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Inhibition of an archaeal protein phosphatase activity by okadaic acid, microcystin-LR, or calyculin A

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Soluble extracts of the methanogenic archaeon, *Methanosarcina thermophila* TM-1, contained a divalent metal ion-stimulated protein–serine phosphatase activity. This activity was sensitive to micromolar concentrations of okadaic acid, microcystin-LR, or calyculin A, three compounds thought to be highly specific inhibitors of the type 1/2A/2B genetic superfamily of eukaryotic protein–serine/threonine phosphatases. The observation that each of these three chemically unrelated compounds inhibited this archaeal protein phosphatase activity suggests the existence of structural homology, and perhaps even common genetic ancestry, with the type 1/2A/2B superfamily of protein–serine/threonine phosphatases found in eukaryotic organisms.

Protein phosphatase; Okadaic acid; Microcystin-LR; Calyculin A; Archaea

1. INTRODUCTION

The modulation of protein function by phosphorylation and dephosphorylation is an ubiquitous regulatory phenomenon employed by organisms of all morphologies, ancestries, and habitats. Despite this universality, the lack of molecular parallels or links between protein kinases or protein phosphatases from eukaryotic and prokaryotic organisms at either the structural, functional, or immunological level (reviewed in [1]) has long been regarded as evidence that the origin and evolution of the protein phosphorylation networks within each of the individual phylogenetic domains were unique. Recently, however, several observations have come to light suggesting that the lines of descent of the protein kinases and protein phosphatases in eukaryotic and bacterial organisms may not have been so completely segregated as previously supposed [2–6].

We have extended the search for molecular parallels to the third phylogenetic domain in nature, the Archaea. Herein we report that extracts of the archaeon, *Methanosarcina thermophila* TM-1 [7], contained a protein–serine phosphatase activity that was sensitive to a set of inhibitors widely thought to specifically target the protein phosphatases of the type 1/2A/2B genetic superfamily in eukaryotes: okadaic acid, microcystin-LR, and calyculin A [8,9]. To our knowledge, this represents the first instance in which sensitivity to these compounds has been observed in a protein phosphatase from a prokaryotic (i.e. archaeal or bacterial) organism of any kind.

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2. MATERIALS AND METHODS

2.1. *Materials*

Purchased materials included the ammonium salt of okadaic acid (LC Services Corp., Woburn, MA); calyculin A and microcystin-LR (Gibco, Bethesda Research Laboratories, Gaithersburg, MD); lysozyme, maleic anhydride, and dioxane (Sigma, St. Louis, MO); and DNase I (Boehringer-Mannheim, Indianapolis, IN). Heat-stable protein phosphatase inhibitor-2 was the gift of Prof. Shirish Shenolikar of Duke University. All other materials were from previously listed sources [10].

2.2. *Growth of organism and preparation of extracts*

Cells of *Methanosarcina thermophila* strain TM-1 [7] were grown anaerobically on acetate in a 10 liter pH auxostat, harvested using a continuous-flow centrifuge, and frozen in liquid nitrogen as described by Sowers et al. [11]. Soluble extracts were prepared anaerobically as described previously [12] with the following modifications: (i) the breakage buffer consisted of 50 mM MOPS, pH 6.8, containing 10 mM $MgCl_2$, 10% (v/v) glycerol, and 0.1 mg/ml DNase I, (ii) the disrupted cells were spun at 4,200 × *g* instead of 27,000 × *g*, and (iii) the resulting supernatant was dialyzed vs. 10 mM MES, pH 6.5. Aliquots of soluble extract were stored frozen at −20°C prior to use.

2.3. *Preparation of DE-52 fraction*

Soluble extract, 10 ml, was thawed and loaded onto a 10–15 ml column of DE-52 cellulose equilibrated in 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5. The column was washed with 10 mM MES, pH 6.5, then developed with a linear salt gradient of 50 ml each of 10 mM MES, pH 6.5, and 10 mM MES, pH 6.5, containing 400 mM KCl. Fractions, 2 ml each, were collected and assayed for protein phosphatase activity as described below. Active fractions were pooled to give the DE-52 fraction, which was then divided into aliquots and stored frozen at −20°C.

2.4. *Protein and phosphate analyses*

Protein concentrations were determined as described by Bradford [13] using pre-mixed reagent and a standardized solution of bovine serum albumin from Pierce (Rockford, IL). Inorganic phosphate was identified by thin-layer electrophoresis or extraction into organic solvents as a molybdic acid complex as previously described [10].

2.5. *Preparation of ^{32}P-labeled phosphoproteins and phosphopeptides*

Casein and mixed histones were phosphorylated using [γ-^{32}P]ATP and the catalytic subunit of the cAMP-dependent protein kinase essentially as described previously [10]. Glycogen phosphorylase *a* was prepared using [γ-^{32}P]ATP and the components of the Protein Phosphatase Assay System from Gibco, Bethesda Research Laboratories, following the manufacturer's protocols. Reduced, carboxyamidomethylated and maleylated lysozyme was prepared and subsequently phosphorylated using [γ-^{32}]ATP and recombinant mouse *lyn* protein tyrosine kinase, as described by Sheng and Charbonneau [14]. The *E. coli* strain expressing the recombinant *lyn* protein–tyrosine kinase was the generous gift of Prof. Marietta Harrison of Purdue University.

2.6. *Assay of protein phosphatase activity*

Protein phosphatase activity was assayed as previously described [10]. Briefly, samples of protein phosphatase were incubated at 25°C in a volume of 30 μl containing 20 mM MES, pH 6.5, 20 mM $MnCl_2$, 0.33 mg/ml BSA, and 2–4 μM casein-bound [^{32}P]phosphate. Reaction was terminated, typically after an incubation period of 60 min, by the addition of 100 μl of 20% (w/v) trichloroacetic acid, mixed, centrifuged for 3 min in a microfuge at maximum speed, and 50 μl of the supernatant liquid removed and counted for ^{32}P radioactivity in 2.0 ml of Eco-Lume liquid scintillation cocktail. An identical procedure was used for the assay of protein phosphatase activity toward glycogen phosphorylase *a* and mixed histones with the exception that, for the last named, the trichloroacetic acid concentration was increased to 32.5% (w/v).

3. RESULTS

Incubation of casein that had been phosphorylated using [γ-^{32}P]ATP and the catalytic subunit of the cAMP-dependent protein kinase with soluble extracts from *Methanosarcina thermophila* TM-1 in the presence of the divalent metal ion, Mn^{2+}, resulted in the release trichloroacetic acid-soluble ^{32}P radioactivity from phosphorylated casein. The reaction product was identified as inorganic phosphate by comparing its migration with that of genuine inorganic phosphate by thin-layer electrophoresis and by quantitatively extracting it into organic solvents as a molybdate complex (data not shown). We observed that soluble extracts also contained what appeared to be an endogenous inhibitor of this phosphocasein protein phosphatase activity. Since this inhibitory factor could be removed by ion-exchange chromatography on DE-52 cellulose, all of the experiments described below were performed using protein phosphatase that had been partially purified in this manner.

In addition to Mn^{2+}, protein phosphatase activity was stimulated by the addition of Ni^{2+} or Co^{2+} (Fig. 1). The addition of any second divalent metal ion in combination with the most effective activator, Mn^{2+}, produced no greater activation than was observed with Mn^{2+} alone (Fig. 1), indicating that only a single type of divalent metal ion-stimulated protein phosphatase was present in these extracts. The chromatographic behavior of protein phosphatase activity on DE-52 ion-exchange columns was also consistent with the presence of a single type of divalent metal ion-stimulated protein phosphatase (data not shown). A survey of a wide range of other potential activators and inhibitors revealed that none of these compounds was able to stimulate protein phosphatase activity significantly above the level produced by divalent metal ions alone (Table I).

In addition to casein, the archaeal protein phosphatase dephosphorylated glycogen phosphorylase *a* as well as mixed histones that had been phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase (Fig. 2). No activity was observed toward RCM-lysozyme that was phosphorylated on tyrosine, suggesting that the enzyme may be phosphoserine- (and per-

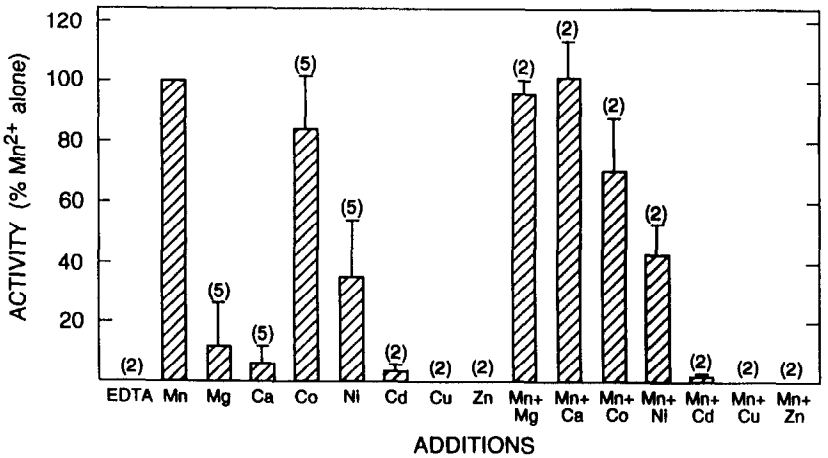

Fig. 1. Stimulation of *M. thermophila* TM-1 protein phosphatase activity by divalent metal ions. The DE-52 fraction, 39 μg, was assayed under standard conditions with the exception that the compounds listed were substituted for the usual activating divalent metal ion, Mn^{2+}. Where present, the concentration of each metal ion was 20 mM and that of EDTA was 5 mM. All results are reported as the percentage of the activity observed with the most potent individual metal ion activator, Mn^{2+}, plus S.E.M. The number of replicates for each measurement is shown in parentheses.

haps phosphothreonine-) specific. Protein phosphatase activity was insensitive to incubation with a wide range of sulfhydryl-modifying reagents, but could be completely eliminated by treatment with diethylpyrocarbonate. Subsequent incubation of diethylpyrocarbonate-treated enzyme with hydroxylamine resulted in partial (approximately 20%) restoration of activity, indicating that this reagent acted, at least in part, through modification of a histidyl residue [15].

Protein phosphatase activity could be completely inhibited by the divalent metal ions, Cd^{2+}, Cu^{2+} or Zn^{2+} (Fig. 1). In addition, the broad-specificity protein phosphatase inhibitors, sodium phosphate, sodium pyrophosphate, and sodium fluoride, all inhibited archaeal protein phosphatase activity (Table I), as did the protein phosphatase 1/2A/2B-specific inhibitors, okadaic acid, microcystin-LR, or calyculin A (Fig. 3). Inhibition by each of the latter three compounds was observed independent of the identity of the phosphoprotein used as substrate, indicating that they most likely were acting upon the protein phosphatase itself. Decreasing the concentration of soluble extract by 10-fold yielded a similar dose–response pattern (data not shown), suggesting that the observed concentration dependence reflected an IC_{50} value in the μM range, and was not the consequence of the titration of a large concentration of protein phosphatase by an extremely avid inhibitor, as has been observed with some samples of mammalian protein phosphatases [9]. Incubation of *M. thermophila* TM-1 in the presence of 10 μM okadaic acid had no discernable effect on either cell growth or methane production. Heparin, a known inhibitor of protein phosphatase 1, also had some inhibitory effect on the enzyme (Table I). Experiments with heat-stable protein phosphatase inhibitor-2 yielded mixed results. Although substantial inhibition, >90%, of the archaeal protein phosphatase was observed when activity toward glycogen phosphorylase *a* was measured, little or no inhibition could be detected when mixed histones or casein were used as substrate (data not shown).

Table I

Effect of potential activators and inhibitors on *M. thermophila* TM-1 protein phosphatase

Addition	Concentration	Activity (% control)
None (control)	–	100
cAMP	1 mM	101 ± 10
cGMP	1 mM	123 ± 16
1,4,5-Inositol trisphosphate	10 μM	126 ± 16
Ca^{2+} plus calmodulin	1 mM and 5 μM	125 ± 7
Spermine	0.1 mg/ml	115 ± 15
Spermidine	0.1 mg/ml	113 ± 14
Poly-L-lysine	0.1 mg/ml	110 ± 11
Poly-L-arginine	0.1 mg/ml	95 ± 6
Heparin	0.1 mg/ml	68 ± 17
	0.6 mg/ml	39 ± 3
Chondroitin sulfate	0.1 mg/ml	106 ± 15
NaF	50 mM	32 ± 20
Tartrate	10 mM	70 ± 12
Tetramisole	1 mM	131 ± 28
p-Nitrophenylphosphate	10 mM	80 ± 18
Sodium phosphate	50 mM	9 ± 6
	10 mM	17 ± 3
Sodium pyrophosphate	10 mM	Not detectable
	1 mM	24 ± 8
Sodium orthovanadate	0.1 mM	74 ± 3
Sodium molybdate	0.1 mM	94 ± 7
β-Glycerol phosphate	10 mM	116 ± 18
Trifluoperazine	150 μM	124 ± 21
ATP	5 mM	102 ± 10
ADP	5 mM	81 ± 15
AMP	5 mM	113 ± 16

The table summarizes the results of several individual experiments. In all cases the DE-52 fraction was assayed for activity under standard conditions with the exception that the compounds listed below were present at the indicated final concentrations. All results represent the average of three or more determinations and are reported as the percent of activity measured in the presence of no added compounds ± S.E.M.

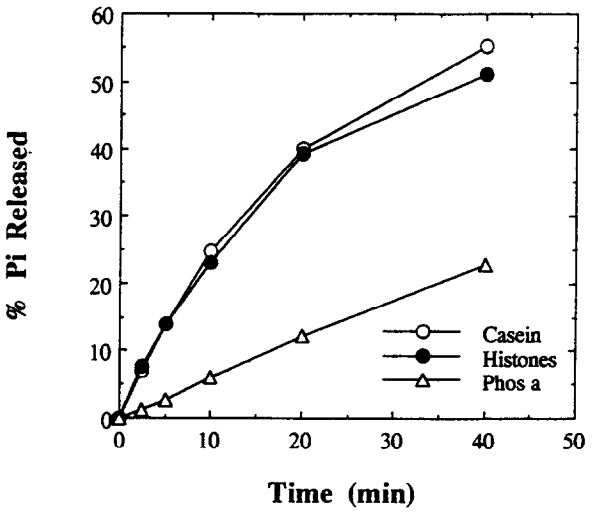

Fig. 2. Comparative rate of dephosphorylation of histones, casein and phosphorylase *a* by *M. thermophila* TM-1 protein phosphatase. The DE-52 fraction, 40 μg, was assayed for activity toward the following phosphoprotein substrates: casein (○), mixed histones (●), and glycogen phosphorylase *a* (△). Standard assay conditions were employed with the exception that (i) the indicated compounds were substituted for the standard substrate, casein, and (ii) the concentration of the trichloroacetic acid solution used to terminate the assay was increased to 32.5% (w/v) to insure complete precipitation of the histones. All substrates were present at a final concentration of 1.7 μM protein-bound phosphate. Shown is the percentage of phosphate released from each substrate as a function of time.

4. DISCUSSION

The methanogenic archaeon, *M. thermophila* TM-1, contained a divalent metal ion-stimulated protein–serine phosphatase activity. With respect to many of its functional properties, i.e. its stimulation by divalent

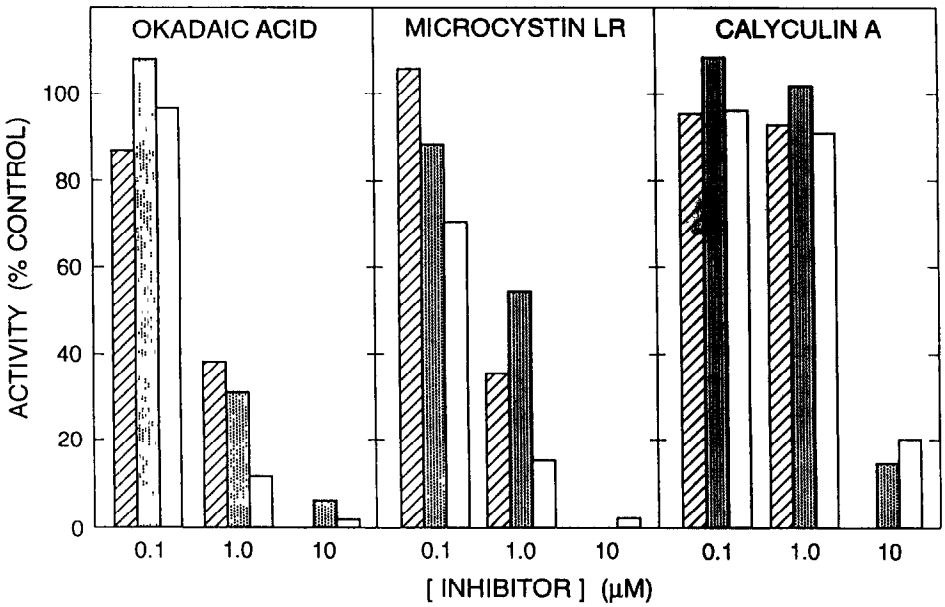

Fig. 3. Inhibition of *M. thermophila* TM-1 protein phosphatase by okadaic acid and related compounds. The DE-52 fraction, 30 μg, was assayed under standard conditions using either casein (hatched bars), histones (stippled bars), or glycogen phosphorylase *a* (open bars), as substrate in the presence of the indicated concentrations of the following inhibitors: okadaic acid (left panel), microcystin-LR (center panel), or calyculin A (right panel). All results are reported as the activity measured relative to that observed in the absence of inhibitors.

metal ions, binding to and elution from hydroxylapatite and DE-52, apparent substrate affinity, and sensitivity to diethylpyrocarbonate, this enzyme strongly resembled the archaeal protein phosphatases that we had previously identified in the extreme acidothermophile, *Sulfolobus solfataricus* [10], and the halophile, *Haloferax volcanii* [16]. Thus, it would appear that these enzymes are members of a common archaeal family of divalent metal ion-stimulated protein–serine/threonine phosphatases. These divalent metal ion-stimulated enzymes represent the only protein phosphatase activities that we have been able to detect thus far in the Archaea using a variety of phosphoserine-containing protein substrates. The protein phosphatase from *M. thermophila* TM-1 different from the other archaeal enzymes, however, in that it could be inhibited by okadaic acid, microcystin-LR, or calyculin A.

Okadaic acid [17], microcystin-LR [18], and calyculin A [19] are three naturally occurring toxins, each of which has been found to be a highly specific and quite potent inhibitor of the type 1/2A/2B superfamily of eukaryotic protein–serine/threonine phosphatases. The observed selectivity of these compounds for the members of this enzyme superfamily is such that sensitivity to them is regarded as their unique functional hallmark [8,9]. Thus, it seems both remarkable and highly significant that all three of these compounds inhibited a protein–serine phosphatase from an organism whose evolutionary pathway diverged from that of the Eucarya eons ago. These three compounds, despite their common inhibitory effects, possess radically different chemical structures: okadaic acid is a polyether fatty acid, microcystin-LR is a cyclic heptapeptide, and calyculin A is a polyhydroxylated fatty acid containing a phosphomonoester and two γ-amino acids. Yet each of them inhibited the *M. thermophila* TM-1 protein–serine phosphatase. While this archaeal enzyme, at least in partially purified form, did not exhibit the spectacular nanomolar affinities for these inhibitors characteristic of the type 1 and 2A protein phosphatases, its observed sensitivity to micromolar concentrations of these compounds was quite comparable to, if not slightly greater than, that exhibited by the other member of this genetic superfamily, protein phosphatase 2B. Since these compounds apparently target a region outside the active site of the eukaryotic enzymes [8], it seems unlikely that the archaeal protein phosphatase's sensitivity would have arisen as a random by-product of its adaptation to the performance of a similar catalytic function.

The implication of our observations is that inhibition by this set of structurally disparate compounds represents the functional manifestation of some degree of structural homology between this archaeal enzyme and a prominent family of eukaryotic protein–serine/threonine phosphatases. Whether this homology is confined to some limited, discrete region or proves to be more global in nature must await the determination of the sequence of the *M. thermophila* TM-1 protein phosphatase. However, it is noteworthy that for those archaeal biomolecules that have been cloned and sequenced to date, homology with their eukaryotic counterparts has proved to be the norm rather than the exception [20].

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