A Salt-Sensitive 3′(2′),5′-Bisphosphate Nucleotidase Involved in Sulfate Activation

Jose Ramón Murguía, Jose María Bellés, Ramón Serrano*

Overexpression of a yeast gene, *HAL2*, allows the cells to tolerate higher than normal extracellular salt concentrations. *HAL2* encodes a 3′(2′)5′-bisphosphate nucleotidase that serves to remove the end products of sulfate transfer during cellular metabolism. The enzyme is inhibited by lithium and sodium and is activated by potassium. Metabolic systems that are sensitive to salt, as well as those governing osmolyte synthesis and ion transport, offer routes by which genetic engineering can be used to improve the tolerance of various organisms to salt.

The progressive salinization of irrigated land has turned the genetic improvement of salt tolerance into an urgent need for the future of agriculture in arid regions (*1*, *2*). The salt tolerance of some crop plants has been improved (*3*, *4*), but knowledge of the molecular basis of salt tolerance will facilitate further progress.

Metabolic systems likely to affect salt tolerance include osmolyte synthesis (*5*, *6*), ion transport (*7*, *8*) at the vacuolar and plasma membranes, and any system sensitive to high intracellular concentrations of salt (*2*). Cellular targets of salt toxicity include protein synthesis and some reactions of sugar metabolism, which are salt-sensitive in vitro (*9*). However, it remains unknown what aspect of cellular metabolism is most sensitive to intracellular salt concentrations in vivo.

We have isolated two yeast genes, *HAL1* and *HAL2*, that when overexpressed improve yeast growth under salt stress (*2*, *10*). *HAL1* confers salt tolerance by modulating cation transport systems (*10*). *HAL2* confers lithium and sodium tolerance and is required for methionine biosynthesis (*11*). These findings suggested that one step of the methionine biosynthetic pathway was especially sensitive to lithium and sodium and that this metabolic route was an important target for salt toxicity. Accordingly, methionine supplementation has been shown to improve salt tolerance in yeast (*11*).

The protein encoded by *HAL2* does not correspond to any of the enzymes required for methionine biosynthesis (*12*). It has homology with inositol phosphatases and with regulatory proteins of unknown function (*11*, *13*). However, the *HAL2* gene product is not an inositol phosphatase, as is demonstrated below.

A hypothesis for the mechanism of action of *HAL2* was suggested by the thermodynamic requirements of sulfate activation (*14*). The reaction catalyzed by adenosine 5′-triphosphate (ATP) sulfurylase to generate adenosine-5′-phosphosulfate (APS) has an overall standard free energy change of +12 kJ/mol, despite the hydrolysis of the liberated pyrophosphate. Therefore, APS does not normally accumulate, and an APS kinase that generates 3′-phosphoadenosine-5′-phosphosulfate (PAPS) is required to drive the reaction and generate appreciable concentrations of activated sulfate. When PAPS is utilized in either sulfate transfer reactions or in reduction to sulfite, 3′-phosphoadenosine-5′-phosphate (PAP) is generated. A specific phosphatase is required to remove the 3′-phosphate and prevent both the trapping of adenine nucleotides as PAP and the product inhibition of PAPS-utilizing reactions by this metabolite. We suggest that *HAL2* encodes the 3′,5′-bisphosphate nucleotidase required for sulfate activation and that this enzyme might be sensitive to lithium and sodium inhibition. The homology between the *HAL2* gene product and inositol monophosphatase (*11*) includes the motifs that form the active site of the latter, as demonstrated by x-ray crystallography (*15*). Given the sensitivity of inositol phosphatases to lithium (*16*), we predicted that the *HAL2* phosphatase would exhibit cation sensitivity.

We overexpressed the *HAL2* gene in yeast by putting its reading frame under control of the strong *PMA1* promoter in a multicopy plasmid (*17*). The *HAL2* protein, as detected with specific antibody, represented 7% of the total protein. Homogenates from *HAL2*-expressing yeast but not from control cells (*hal2* null mutant) exhibited phosphohydrolytic activity with PAP as substrate (*18*). In addition, the *HAL2* protein could be purified by affinity chromatography on a PAP-agarose column (Fig. 1). Analysis of the reaction product by high-performance liquid chromatography (HPLC) demonstrated the stoichiometric conversion of PAP to adenosine monophosphate (AMP) by hydrolysis of the 3′-phosphate (Fig. 2). The *HAL2* phosphatase has a narrow substrate specificity (Table 1), with PAP and PAPS serving best as substrates, followed by 2′-phosphoadenosine-5′-phosphate. The phosphatase has no de-

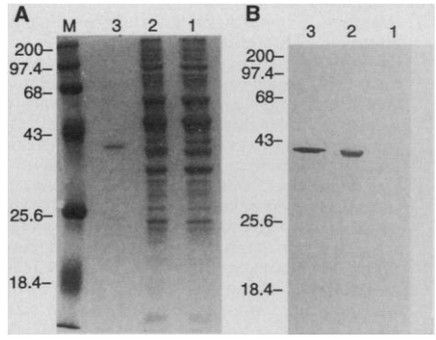

Fig. 1. Expression and purification of the *HAL2* gene product. Protein extracts from exponentially growing RS-1051 cells [containing a disruption of the *HAL2* gene (*11*)] and from yeast cells overexpressing the *HAL2* gene were prepared as described (*25*). Purification of *HAL2* protein from the homogenates of *HAL2*-overexpressing strain was carried out in a PAP-agarose (Sigma) affinity chromatography step as described (*20*). SDS–polyacrylamide gel elecrophoresis (PAGE) in 10% acrylamide gels and immunoblotting with the alkaline phosphatase system were performed by standard procedures (*26*). (**A**) Coomassie blue staining of proteins. (**B**) Protein immunoblot analysis with specific antibody against the *HAL2* gene product (*11*). Lane 1, total homogenate from RS-1051 strain (10 μg); lane 2, total homogenate from yeast cells overexpressing the *HAL2* gene (10 μg); and lane 3, purified *HAL2* protein (1 μg).

Table 1. Substrate specificity of the *HAL2* phosphatase. The activity (*18*) obtained with different substrates (concentration, 1 mM; 0.2 mM in the case of PAPS) is expressed as the percent activity observed with PAP (2.7 μmol min^{-1} per milligram of protein). Results are given as the mean of two independent experiments with values differing by <10%.

Substrate*	Activity
PAP	100
PAPS†	100
2′-PAP	23
3′-AMP	<1
AMP	<1
ADP	<1
ATP	<1
NADP	<1
Ins-1-P	<1
Ins-1,4-diP	<1
p-Nitrophenylphosphate	<1

*PAP: 3′-Phosphoadenosine-5′-phosphate; PAPS: 3′-phosphoadenosine-5′-phosphosulfate; 2′-PAP: 2′-phosphoadenosine-5′-phosphate; 3′-AMP: adenosine 3′-monophosphate; AMP: adenosine 5′-monophosphate; Ins-1-P: D-myo-inositol 1-monophosphate; Ins-1,4-diP: D-myo-inositol 1,4-bis-phosphate. †PAPS (Sigma) is supplied with 2.5 mol of lithium per mole. Given the instability of this compound, removal of lithium is not recommended. To correct for lithium inhibition, we assayed the reference substrate PAP with the same quantity of lithium (0.5 mM for 0.2 mM substrate).

Instituto de Biologia Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas, Universidad Politécnica de Valencia, Camino de Vera, 46022 Valencia, Spain.

*To whom correspondence should be addressed.

tectable effect on inositol mono- and diphosphates, 3′-AMP, AMP, nicotinamide adenine dinucleotide phosphate (NADP), and *p*-nitrophenylphosphate. Therefore, the *HAL2* gene product qualifies as a 3′(2′),5′-bisphosphate nucleotidase (E.C. 3.1.3.7) (*19*).

The *HAL2* phosphatase is very sensitive to lithium [half-maximal inhibition concentration (IC_{50}) = 0.1 mM] and moderately sensitive to sodium (IC_{50} = 20 mM) (Fig. 3A). Inhibition by lithium and sodium was not competitive because an excess of PAP substrate did not reverse the inhibition. However, we could not discriminate between uncompetitive and noncompetitive inhibition because the Michaelis constant (K_m) for PAP is <20 μM, the sensitivity limit of our assay procedure. Tetramethylammonium had no significant effect. Potassium produced a small activation (40%), as did rubidium and ammonium. Magnesium was required for activity, with an optimal concentration of 0.5 mM, although excess magnesium (>2 mM) was slightly inhibitory. 3′(2′),5′-Bisphosphate nucleotidases have been described in liver (*20*) and in the alga *Chlorella* (*21*), but the effects of lithium and sodium have not been reported.

The inhibitory effects of lithium and sodium were overcome by high concentrations of potassium (Fig. 3B). Inhibition by lithium or sodium exhibited hyperbolic dependence on concentration, but the activation effect of potassium followed sigmoidal kinetics, with a Hill coefficient of about 2. The reversal of sodium inhibition by potassium is in accordance with physiological observations suggesting that salt toxicity is influenced by the intracellular Na^+/K^+ ratio (*9*, *10*).

Animal inositol phosphatases are also inhibited by lithium and activated by potassium. Sodium, however, activates inositol phosphatases in the same way as potassium (*16*), but inhibits the *HAL2* phosphatase. The molecular basis for lithium inhibition in inositol phosphatases remains unknown because lithium cannot be visualized by x-ray crystallography (*15*).

We have partially purified 3′,5′-bisphosphate nucleotidase from tomato leaves (*22*) and find that half-maximal inhibition by lithium and sodium occurs at 0.4 mM and 50 mM, respectively. The enzyme from rat liver, partially purified by a similar procedure, is also sensitive to lithium (IC_{50} = 0.4 mM).

Our results identify a target for lithium and sodium toxicity that is a general participant in cellular metabolism. In the case of yeast, both overexpression of *HAL2* and methionine supplementation improve lithium and sodium tolerance (*11*). Given the cation sensitivity of the 3′,5′-bisphosphate nucleotidase encoded by *HAL2* and its participation in methionine biosynthesis, it can be concluded that this enzyme is an important determinant of the growth inhibition produced by lithium and sodium. Accordingly, incubation of yeast cells with NaCl or LiCl (but not with KCl) increased the intracellular concentration of PAP from <0.1 mM to between 1 and 1.3 mM. Because this value represented only 15 to 20% of the total pool of adenine nucleotides, lithium and sodium inhibition of 3′,5′-bisphosphate nucleotidase might be toxic for cells mostly as result of the accumulation of PAP, with the subsequent inhibition of PAPS-utilizing enzymes (*20*).

The mechanism of salt toxicity de-

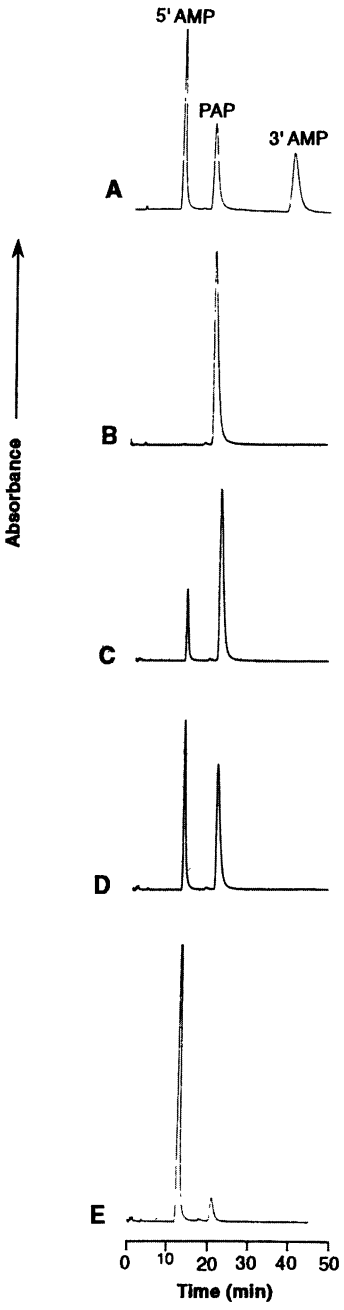

Fig. 2. Identification by HPLC of the product of PAP hydrolysis catalyzed by the *HAL2* gene product. Purified *HAL2* protein (200 ng) was incubated at 30°C in a buffer containing 0.2 M potassium bicine (pH 8), 0.5 mM magnesium acetate, and 2 mM PAP as substrate. At the indicated times, 20-μl samples were taken from the reaction mixture and subjected to HPLC analysis in a Waters 600E liquid chromatograph. Samples were ultrafiltered through Millipore (Molsheim, France) HV filters (0.45 μm), injected onto a reversed-phase C18 column (LiChrosphere 100, 4 mm by 250 mm, 5-μm particle size, Merck RT), eluted, and detected as described (*27*). The results were quantified with a Waters 746 integrator. (**A**) Standards of AMP, PAP, and 3′-AMP. Standards were dissolved in water (Milli Q plus) at a final concentration of 20 mM each, and 2.5 μl were injected for analysis. (**B**) Reaction mixture at time zero. (**C**) Reaction mixture at 30 min. (**D**) Reaction mixture at 1 hour 30 min. (**E**) Reaction mixture at 3 hours 30 min.

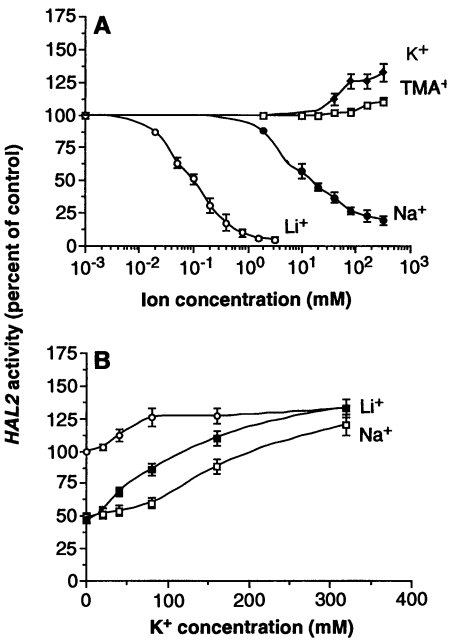

Fig. 3. Effect of cations on the *HAL2* 3′,5′-bisphosphate nucleotidase. The standard reaction conditions were as described (*18*). (**A**) Inhibition of *HAL2* phosphatase by sodium and lithium. Increasing amounts of lithium (open circles), sodium (filled circles), tetramethyl ammonium (TMA^+, open squares), and potassium (filled rhombs) were used. (**B**) Reversal of lithium and sodium inhibition by potassium. Activity was assayed in the absence of inhibitory cations (open circles) or in the presence of 0.1 mM lithium (filled squares) or 10 mM sodium (open squares) at different potassium concentrations. The results are expressed as percent activity observed in the absence of cations and are the means (± standard deviation) of at least three independent experiments, each performed in duplicate. Error bars correspond to the standard deviation. Cations were added as chloride salts.

scribed here may be relevant for understanding salt tolerance in crop plants because plant 3′,5′-bisphosphate nucleotidase is also sensitive to lithium and sodium and is required for sulfate activation in sulfotransferase reactions (*23*). Lithium therapy in humans, known to affect inositol phosphatases (*24*), should also be considered in light of the lithium sensitivity of 3′,5′-bisphosphate nucleotidase and given the multitude of sulfotransferase reactions involved in detoxification and in modification of carbohydrates, peptides, and proteins (*23*).

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18. Phosphatase assays were carried out in a total volume of 100 μl containing 50 mM tris-MES (pH 7.5), 0.5 mM magnesium acetate, 1 mM PAP, and 0.2 μg of purified *HAL2* protein. After 30 min at 30°C, the liberated inorganic phosphate was quantified by the malachite green procedure [A. A. Baykov, O. A. Evtushenko, S. M. Avaeva, *Anal. Biochem.* **171**, 266 (1988)].
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22. Fully expanded tomato leaves (30 days old after sowing) were homogenized in liquid nitrogen at a ratio of 1 g per 3 ml of 50 mM tris-HCl (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride and 30 mM β-mercaptoethanol. Insoluble polyvinyl-polipyrrolidone was added to the crude extract at 0.1 g per gram of tissue to adsorb phenolics and centrifuged for 2 hours at 100,000*g*. The resulting supernatant was adjusted to 60% saturation with solid $(NH_4)_2SO_4$, allowed to stand for 1 hour, and centrifuged at 20,000*g* for 20 min. The pellet was resuspended in 50 mM tris-HCl (pH 7.5), 10 mM β-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride and dialyzed against the same buffer for 24 hours. Affinity chromatography with PAP-agarose was as described (*20*). All purification steps were carried out at 4°C.
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Identification of Bases in 16*S* rRNA Essential for tRNA Binding at the 30*S* Ribosomal P site

Uwe von Ahsen* and Harry F. Noller

Previous studies suggest that the mechanism of action of the ribosome in translation involves crucial transfer RNA (tRNA)–ribosomal RNA (rRNA) interactions. Here, a selection scheme was developed to identify bases in 16S rRNA that are essential for tRNA binding to the P site of the small (30S) ribosomal subunit. Modification of the N-1 and N-2 positions of 2-methylguanine 966 and of the N-7 position of guanine 1401 interfered with messenger RNA (mRNA)–dependent binding of tRNA to the P site. Modification of the same positions as well as of the N-1 and N-2 positions of guanine 926 interfered with mRNA-independent binding of tRNA at high magnesium ion concentration. These results suggest that these three bases are involved in intermolecular contacts between ribosomes and tRNA.

Central to the process of translation is the ribosome-mediated interaction between mRNA and tRNA. In addition to base pairing to the codon by the tRNA anticodon, tRNAs also interact with the ribosome itself. This is particularly evident at the P site, where tRNA can be bound, at high Mg^{2+} concentrations in the absence of mRNA (*1*, *2*). Earlier studies suggested that specific bases in 16S rRNA participate in this interaction (*3*). Modification with kethoxal of a limited number of guanine residues in the 16S rRNA of 30S subunits blocks tRNA binding to the P site. Loss of activity was prevented when tRNA was bound before modification, suggesting that the inactivation was due to modification of the binding site itself rather than to general disruption of ribosome structure. More recently, a subset of the bases in 16S rRNA that react with chemical probes was found to be protected from modification by tRNA bound to the 30S P site (*2*). Most of the protected bases are located in universally conserved sequences in small subunit rRNA, consistent with a potential role in tRNA binding.

In the experiments described here, we ask which bases in 16S rRNA are the targets of functional inactivation by chemical probes of all four nucleotide bases. Our strategy was to chemically modify 30S subunits at a low level, so that a subset of the 30S population retains its ability to bind tRNA at the P site. The binding-competent subunits are then bound to tRNA derivatized at its 3′ end with biotin and selectively removed by capture with streptavidin beads (Fig. 1) (*4*). This method is made possible by the fact that only a 15-nucleotide region of the anticodon stem-loop of tRNA is involved in 30S P site binding (*2*, *5*, *6*), leaving its 3′ end, which normally interacts only with the 50S subunit, available for interaction with streptavidin.

We tested the ability of the streptavidin beads to capture 30S subunits by means of their interaction with biotinylated tRNA. Biotinylated *Escherichia coli* $tRNA^{Phe}$ or $tRNA_2^{Leu}$ was bound to 30S ribosomal subunits (labeled with [^{32}P]pCp at the 3′ end of 16S rRNA) in the presence of polyuridylate [poly(U)] or a random copolymer of U and C [poly(U,C)], respectively. Magnetic streptavidin beads were added to the tRNA-30S complexes, and the captured complexes were removed (*4*). Increasing amounts of small ribosomal subunits were captured by the streptavidin matrix with increasing magnesium ion concentration (Table 1), in accordance with the known dependence of nonenzymatic P site binding on Mg^{2+} concentration (*1*, *7*). The similar behavior of two different tRNA species

Sinsheimer Laboratories, University of California, Santa Cruz, CA 95064, USA.

*Present address: Institute of Microbiology and Genetics, University of Vienna, Dr. Bohr-Gasse 9, A-1030 Vienna, Austria.