of ATP at 65 °C. Lanes 1A, 1B and 1C: dsDNA, pDNA and linearized pDNA with a restriction enzyme incubated without enzyme for 1 h (control). Lanes 2A, 2B and 2C: dsDNA, pDNA and linearized pDNA with a restriction enzyme incubated with the alkaline phosphatase (9 units) for 30 min and lanes 3A, 3B: dsDNA and pDNA were incubated for 60 min. All these experiments were performed at least in duplicate

(band 2), respectively. This was accompanied by the corresponding decrease of the amount of the plasmid bands (supercoiled and relaxed) on the agarose gel due to its migration. These observations indicated the interaction of the DING protein with DNA resulting to the formation of stable protein-DNA complexes. In addition, a small amount of a low molecular weight DNA band appeared (band 5) corresponding to degradation products. Similar results were obtained with linearized pDNA (Fig. 5C). The degradation products estimated to be 23% of the total amount of the linearized pDNA (Fig. 5C, lane 2, band 3).

To confirm the interaction of the DING protein with dsDNA, a gel retardation assay was performed with a short 93-bp linear duplex DNA. In the presence of high concentration of the *T. thermophilus* DING protein a strong band-shifting appeared indicating the formation of stable DING protein-DNA complexes (data not shown) However, complexes of the DING protein with high-polymerized dsDNA did not produce well-spaced bands with the 93-bp linear duplex DNA indicating that the result may have been affected either by the heterogeneity of DNA structure or the high molecular weight or the instability of the complexes.

Furthermore, the degree of DNA degradation was assessed by the release of acid-soluble oligonucleotides in the absence or presence of ATP. Upon incubation of either ssDNA or dsDNA with the DING enzyme a significant release of acid-soluble oligonucleotides was obtained above the background levels. In both assays the presence of 40 μM ATP stimulated enzyme activity by approximately 35–40% (data not shown). In all these cases appropriate control experiments were performed to monitor the presence of DNA in the enzyme preparation (data not shown). Thus, the DING enzyme exhibited ability to hydrolyze native dsDNA and ssDNA.

Cation requirements and effect of putative inhibitors

The activity of the DING purified enzyme (Lysine-sepharose step) was assayed under standard conditions in the

Table 5. Effect of various activators and inhibitors on the DING phosphorolytic activity

Activator/inhibitor	Concentration used (mM)	Enzyme activity (%)
Enzyme dialyzed (control)		100
$MgCl_2$	1	104
$CaCl_2$	1	113
$MnCl_2$	1	97
$ZnCl_2$	1	34
$NiCl_2$	1	35
$CoCl_2$	1	70
$CdCl_2$	1	21
$PbCl_2$	1	32
$HgCl_2$	1	16
EDTA	1	26
Molybdate	10	56
Pyrophosphate	1	14
Tartrate	50	96
Vanadate	0.010	52
NaF	5	89

presence of 1 mM of various bivalent cations, which were added directly to the purified enzyme. Of the metal ions tested, only Ca^{2+} showed limited activation (13%) of phosphorolytic activity (Table 5). All other cations either had limited effect on enzyme activity (Mg^{2+}, Mn^{2+}) or showed inhibitory action in the order Co^{2+} (30%) < Ni^{2+} (65%) < Zn^{2+} (66%) < Cd^{2+} (79%) < Hg^{2+} (84%). In addition, the activity of the DING enzyme was assayed in the presence of various putative inhibitors. The enzyme was pre-incubated at room temperature for 10 min in the presence of each putative inhibitor. The residual activity was assayed under standard conditions. The enzyme barely functioned in the presence of 1 mM of pyrophosphates and it was inhibited significantly by the presence of 1 mM EDTA. Among the other widely recognized inhibitors tested, vanadate and molybdate inhibited enzyme activity by approximately 48 and 44%, respectively, while fluoride and tartrate inhibited to a lesser extent (Table 5).

Effect of fatty acids

In Gram-positive bacteria, the substrate binding protein (SBP) of ABC transporters possesses a membrane lipoprotein attachment site next to a hydrophobic signal sequence. In Gram-negative bacteria the SBP is located in the periplasm (Braibant et al., 2000). As shown in Fig. 6, a certain chain-length of fatty acids is required for DING enzyme activation. Fatty acids with chain length of C6–C10 activated the enzyme by approximately 30% at 1 mM. Palmitic or stearic acid with C16 or C18 respectively inhibited dramatically phosphorolytic activity at

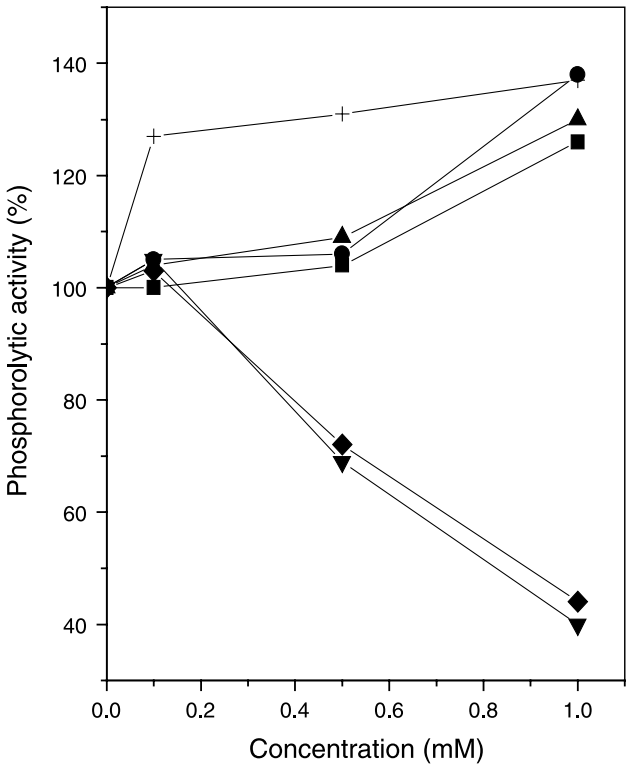

Fig. 6. Effect of fatty acids on DING phosphorolytic activity. Enzyme preparations (0.05 units) were incubated at 70 °C at pH 12.3 for 30 min with different amounts of fatty acids. Caproic acid C6 (■), caprylic acid C8 (●), capric C10 (▲), palmitic C16 (▼), steatic C18 (◆) and oleic acid (+)

the same concentration. However, oleic acid with a chain length of C18 and one double bond increased the phosphorolytic activity by approximately 30% at 0.1–1mM, suggesting the requirement for unsaturated fatty acids for the activation of DING phosphorolytic activity.

In the presence of 20 μg/ml of phosphatidyl ethanolamine, the phosphorolytic activity was increased by approximately 15%, while higher concentrations (of 40, 60 and 100 μg/ml) caused smaller increase of approximately 9, 7 and 4% respectively, (data not shown). The smaller induction of 6% by 20 μg/ml of L-lysophosphatidyl choline may be attributed to the fact that phospholipids are physiological cellular constituents accounting for 30% of the total lipids, identified as phosphoethanolamine (4%), phosphatidylglycerol (3%), phospahtidylinositol (10%), cardiolipin (3%), phosphatidic acid (1%) and other lipids, in the closely relative strain *T. aquaticus*. The increased *T. thermophilus* DING phosphorolytic activity by the aforementioned fatty acids, and phospholipids suggested that the enzyme might have a requirement for lipids that reconstitute a physiological hydrophobic environment, similar to the one existing *in vivo*.

Effect of okadaic acid

The sensitivity of purified DING phosphatase to okadaic acid was studied. Okadaic acid is a polyether derivative of a C38 fatty acid, produced by toxigenic dinoflagellates of the *Dinophysis* and *Prorocentrum* species (Meštrović and Pavela-Vrančič, 2003). Okadaic acid did not inhibit *T. thermophilus* DING phosphorolytic activity up to 1 μM (data not shown).

Discussion

The *T. thermophilus* alkaline phosphatase characterized here represents one of the sparse phosphatases purified so far from thermophilic microorganisms. The enzyme possessed the characteristic DING N-terminal amino acid sequence and showed multiple nucleotide-processing activities, including ATPase, dsDNA endonuclease and 3′-exonuclease activity as well as capability of aggregating pDNA, functioning optimally at high temperature and pH values thus being thermostable and hyperalkaline.

The DING proteins present functional similarities between pro- and eukaryotes (Berna et al., 2002). Only a few bacterial strains are known to possess a eukaryotic type DING protein (Berna et al., 2002), arguing against the very early evolution of the mature DING protein with a fundamental role in a common ancestor of all three kingdoms. Microbial DING proteins may have functional and structural similarities with various animal proteins, involved in the generation and immobilization of free phosphate, an essential part of calcification in animal systems (Berna et al., 2002). This occurs in arthritic synovial fluid where nucleoside triphosphate pyrophosphatase is thought to be a key enzyme (see Table 2, human synovial stimulatory protein) (Pattrick et al., 1991; Ryan et al., 1991). The investigation of substrate specificity and kinetic properties of *T. thermophilus* DING protein showed that the enzyme displayed adenosine triphosphate pyrophosphatase activity, exhibiting the lowest K_m value of 0.006 mM for ATP, amongst the substrates tested.

Interestingly, in spite of the significant sequence homology of *P. fluorescens* (Pflu) DING protein with periplasmic phosphate-binding proteins from other Gram-negative bacteria, and the great similarity of the N-terminal sequence with *T. thermophilus* DING enzyme (Table 2), the Pflu DING protein, does not exhibit detectable phosphatase activity and binds phosphate, with relatively low affinity (Scott and Wu, 2005).

In addition, the *T. thermophilus* DING enzyme demonstrated phosphodiesterase activity besides its phosphomonoesterase activity; a feature common to the homologue *P. aeruginosa* L-AP (Tan and Worobec, 1993), which is indicative of the capability for DNA affinity, binding and hydrolysis. Secondary nucleolytic activities such as ATPase and prokaryotic DNA polymerase is displayed by the DING of *E. coli* (Koonin, 1993; Voloshin et al., 2003), while a phosphatase purified from *E. coli* was able to hydrolyze phosphomono- or diester bridges between the phosphate and the 3′-hydroxyl group of a DNA chain, with the characteristics of a DNA phosphatase-exonuclease (Richarson et al., 1964). An ATP-dependent deoxyribonuclease from *B. subtilis* possesses three activities such as DNase on linear single and dsDNA, DNA unwinding and ATPase activity (Shemyakin et al., 1979).

T. thermophilus DING protein isolated from the cytoplasm lacked its signal peptide present to other homologues, such as *P. aeruginosa* L-AP (Tan and Worobec, 1993), indicating that the DING protein transport from the periplasmic space towards the cytoplasm may takes place after truncation of its signal peptide. Moreover, its activation by oleic acid could be attributed to a hydrophobic origin of the enzyme that may initially be localized in the periplasm, or to its stabilization at high temperatures. Periplasmic expression of DING proteins appears to be related to signalling components that are able to interact with signal molecules only after passing through the bacterial outer membrane (Scott and Wu, 2005). Some related signalling pathways are linked to the activation of cell growth, and appear to contribute to abnormal cell proliferation, which is a feature in both cancer and RA (Lukashev and Werb, 1998), in which DING proteins are present (see related proteins in Table 2). In addition, the component of the SOS regulon *Din*G of *E. coli* is transcriptionally induced in response to DNA damage by treatments like the heat shock adaptive response (Lewis et al., 1992). In Gram-negative bacteria DING proteins are periplasmic binding proteins, which concentrate ions and other metabolites from the environment, prior to uptake by cytoplasmic membrane transporters (Scott and Wu, 2005). This is consistent with expression of the *P. aeruginosa* L-AP after exposure to phosphate poor media.

T. thermophilus phosphatase activity was reduced to about 26% in the presence of the chelating agent EDTA attributing a metalloenzyme character to the protein. However, although the presence of metal ions such as Mg^{2+} or Ca^{2+}, slightly increased the enzyme activity, the thermostability of the enzyme was affected only at high temperatures. Similarly, the thermostability of the *T. neapolitana* alkaline phosphatase was increased in the presence of Co^{2+} and Mg^{2+} (Dong and Zeikus, 1997), while Mg^{2+}

has been reported to play an important role in thermostability and structural stabilization of the catalytically active form of *E. coli* alkaline phosphatase metalloenzyme (Janeway et al., 1993). Thus, Ca^{2+} may replace Mg^{2+} in the *T. thermophilus* apoenzyme. Moreover, *T. thermophilus* alkaline phosphatase was inhibited by Zn^{2+} exhibiting similar behavior to the Tca alkaline phosphatase precursor from *Thermus caldophilus* GK24, for which it was suggested a direct involvement in some of the different metal binding sites (Park et al., 1999). The inhibition of *T. thermophilus* DING enzyme by vanadate, a putative inhibitor of phosphatases, ATPase activity and DNA binding capability of the ABC ATPases superfamily (Pezza et al., 2002) might suggest that this enzyme may involved to DNA recombination and repair mechanisms. On the other hand, *T. thermophilus* DING enzyme was not affected by okadaic acid an inhibitor of protein phosphatases 1 (PP1) and 2A (PP2A).

Alongside to this background, the high degree of structural conservation of microbial, animal and plant DING proteins argues for a fundamentally important role of *T. thermophilus* DING phosphatase in a signal transduction mechanism and in the DNA repair/DNA metabolism, which deserves elucidation. The physiological mechanisms in which are involved may be related with stress conditions like phosphate limitation, mutagenesis or DNA damage.

Acknowledgments

The authors are grateful to Pr. J. G. Georgatsos for his valuable discussion and C. Papaneophytou for his technical assistance on the figure presentation.

References

Adams L, Davey S, Scott K (2002) The DING protein: an autocrine growth-stimulatory protein related to the human synovial stimulatory protein. Biochim Biophys Acta 1586: 254–264

Albers SV, Van de Vossenberg JLCM, Driessen AJM, Konings WN (2001) Bioenergetics and solute uptake under extreme conditions. Extremophiles 5: 285–294

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402

Bairoch A, Boeckmann B (1992) The SWISS_PROT protein sequence data bank. Nucleic Acids Res 20: 2019–2022

Baykov AA, Evtushenko OA, Avaeva SM (1988) A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay. Anal Biochem 171: 266–270

Belenky M, Prasain J, Kim H, Bames S (2003) DING, a genistein target in human breast cancer: a protein without a gene. J Nutr 133: 2497S–2501S

Berna A, Bernier F, Scott K, Stuhlmuller B (2002) Ring up the curtain on DING proteins. FEBS Lett 524: 6–10

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Braibant M, Gilot P, Content J (2000) The ATP binding cassette (ABC) transport systems of *Mycobacterium tuberculosis*. FEMS Microbiol Rev 24: 449–467

Cava F, de Pedro MA, Schwarz H, Henne A, Berenguer J (2004) Binding to pyruvylated compounds as an ancestral mechanism to anchor the outer envelope in primitive bacteria. Mol Microbiol 52: 677–690

Dong G, Zeikus JG (1997) Purification and characterization of alkaline phosphatase from *Thermotoga neapolitana*. Enzyme Microb Technol 21: 335–340

Hain NA, Stuhlmuller B, Hahn GR, Kalden JR, Deutzmann R, Burmester GR (1996) Biochemical characterization and microsequencing of a 205-kDa synovial stimulatory protein for T cells and reactive with rheumatoid factor containing sera. J Immunol 157: 1773–1780

Huang C-T, Xu KD, McFeters GA, Stewart PS (1998) Spatial patterns of alkaline phosphatase expression within bacterial colonies and biofilms in response to phosphate starvation. Appl Environ Microbiol 64: 1526–1531

Janeway CM, Xu X, Murphy JE, Chaidaroglou A, Kantrowitz ER (1993) Magnesium in the active site of *Escherichia coli* alkaline phosphatase is important for both structural stabilization and catalysis. Biochemistry 32: 1601–1609

Kerr ID (2002) Review: structural and association of ATP-binding cassette transporter nucleotide-binding domains. Biochim Biophys Acta 1561: 47–64

Koonin EV (1993) *Escherichia coli din*G gene encodes a putative DNA helicase related to a group of eukaryotic helicases including Rad3 protein. Nucleic Acids Res 21: 1497

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lewis LK, Jenkins ME, Mount DW (1992) Isolation of DNA damage-inducible promoters in *Escherichia coli*. Regulation of *pol*B (*din*A), *din*G, and *din*H by LexA repressor. J Bacteriol 173: 3377–3385

Lioliou EE, Pantazaki AA, Kyriakidis DA (2004) *Thermus thermophilus* genome analysis: benefits and implications. Microb Cell Fact 3: 5–7

Lukashev ME, Werb Z (1998) ECM signalling: orchestrating cell behaviour and misbehaviour. Trends Cell Biol 8: 437–441

Meštrović V, Pavela-Vrančič M (2003) Inhibition of alkaline phosphatase activity by okadaic acid, a protein phosphatase inhibitor. Biochimie 85: 647–650

Oshima T, Imahori K (1974) Description of *Thermus thermophilus*, com. nov., a non sporulating thermophilic bacterium from a Japanese thermal spring. Int J Syst Bacteriol 24: 102–112

Pantazaki AA, Karagiorgas AA, Liakopoulou-Kyriakides M, Kyriakidis DA (1998) Hyperalkaline and thermostable phosphatase in *Thermus thermophilus*. Appl Biochem Biotechnol 75: 249–259

Pantazaki AA, Pritsa AG, Kyriakidis DA (2002) Biotechnologically relevant enzymes from *Thermus thermophilus*. Appl Microbiol Biotechnol 58: 1–12

Park T, Lee JH, Kim HK, Hoe HS, Known ST (1999) Nucleotide sequence of the gene for alkaline phosphatase of *Thermus caldophilus* GK24 and characteristics of the deduced primary structure of the enzyme. FEMS Microbiol Lett 180: 133–139

Pattrick M, Hamilton E, Hornby J, Doherty M (1991) Synovial fluid pyrophosphate and nucleoside triphosphate pyrophosphatase: comparison between normal and diseased and between inflamed and non-inflamed joints. Ann Rheum Dis 50: 214–218

Peterson ME, Eisenthal R, Danson MJ, Spence A, Daniel RM (2004) A new intrinsic thermal parameter for enzymes reveals true temperature optima. J Biol Chem 279: 20717–20722

Pezza RJ, Villarreal MA, Montich GG, Argarana CE (2002) Vanadate inhibits the ATPase activity and DNA binding capability of bacterial MutS. A structural model for the vanadate-MutS interaction at the Walker A motif. Nucleic Acids Res 30: 4700–4708

Razzell WE, Khorana HG (1959) Studies on polynucleotides III. Enzymic degradation. Substrate specificity and properties of snake venom phosphodiesterase. J Biol Chem 234: 2105–2113

Richarson CC, Lehman IR, Kornberg A (1964) A deoxyribonucleic acid phosphatase-exonuclease from *E. coli*. II. Characterization of the exonuclease activity. J Biol Chem 239: 251–258

Ryan LM, Rachow JW, McCarty DJ (1991) Synovial fluid ATP: a potential substrate for the production of inorganic pyrophosphate. J Reumatol 18: 716–720

Sander M (1997) In situ activity gel for DNA repair 3′-phosphodiesterase. Nucleic Acids Res 25: 1868–1869

Scott K, Wu L (2005) Functional properties of a recombinant bacterial DING protein: comparison with a homologous human protein. Biochim Biophys Acta 1744: 234–244

Shemyakin MF, Grepachevsky AA, Chestukhin AV (1979) Properties of *Bacillus subtilis* ATP-dependent deoxyribonuclease. Eur J Biochem 98: 417–423

Tan AS, Worobec EA (1993) Isolation and characterization of two immuno-chemically distinct alkaline phosphatases from *Pseudomonas aeruginosa*. FEMS Microbiol Lett 106: 281–286

Voloshin ON, Vanevski F, Khil PP, Camerini-Otero RD (2003) Characterization of the DING damage-inducible helicase *Din*G from *Escherichia coli*. J Biol Chem 278: 28284–28293

Walker JE, Saraste M, Runswick MJ, Gay NJ (1982) Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J 1: 945–951

Werner A, Kinne RKH (2001) Evolution of the Na-Pi cotransport systems. Am J Physiol Regul Integr Comp Physiol 280: R301–R312

Authors' address: Assist. Prof. A. A. Pantazaki, Laboratory of Biochemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece,
Fax: + 30-2310-997689, e-mail: natasa@chem.auth.gr