IDENTIFICATION OF THE ACID PHOSPHATASE (OPTIMUM pH 2.5) OF *ESCHERICHIA COLI*

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1. Introduction

A 1 M formic acid supernatant of a crude acellular extract of *Escherichia coli* K12 has been reported to catalyze the hydrolysis of the γ phosphorus ester bond of UTP and to a lesser extent of GTP, while in CTP and ATP, this reaction was much slower [1]. The corresponding β residues were about ten times more resistant and the α bonds in nucleoside monophosphates were unaffected. The regulatory nucleotide guanosine 5′-diphosphate 3′-diphosphate (ppGpp) however, was sequentially degraded into pGpp and finally pGp. The enzyme present in this supernatant and responsible of these degradations was also able to hydrolyze paranitrophenyl phosphate (PNP-P) with a good efficiency.

About 12% of the activity present in bacteria could be extracted by a single wash of whole cells with Tris buffer (pH 7.8) containing 1 mM EDTA. The specific activity of this fraction was 90 times higher than in crude extracts.

The identity of this peculiar phosphatase with the acid phosphatase activity described earlier [2] was postulated. None of these enzymes, however, have presently been identified or characterized.

The present report is concerned both with the subcellular location of the enzyme and with its identification. Some of its essential characteristics are described and discussed.

2. Materials and methods

2.1. *Strain and media*

Escherichia coli K12 strain K10 (*HfrC; relA*) was used throughout this work. Cells were grown in MLT rich medium [3].

2.2. *Preparation of bacterial fractions*

Bacteria were disrupted by sonic oscillation and unbroken cells were removed by low-speed centrifugation. The lysate was first spun down at 30 000 × *g* and the pellet constituted the 'crude membrane' fraction. The supernatant was re-centrifuged at 100 000 × *g*; the pellet of this second centrifugation was named 'crude ribosomes' and the supernatant 'cytoplasm'. Osmotic shock was performed according to [4]; membranes of osmotically shocked bacteria were prepared according to [5]. An 'EDTA wash' of whole cells was prepared and concentrated as previously described [1].

2.3. *Enzyme assays*

The phosphomonoesterase activity was measured by its ability to hydrolyse PNP-P in glycine-HCl buffer (85 mM pH 2.5). The concentration of PNP-P was, unless otherwise stated, 20 mM and the volume of enzymatic extract never exceeded 10% of the total volume. The reaction was stopped by diluting aliquots into 10 vol. of 0.1 N NaOH, allowing the determination of PNP-P concentration by absorbance at 410 nm. One unit of enzyme was defined as the activity able to hydrolyze 1 nmol of the synthetic substrate paranitrophenylphosphate (PNP-P)/min at 37°C under our standard assay conditions as described above.

3. Results

The activity present in crude extracts or in the EDTA wash of whole cells, which hydrolyzed the phosphate bonds of some nucleoside polyphosphates and PNP-P, did not split bis-paranitrophenyl phosphate (bis-PNP-P) under the same conditions. Inorganic pyrophosphate was not degraded and monophosphorylated sugars such as glucose-6-phosphate, were a

hundred times less susceptible than PNP-P (data not shown). Hence it behaved as a phosphomonoesterase (EC 3.1.3.2) with a restricted substrate specificity. For these reasons, PNP-P was used as substrate in all the following experiments.

3.1. *Subcellular distribution of the enzyme*

The repartition of the acid phosphomonoesterase among some fractions recovered after cell disruption is indicated in table 1. Its absence from the sedimentable material and its complete solubility in 1 M formic acid [1] apparently argues in favor of a soluble enzyme. Most of the activity, however, originally lay at the periphery of the intact cell: this was suggested by an important recovery in the 'shock fluid' obtained by the procedure of Neu and Heppel [4], which is known to remove a number of so-called periplasmic proteins. The activity remaining within the cells was mostly recovered in the 30 000 × *g* supernatant of broken bacteria.

3.2. *Characterization of the acid phosphatase*

As shown in table 1, the enzymatic specific activity of the EDTA extract was higher than in the other acellular fractions. Consequently this 'EDTA wash', although containing only a small part of the total activity, was considered as a convenient material for a possible direct identification of the enzyme by two-dimensional slab gel electrophoresis, according to [6]. The enzymatic activity was demonstrated in the first dimension by incubating a longitudinal section of the gel strip in the presence of PNP-P followed by soaking in 1 M NaOH. After the second run in the presence of SDS, only one polypeptide of apparent MW 45 000 could be seen on the slab, in the region of the strip corresponding to the activity (fig.1). This protein had an isoelectric point of 6.3 and was a minor constituent of the EDTA extract.

The same concentrated 'EDTA wash' was applied to a column of Biogel P100 equilibrated with Tris buffer (pH 7.8). The activity eluted with an apparent MW of 43 000 (fig.2).

The pooled active fractions collected from this column were used for the following experiments.

The dependence of the activity on pH was assayed both in 100 mM HCl containing various amounts of pure glycine to obtain the required final pH, and in 85 mM glycine adjusted for pH with concentrated HCl (fig.3). The optimum was pH 2.5 in both systems although the enzyme was more active at this pH in the second system.

The enzyme which eluted from the Biogel P100 column had no salient requirements for divalent cations (data not shown); its K_m for PNP-P at 37°C and at pH 2.5 was found from the Lineweaver and Burk representation to be 5 mM (fig.4).

4. Discussion

Five different acid phosphatase activities have been distinguished in *E. coli* [7] and among those, one had an optimal pH identical to the enzyme studied here. This unusually low pH allowed Hafkenscheid to compare the susceptibility of various substrates for this enzyme, directly in crude extracts [2]. Mono- and diphosphorylated sugars, adenosine mono- and triphosphate, phosphorylethanolamine and phosphoserine were all found by the last author to be less efficiently hydrolyzed than the synthetic substrate PNP-P. The

Table 1
Selective extraction of the pH 2.5 phosphomonoesterase by a non-disruptive method (the data refer to a 1-liter culture in MLT broth)

Fractions	Total activity (nmol PNP/min)	% total activity	Specific activity (nmol PNP/min/mg protein)
Crude sonicate	3875	100	13.8
'shock fluid'	2600	66.7	200
Cytoplasm (30 000 × *g* supernatant)	855	21.9	10.2
'Membranes'	330	8.5	11.1
Independent 1 mM EDTA wash of whole bacteria	620	16	552

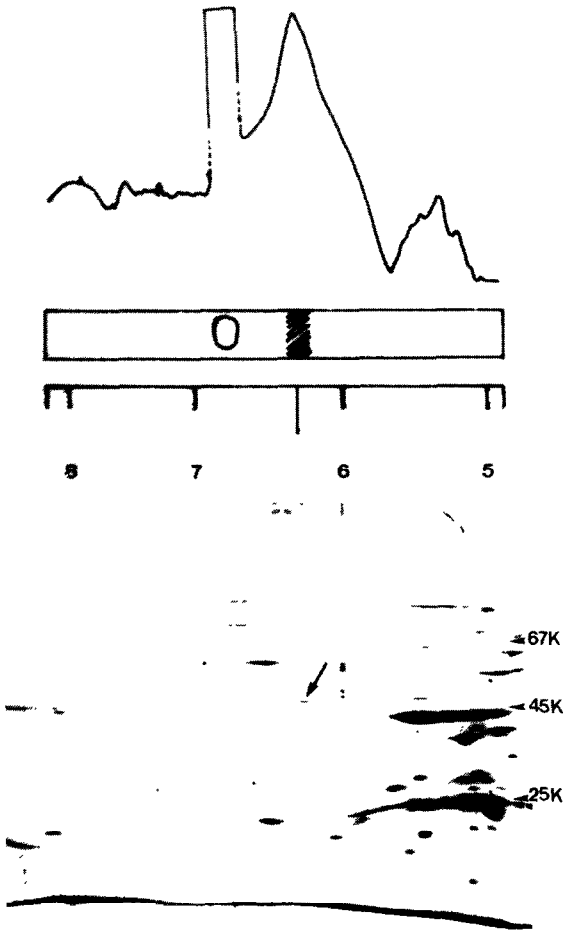

Fig.1. Two dimensional electrophoretic pattern of the proteins present in the 'EDTA wash'. From top to bottom of the figure are represented: a densitometric trace recording of a gel strip of the isoelectric focusing (first dimension); a diagrammatic representation of this gel strip showing the place where the sample was deposited (circle) and the region of maximal yellow coloration (hatched zone); the pH distribution measured in the gel strip; a photograph of the gel slab run in SDS, after staining with Coomassie blue. The protein found in the region corresponding to the activity is indicated by an arrow.

identity of the present phosphatase, whose unusual properties on nucleoside polyphosphates have been reported [1], with that described by the authors mentioned above was strongly suggested by both its low activity on monophosphorylated sugars or adenosine polyphosphates, and its presence in large amounts in the 'shock fluid' of bacteria.

The enzyme studied here which consisted of a single polypeptide chain of apparent MW 45 000, had

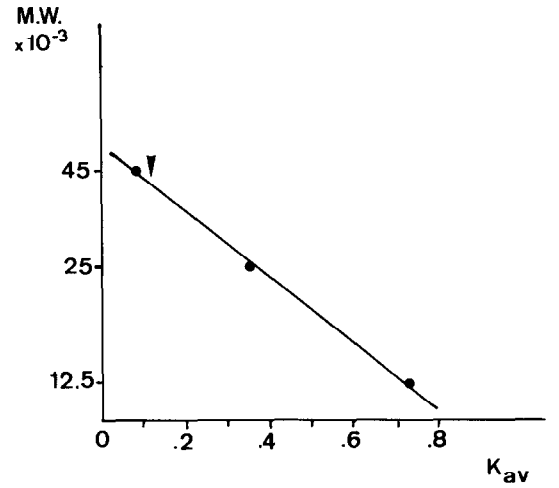

Fig.2. Pattern of elution of the acid phosphatase from a column of Biogel P2. The points refer to the filtration of the following MW standards: ovalbumin (45 000); chymotrypsinogen (25 000); and cytochrome *c* (12 500). The arrow indicates the position corresponding to the activity.

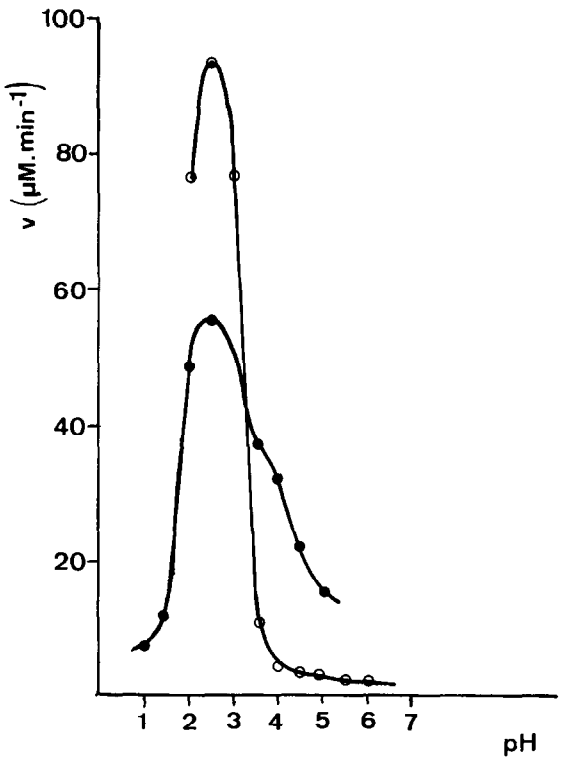

Fig.3. Effect of pH on the activity of the acid phosphatase. 100 units of acid phosphatase were incubated at 37°C with PNP-P (20 mM) either in 85 mM glycine adjusted with concentrated HCl to obtain the required pH (–○–) or in 50 mM HCl adjusted for pH with the pure glycine (–●–).

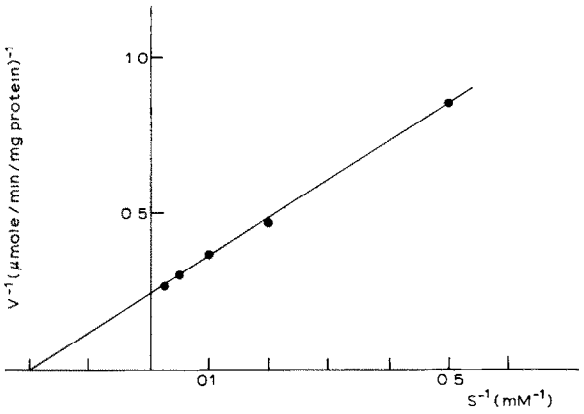

Fig.4. Lineweaver and Burk representation of the initial velocity at 37°C. The experiments were performed with 400 units of enzyme partially purified on Biogel P100 (pooled active fractions).

an isoelectric point of 6.3. It was soluble at pH 2.0, a property shared by few proteins of such MW. It could be detached from the cells by a wash in presence of EDTA, a procedure known to remove not only part of the lipopolysaccharide [8] but also some enzymes normally bound to the cytoplasmic membrane such as the ATPase [9]. This may be an indication of a weak association in vivo with either the outer or the cytoplasmic membrane. The enzyme was not recovered, however, among the 'intrinsic' proteins of both membranes (see table 1).

The unusually low optimal pH displayed by the enzyme and its presence in the shock fluid in fact raised many questions about the existence of the periphery of the cell of a microenvironment where it might function. According to Mitchell's chemiosmotic hypothesis, a difference of two pH units between the inside of the cell and the external medium may be recorded [10]. It is questionable whether newly extruded protons immediately diffuse across the outer membrane, or whether a much slower process does not favor a pH heterogeneity inside the periplasmic space, allowing, for example, a microlayer of high proton density on the outside of the cytoplasmic membrane. This hypothesis is supported by one observation of Tsuchiya and Rosen, that an optimal proton-driven ATP synthesis in membrane vesicles of *E. coli* was obtained for an external pH of exactly 2.5 (the internal pH being 8.2) [11]. Additional evidence for such a mechanism has recently been reported [12].

More information is needed to understand the physiological function of this atypical phosphatase and work is in progress to determine the effects of its genetic alteration.

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