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A Lysine Substitute for K^+

A460K MUTATION ELIMINATES K^+ DEPENDENCE IN H^+-PYROPHOSPHATASE OF *CARBOXYDOTHERMUS HYDROGENOFORMANS**

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The H^+ proton-translocating inorganic pyrophosphatase (H^+-PPase) family is composed of two phylogenetically distinct types of enzymes: K^+-dependent and K^+-independent. However, to date, the sequence criteria governing this dichotomy have remained unknown. In this study, we describe the heterologous expression and functional characterization of H^+-PPase from the thermophilic bacterium *Carboxydothermus hydrogenoformans*. Both PP_i-hydrolyzing and PP_i-energized H^+ translocation activities of the recombinant enzyme in *Escherichia coli* inner membrane vesicles are strictly K^+-dependent. Here we deduce the K^+ requirement of all available H^+-PPase sequences based on the K^+ dependence of *C. hydrogenoformans* H^+-PPase in conjunction with phylogenetic analyses. Our data reveal that K^+-independent H^+-PPases possess conserved Lys and Thr that are absent in K^+-dependent H^+-PPases. We further demonstrate that a A460K substitution in *C. hydrogenoformans* H^+-PPase is sufficient to confer K^+ independence to both PP_i hydrolysis and PP_i-energized H^+ translocation. In contrast, a A463T mutation does not affect the K^+ dependence of H^+-PPase.

H^+ Proton-pumping inorganic pyrophosphatase (H^+-PPase)[1] is an integral membrane protein that utilizes the energy released upon hydrolysis of pyrophosphate (PP_i) to transport protons across the membrane against the electrochemical potential gradient (1–3). H^+-PPases represent a distinct class of ion translocases with no sequence similarity to ubiquitous ATP-energized pumps such as F-, V-, and P-type ATPases or ABC transporters (4). Both PPase and proton translocation activities are associated with a single 66–90-kDa polypeptide (5–7), which possibly forms a dimer (8, 9). H^+-PPase is a highly hydrophobic protein as estimated from the 14–16 transmembrane spans predicted by computer modeling (10, 11).

The existence of K^+-independent and K^+-dependent H^+-PPases has been known for some time (12, 13), although only recent heterologous expression studies document the attribution of K^+ requirement to a particular sequence. Enzymes from the archaeon *Pyrobaculum aerophilum* (14), the plant *Arabidopsis thaliana* (AVP2) (15), and the bacterium *Rhosospirillum rubrum* (16) are capable of PP_i hydrolysis in the presence of Mg^{2+}, whereas those from the plants *A. thaliana* (AVP1) (7), *Vigna radiata* (11), and the bacterium *Thermotoga maritima* (17) additionally require millimolar concentrations of K^+ for activity. Based on phylogenetic analyses of a large set of H^+-PPase sequences, it was suggested that K^+-dependent and K^+-independent enzymes form two independently evolving groups (17). However, there were insufficient experimental data to precisely define the boundary between K^+-dependent and independent H^+-PPases.

In a previous study, we noted that K^+ dependence in H^+-PPases correlates with a cysteine conservation pattern and suggested that the enzyme of the bacterium *C. hydrogenoformans* (Ch-PPase) is a K^+-dependent H^+-PPase that is closely related to K^+-independent H^+-PPases (16). Here we report the high yield expression of fully functional Ch-PPase in *Escherichia coli*. The K^+ dependence of this enzyme in conjunction with phylogenetic analyses permitted the inference of the K^+ requirement of all available H^+-PPase sequences. Site-directed mutagenesis experiments revealed that a single Lys residue that is specifically conserved in K^+-independent H^+-PPases abolishes K^+ dependence when introduced in Ch-PPase.

EXPERIMENTAL PROCEDURES

Materials—Tetramethylammonium (TMA) pyrophosphate was prepared by passing a solution of tetrasodium pyrophosphate through a column packed with Dowex 50W-X8 (Fluka, Switzerland) charged with TMA^+. The concentration of the resulting TMA_4PP_i solution was determined by measuring P_i after boiling in 1 M HCl. All other reagents were from commercial sources.

Plasmid Construction—The 2-kilobase open reading frame (corresponding to positions 3264–1204 of TIGR_129958 contig2356, NC_002972) identified by BLAST searches of preliminary sequence data collated by the Institute for Genomic Research (www.tigr.org) as encoding putative H^+-PPase was amplified from the genomic DNA of *C. hydrogenoformans* (DSM 6008) (18) by PCR using *Pfu* Turbo DNA polymerase (Stratagene). The primers employed incorporated artificial *Nde*I and *Xho*I restriction sites. The PCR product was digested with the above restriction enzymes and inserted into the corresponding restriction sites of the pET36b(+) multiple cloning site (Novagen). Mutagenesis was performed using an inverse PCR technique (19). Ch-PPase encoding regions of the constructs were sequenced to confirm the presence of the required mutations and/or the absence of secondary substitutions.

Ch-PPase Expression—Ch-PPase was expressed in *E. coli* C43(DE3) cells (20) essentially as described previously for *R. rubrum* H^+-PPase (16).

Isolation of Inner Membrane Vesicles (IMV)—IMV were prepared by modification of the procedure originally described by Rosen (21). The cell pellet (6 g wet weight) was resuspended in 20 ml of buffer A (10 mM TMA-MOPS, pH 7.2, 0.15 M sucrose, 1 mM $MgCl_2$, 5 mM dithiothreitol, 50 μM TMA-EGTA) supplemented with DNase I (20 μg/ml) and disrupted by a single passage through a French pressure cell at 14,000 p.s.i. (96.5 MegaPascals). Unbroken cells and cell debris were removed

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[1] The abbreviations used are: H^+-PPase, proton-translocating inorganic pyrophosphatase; PP_i, inorganic pyrophosphate; Ch-PPase, *C. hydrogenoformans* H^+-PPase; MOPS, 4-morpholinepropanesulfonic acid; TMA, tetramethylammonium; IMV, inner membrane vesicles.

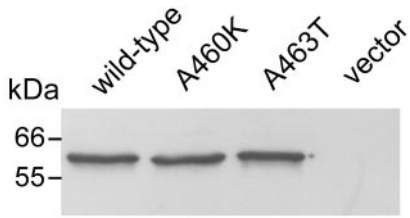

FIG. 1. **Western analysis of IMV prepared from C43(DE3) cells expressing wild-type and mutated Ch-PPases or transformed with vector only.** Triton X-100 pre-solubilized IMV (1.2 μg of protein) was subjected to SDS-PAGE, electrotransferred, and probed with *R. rubrum* H^+-PPase polyclonal antiserum. The bands shown were the only immunoreactive species detected.

by centrifugation at 38,000 × *g* for 30 min at 4 °C. The supernatant was transferred to an ultracentrifuge bottle, underlayered with 4 ml buffer A supplemented with 0.75 M sucrose, and centrifuged at 150,000 × *g* for 3 h at 4 °C. The IMV suspension was withdrawn from the bottom of the bottle, frozen in liquid nitrogen, and stored at −70 °C until use. Protein concentrations in IMV suspensions were measured by the Bradford assay (22). IMV quantities were calculated in terms of protein content.

Western Analyses—Electrophoresis was performed with 12% gels containing 0.1% SDS (23). Samples for electrophoresis were prepared as described previously (16). Electrophoresed samples were transferred to a nitrocellulose Hybond™ ECL membrane (Amersham Biosciences) in standard Towbin buffer (24) containing 20% (v/v) methanol for 1 h at 100 V in a Mini Trans-Blot apparatus (Bio-Rad). Transferred protein bands were stained with Ponceau S, and *R. rubrum* H^+-PPase antiserum-reactive bands (25) were visualized using an ECL kit.

PP_i Hydrolysis Measurements—PP_i hydrolysis was assayed by continuously recording P_i liberation with an automatic P_i analyzer (26) at 40 °C. The reactions were performed in an initial volume of 25 ml containing 100 mM TMA-MOPS, pH 7.2, 5.26 mM $MgCl_2$, 163 μM TMA-PP_i, 50 μM TMA-EGTA, and 0.25 mM TMA fluoride. KCl and TMA chloride were added to achieve the desired concentration of K^+ and ionic strength of 0.22 M. The reaction was initiated by the addition of suitable aliquots (5–50 μl) of IMV suspension, and P_i liberation was monitored for 3 min.

Proton Translocation Measurements—H^+ translocation across the IMV membrane was assayed fluorometrically at 25 °C in 2 ml buffer A supplemented with 0.15 mM sucrose, 50 mM $MgCl_2$ and 100 mM KCl where indicated using 500 μg of IMV and 3 μM acridine orange as a ΔpH indicator (21). Excitation and emission wavelengths were set at 495 and 540 nm, respectively. H^+ translocation was initiated by the addition of 1 mM TMA-PP_i and measured with a PerkinElmer MPF-2A fluorometer.

Control over K^+ and Na^+ Contamination—The background concentrations of K^+ and Na^+ in assay media were below 5 μM and 20 μM, respectively, as determined by atomic absorption spectrometry (SpectrAA-300, Varian Techtron, Mulgrave, Australia).

RESULTS AND DISCUSSION

Recombinant Ch-PPase Is K^+-dependent and Fully Functional—We initially expressed Ch-PPase in *E. coli* C43(DE3) using the method previously established for *R. rubrum* H^+-PPase (16). SDS-PAGE and Western blot analyses revealed an intense H^+-PPase antiserum-reactive band in IMV prepared from C43(DE3) cells expressing Ch-PPase (Fig. 1). Although the molecular mass of Ch-PPase calculated from the amino acid sequence is 71 kDa, the 60-kDa band was attributed to the full-length enzyme based on consistent reports of anomalous electrophoretic mobility for H^+-PPases in SDS-PAGE (5, 16, 27). Because *E. coli* IMV lack endogenous pyrophosphatase, both PP_i hydrolysis and PP_i-energized H^+ translocation by Ch-PPase can be assayed directly in these vesicles. Although the optimum temperature range for the growth of *C. hydrogenoformans* is 65–70 °C, the PP_i hydrolysis by Ch-PPase was measured at 40 °C because at this temperature *E. coli* lipids still form a bilayer, the natural environment of membrane proteins. TMA fluoride (0.25 mM) was added to abolish any contribution of contaminating soluble *E. coli* pyrophosphatase. Under these conditions, the PPase activity in IMV containing Ch-PPase was negligible in K^+-free (<5 μM K^+) medium and

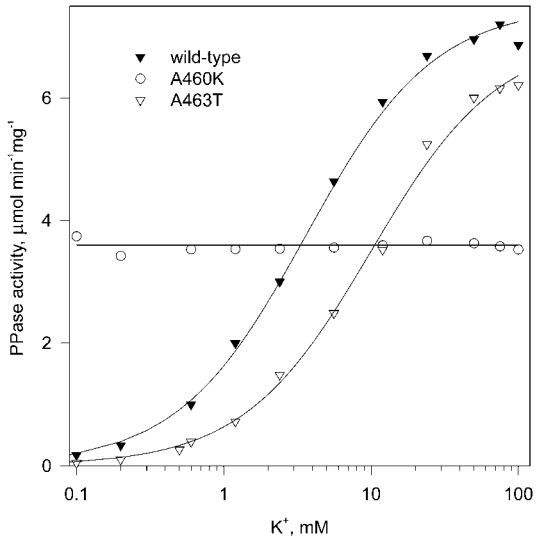

FIG. 2. **Rate of PP_i hydrolysis by IMV prepared from C43(DE3) cells expressing wild-type (▼), A460K (○), and A463T (▽) Ch-PPases as a function of K^+ concentration.** PP_i hydrolysis was assayed by continuously recording P_i liberation at 40 °C. Lines of best fit were computed by non-linear regression analysis (28) to yield dissociation constant values for K^+ binding of 3.6 ± 0.2 mM (▼) and 10 ± 0.5 mM (▽) and limiting values for activity of 7.5 ± 0.2 μmol $min^{-1}mg^{-1}$ (▲) and 7.0 ± 0.2 μmol $min^{-1}mg^{-1}$ (▽), respectively. The line corresponding to constant activity of 3.6 ± 0.1 μmol $min^{-1}mg^{-1}$ (○) was computed by linear regression.

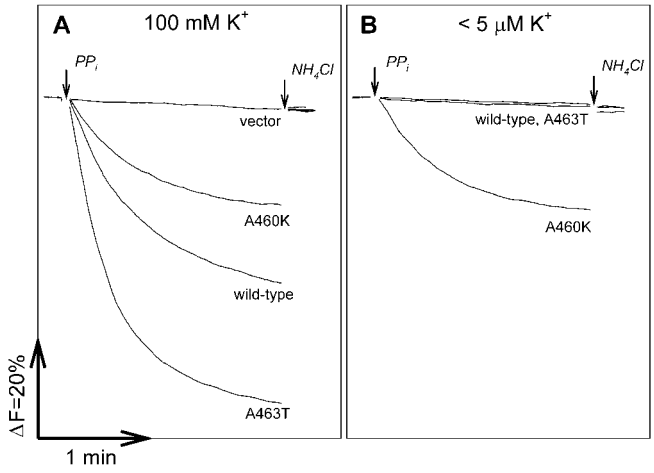

FIG. 3. **PP_i-dependent H^+ translocation by IMV prepared from C43(DE3) cells expressing wild-type and mutated Ch-PPase or transformed with vector only.** H^+ translocation was assayed at 25 °C with the fluorescent ΔpH indicator, acridine orange, in the presence of 100 mM KCl (*A*) or in K^+-free (*B*) medium. Intravesicular acidification was initiated and terminated at the times indicated by the addition of 1 mM TMA-PP_i and 10 mM NH_4Cl, respectively. For each variant, quantitatively similar data were obtained with three IMV preparations. Data from a single experiment are shown. The A463T variant hydrolyzed PP_i twice as fast as the wild-type enzyme when assayed at 25 °C (data not shown), which accounts for the surprisingly high rate of fluorescence quenching in IMV containing A463T.

increased as a simple hyperbolic function of K^+ concentration, consistent with the presence of a single K^+ binding site (Fig. 2, *closed triangles*). Heterologously expressed Ch-PPase was also active in PP_i-energized H^+ translocation into *E. coli* IMV as monitored by acridine orange fluorescence quenching. The latter assay was most reproducible at 25 °C where *E. coli* IMV are resistant to aggregation and their passive permeability to H^+ is low. In agreement with data on PP_i-hydrolyzing activity, PP_i-energized H^+-translocation was observed at 100 mM K^+ (Fig. 3*A*) but not in K^+-free medium (Fig. 3*B*). We conclude that

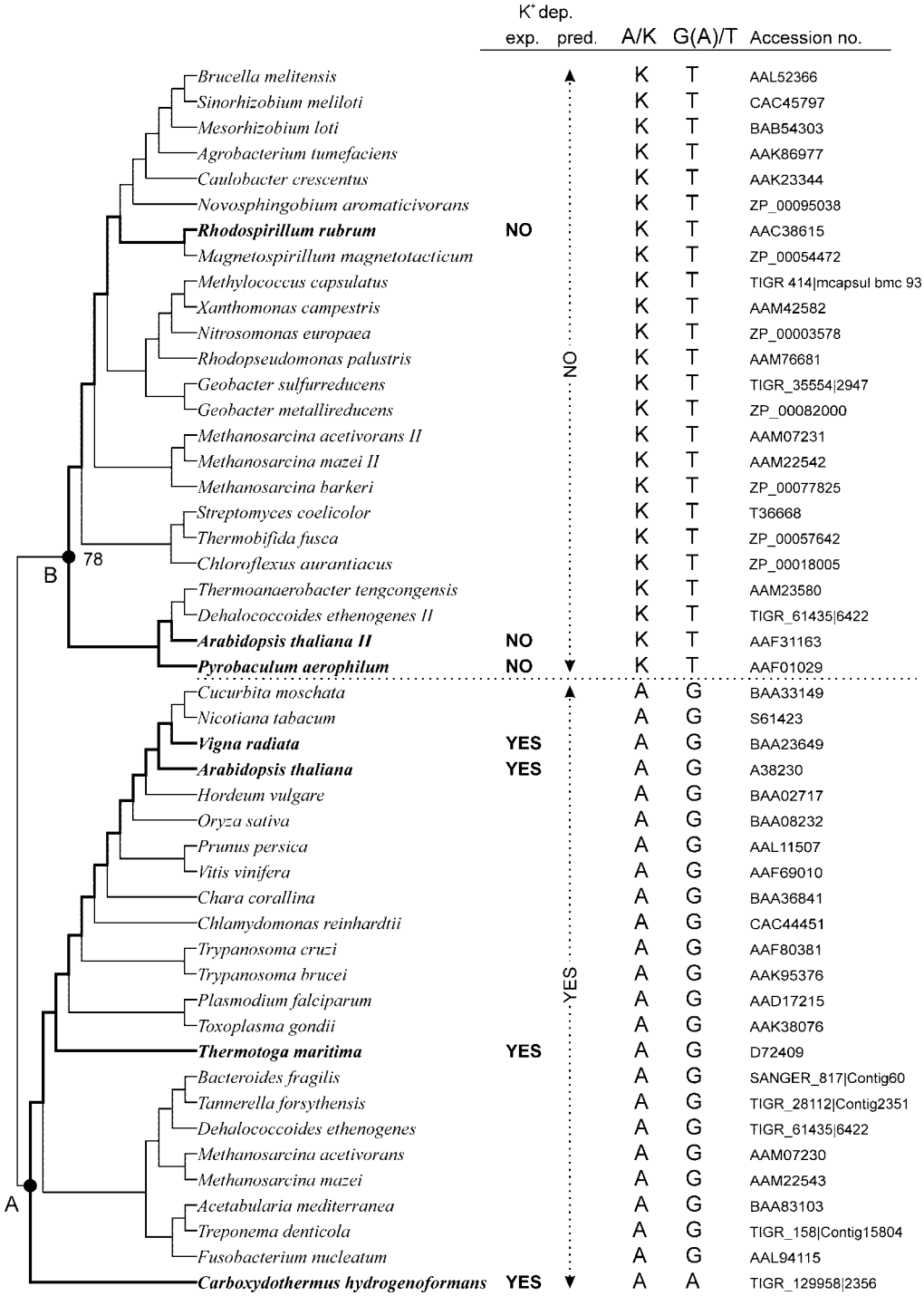

FIG. 4. **K^+ requirement of functionally characterized H^+-PPases and all available H^+-PPase sequences on the basis of phylogenetic analyses.** A multiple sequence alignment was generated by ClustalX version 1.81 (29), and the block of sequence corresponding to positions 8–684 of Ch-PPase was subjected to phylogenetic analysis using both maximum likelihood and distance (neighbor-joining) methods (PHYLIP, version 3.6a3) (30). Qualitatively, similar results were obtained with both procedures, but only the tree retrieved from the distance analysis is shown. The tree, presented as cladogram, was arbitrarily rooted with the *C. hydrogenoformans* sequence. Functionally characterized H^+-PPases are indicated in *boldface*, and their K^+ requirement is *traced* back along the branches (*boldface lines*) to nodes A and B that are accentuated by *black circles*. For the node B, bootstrap value is indicated as percentage of 1000 replications. The *columns* to the *right* of the tree display K^+ requirement of functionally characterized H^+-PPases (*K^+ dep.*, *exp. column*) inferred K^+ requirement of all H^+-PPase sequences (*K^+ dep.*, *pred. column*), residues in Ala/Lys (*A/K column*) and Gly(Ala)/Thr (*G(A)/T column*) positions in the multiple sequence alignment and protein sequence accession numbers in the GenBank™ or DNA contig numbers for preliminary sequences obtained from The Institute for Genomic Research (www.tigr.org) and The Sanger Center (www.sanger.ac.uk).

heterologous expression of Ch-PPase results in fully functional and strictly K^+-dependent enzyme.

K^+ Requirement of Functionally Characterized H^+-PPases and All Available H^+-PPase Sequences from Phylogenetic Analyses—If we consider the phylogenetic relationships of H^+-PPases (Fig. 4, phylogenetic tree), the K^+ requirement of seven heterologously expressed sequences (Fig. 4, *K^+ dep.*, *exp.*), and assume that K^+ dependence was eliminated or acquired at a distinct point in evolution, the K^+ requirements of all available H^+-PPase sequences may be resolved. As traced back from the

phylogenetic tree in Fig. 4, the K^+-dependent H^+-PPases from *A. thaliana* (7), *V. radiata* (11), *T. maritima* (17), and *C. hydrogenoformans* have a common ancestor at node A, whereas K^+-independent H^+-PPases from *P. aerophilum* (14), *A. thaliana II* (15), and *R. rubrum* (16) have a common ancestor at node B. Any currently available H^+-PPase sequence is the descendant of either the ancestor at node A or at node B and is hence classified as K^+-dependent (from *Cucurbita moschata* to *C. hydrogenoformans*) or K^+-independent (from *Brucella melitensis* to *P. aerophilum*), respectively.

The Structural Basis for K^+ Requirement—A comparison of the amino acid conservation patterns in K^+-dependent and K^+-independent H^+-PPases revealed only two sequence positions where a residue conserved in one type is absent from the equivalent position in all the sequences from the other type. The Ala/Lys position is occupied by Ala in K^+-dependent H^+-PPase and Lys in K^+-independent H^+-PPase (Fig. 4, *A/K column*). Similarly, the Gly(Ala)/Thr position is occupied by Gly or Ala in K^+-dependent H^+-PPase and Thr in K^+-independent H^+-PPase (Fig. 4, *G(A)/T column*). These Ala/Lys and Gly(Ala)/Thr positions are located two amino acids apart in the conserved region at the interface between the predicted cytosolic loop and its adjoining transmembrane span and correspond to Ala^{460} and Ala^{463} of Ch-PPase.[2] To determine the effects of these residues on K^+ requirement, we replaced Ala^{460} with Lys and Ala^{463} with Thr in Ch-PPase. Both A460K and A463T variants were expressed with the same yield as wild-type enzyme as judged from the intensities of the H^+-PPase antiserum-reactive bands (Fig. 1). The A463T substitution resulted in strictly K^+-dependent H^+-PPase but with three times reduced affinity for K^+ compared with wild-type enzyme (Fig. 2, *open triangles*). In contrast, both PP_i-hydrolyzing (Fig. 2, *open circles*) and PP_i-energized H^+ translocation (Fig. 3, *A* and *B*) activities of A460K variant were entirely K^+-independent and half that of wild-type enzyme. The Lys in the Ala/Lys position was thus identified as the sole primary determinant of K^+ independence in H^+-PPases.

The transition from K^+-dependent to K^+-independent H^+-PPase may be viewed simply as the substitution of K^+ with the NH_3^+ group of Lys. However, the residues forming the K^+ binding site remain to be determined. Although Ala in the Ala/Lys position and Gly in the Gly(Ala)/Thr position are specifically conserved in K^+-dependent H^+-PPases, their direct participation in K^+ binding is neither evident, nor is it likely that these residues are the only ones forming the K^+ binding site. The current hypothesis is that the K^+ binding site is located within the area occupied by the NH_3^+ group of conserved Lys.

In summary, phylogenetic analyses and the primary sequence criterion of K^+ requirement established in this work provide a means for accurate annotation of any H^+-PPase sequence. With the number of available H^+-PPase sequences doubling every year, this approach should be of great assistance in classification.

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[2] The Ala/Lys and Gly(Ala)/Thr positions also correspond to Lys^{465} and Thr^{468} of *R. rubrum* H^+-PPase (GenBank™ accession number AAC38615) and Ala^{541} and Gly^{544} of *A. thaliana* H^+-PPase (GenBank™ accession number A38230).

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