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Constant Enthalpy Change Value during Pyrophosphate Hydrolysis within the Physiological Limits of NaCl*

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Satoshi Wakai[‡1], **Shun-ichi Kidokoro**[§], **Kazuo Masaki**[¶], **Kaoru Nakasone**[‖], **and Yoshihiro Sambongi**[‡2]

From the [‡]*Graduate School of Biosphere Science, Hiroshima University, Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan, the* [§]*Department of Bioengineering, Nagaoka University of Technology, Kamitomioka, Nagaoka, Niigata 940-2188, Japan, the* [¶]*Applications Research Division, National Research Institute of Brewing, Kagamiyama, Higashi-Hiroshima, Hiroshima 739-0046, Japan, and the* [‖]*Department of Chemistry and Environmental Technology, School of Engineering, Kinki University, Umenobe Takaya, Higashi-Hiroshima, Hiroshima 739-2116, Japan*

Background: Decreased water activity was thought to result in smaller enthalpy change values during pyrophosphate hydrolysis.
Results: The enthalpy change in pyrophosphate hydrolysis caused by halophilic and non-halophilic enzymes is constant up to 4.0 M NaCl.
Conclusion: Water activity with high NaCl does not affect the pyrophosphate hydrolysis enthalpy change.
Significance: Biological energy conversion is enthalpically driven within the physiological limits of NaCl.

A decrease in water activity was thought to result in smaller enthalpy change values during PP_i hydrolysis, indicating the importance of solvation for the reaction. However, the physiological significance of this phenomenon is unknown. Here, we combined biochemistry and calorimetry to solve this problem using NaCl, a physiologically occurring water activity-reducing reagent. The pyrophosphatase activities of extremely halophilic *Haloarcula japonica*, which can grow at ~4 M NaCl, and non-halophilic *Escherichia coli* and *Saccharomyces cerevisiae* were maximal at 2.0 and 0.1 M NaCl, respectively. Thus, halophilic and non-halophilic pyrophosphatases exhibit distinct maximal activities at different NaCl concentration ranges. Upon calorimetry, the same exothermic enthalpy change of −35 kJ/mol was obtained for the halophile and non-halophiles at 1.5–4.0 and 0.1–2.0 M NaCl, respectively. These results show that solvation changes caused by up to 4.0 M NaCl (water activity of ~0.84) do not affect the enthalpy change in PP_i hydrolysis. It has been postulated that PP_i is an ATP analog, having a so-called high energy phosphate bond, and that the hydrolysis of both compounds is enthalpically driven. Therefore, our results indicate that the hydrolysis of high energy phosphate compounds, which are responsible for biological energy conversion, is enthalpically driven within the physiological limits of NaCl.

Previous studies showed that a decrease in water activity leads to a smaller enthalpy in PP_i hydrolysis (1–4). Although these previous studies suggested the importance of solvation as the energy origin of high energy phosphate compounds, including PP_i and ATP, its physiological significance remained unknown. This is because the previous results were obtained by theoretical calculations considering the gas phase with water activity to be zero or from experiments involving non-physiological solvents to reduce water activity, not by biologically relevant methods.

A variety of microbes adapt to natural environments containing up to 4 M NaCl. For example, extremely halophilic archaea require a nearly saturated level of NaCl for their growth. Such halophiles accumulate NaCl or KCl within their cells to a concentration equivalent to that in the external environment (5). An environmental decrease in the salt concentration causes a decrease in the cellular salt concentration in such archaea (6). Therefore, in extremely halophilic archaeal cells, water activity might change in accordance with the amount of salt present. We wondered whether or not naturally occurring physiological salt conditions affect the PP_i hydrolysis enthalpy change value.

PP_i is an ATP analog, being a so-called high energy phosphate compound, and the hydrolysis of both compounds is enthalpically driven (7). If the enthalpy change in PP_i hydrolysis becomes smaller with increasing NaCl concentrations, analogous to the previous suggestion made for non-physiological conditions, the decrease in the energy derived from PP_i and ATP would have deleterious effects on the growth of halophilic organisms. However, the effect of salt on PP_i hydrolysis enthalpy is unknown.

The aim of this study was to determine the PP_i hydrolysis enthalpy change values within the physiological limits of NaCl, which is one of the naturally occurring solutes that decrease water activity. First, we investigated the salt dependence of the biochemical pyrophosphatase (PPase)[3] activities of the extremely halophilic archaeon *Haloarcula japonica* and the non-halophiles *Escherichia coli* and *Saccharomyces cerevisiae*. We also carried out biophysical measurements to examine the

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[1] To whom correspondence may be addressed: Organization of Advanced Science and Technology, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan. Tel.: 81-78-803-6462; Fax: 81-78-803-6462; E-mail: wakaists@pegasus.kobe-u.ac.jp.
[2] To whom correspondence may be addressed. E-mail: sambongi@hiroshima-u.ac.jp.

[3] The abbreviations used are: PPase, pyrophosphatase; ITC, isothermal titration calorimetry.

NaCl dependence of these PPase activities using isothermal titration calorimetry (ITC). The data obtained were compared.

EXPERIMENTAL PROCEDURES

Microorganism, Medium, and Cultivation—*H. japonica* TR-1 was cultivated in 6 liters of CM medium (8) at 37 °C for 4 days. The cells were harvested by centrifugation at 8000 × *g* for 10 min and suspended in buffer containing 10 mM Tris-HCl (pH 8.0), 3.4 M NaCl, 140 mM KCl, and 10% (v/v) glycerol.

Preparation of a H. japonica Soluble Extract—The procedure for the *H. japonica* soluble extract preparation was carried out in the presence of 3.4 M NaCl to maintain high salt conditions. *H. japonica* cells were disrupted with a French pressure cell (Thermo Fisher Scientific) at 138 megapascals, followed by centrifugation at 12,000 × *g* to obtain a cell-free extract. This extract was then ultracentrifuged at 200,000 × *g*, and the supernatant was used as the soluble extract and was stored at −20 °C. Protein concentrations were determined by the Lowry method (9) using bovine serum albumin as a standard.

Conventional Biochemical PPase Activity—The conventional biochemical PPase activity of the *H. japonica* extract (100 μg of protein) was measured at 37 °C in a mixture (0.6 ml) containing 10 mM Tris-HCl (pH 8.0), 0–4.0 M NaCl, and 10 mM $MgCl_2$. The reaction was initiated by the addition of 4 mM disodium PP_i and terminated by the addition of 0.33 N trichloroacetic acid. PPase activity was calculated using a colorimetric assay (10) that determines the amount of P_i liberated from PP_i. Similarly, the purified PPases from the non-halophiles *E. coli* and *S. cerevisiae* (purchased from Sigma) were assayed at 25 °C. The incubation time and the protein amount were adjusted so that the PPase activity was linear with time. One unit of PPase activity was defined as the amount of enzyme that liberated 1 μmol of P_i/min.

Hydrolysis Enthalpy Measured by ITC—The calorimetric output during PP_i hydrolysis by the *H. japonica* extract was measured with an isothermal titration calorimeter (VP-ITC, MicroCal, Northampton, MA). The resulting data were analyzed using Origin 7.0 software (OriginLab, Northampton, MA) with a MicroCal ITC data analysis add-on. All experiments were performed at 25 °C. The reference cell was filled with distilled deionized water. The stirring speed used was 310 rpm, and the reference offset value was set at 20% full power. The volume of the ITC cell was 1.444 ml.

The calorimeter cell was filled with 10 mM Tris-HCl (pH 8.0 at 25 °C), 0.1–4 M NaCl, 10 mM $MgCl_2$, and *H. japonica* extract (0.5 mg of protein/ml). The injection syringe was filled with the same buffer without the extract but with 20 mM PP_i. A single injection was carried out sequentially at least six times, with 60 nmol of PP_i constantly introduced into the cell per injection and with a time interval between injections of 10–120 min. This interval was sufficient for completion of the PP_i hydrolysis reaction in each titration; thus, the calorimetric output returned to the base level in each case.

The same ITC measurements were carried out using the purified *E. coli* and *S. cerevisiae* PPases instead of the *H. japonica* extract. The time interval between injections for the enzyme assay was 5–60 min, which was sufficient for completion of the PP_i hydrolysis reaction with these enzymes.

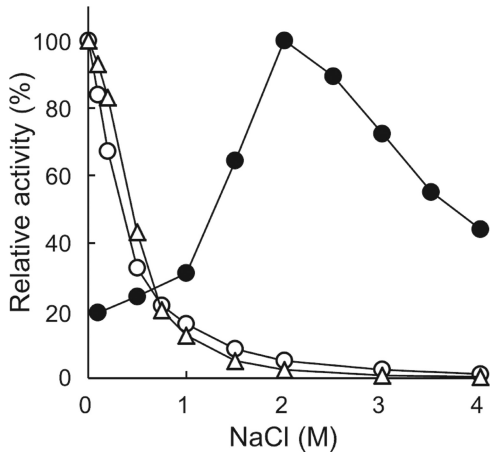

FIGURE 1. **Effects of NaCl on PP_i hydrolysis activity.** Shown is the NaCl dependence of the PPase activities of a *H. japonica* soluble extract (●), purified *E. coli* PPase (○), and purified *S. cerevisiae* PPase (△). The specific activity data are normalized to the maximal activity observed for each enzyme source. The *symbols* represent the mean values obtained for at least three independent measurements.

Determination of Water Activity—The water activities of the NaCl solutions of a range of concentrations used for calorimetric analysis were determined at 25 °C using a Rotronic Hygro-Palm water activity machine (GSI Creos). This equipment was calibrated using a saturated NaCl solution of known water activity (0.753).

RESULTS AND DISCUSSION

Conventional Biochemical PPase Activity with NaCl—We first examined the NaCl dependence of the conventional PPase activity in a soluble extract of extremely halophilic *H. japonica* (8). This was then compared with those of PPases purified from the non-halophiles *E. coli* and *S. cerevisiae*.

The PPase activity of the halophilic *H. japonica* extract increased with NaCl concentration to reach a maximum at 2.0 M (Fig. 1), giving a specific activity of 1.39 units/mg. In the NaCl concentration range of 1.5–4.0 M, at least 40% of the maximal PPase activity value remained in the halophilic *H. japonica* extract, indicating that a cellular NaCl concentration change, if one occurs, does not have a severe effect on the PPase activity. This might be one of the adaptive mechanisms of this archaeon against hyposaline stress. A similar NaCl dependence was also observed for other enzymes from halophiles (11, 12). In contrast, the PPases purified from non-halophilic *E. coli* and *S. cerevisiae* exhibited maximal activity at 0–0.1 M NaCl (Fig. 1), with specific activities of 1120 units/mg for the *E. coli* enzyme and 438 units/mg for the *S. cerevisiae* enzyme. These non-halophilic activities were inhibited by higher NaCl concentrations, *e.g.* they were <10% of the maximal values with 1.5 M NaCl.

We are now able to examine both halophilic and non-halophilic PPases, which show distinct maximal activities at different NaCl concentration ranges. In particular, a halophilic source was useful for this study because a high salt concentration generally causes problems regarding the stability and structure of non-halophilic proteins; a halophilic source overcomes these problems.

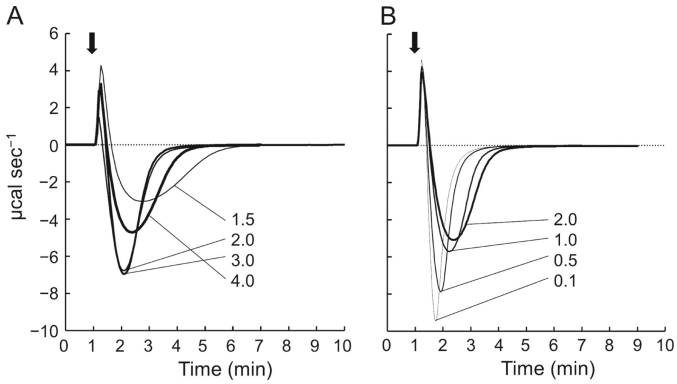

FIGURE 2. **ITC measurement of PP_i hydrolysis by the *H. japonica* extract and a purified *E. coli* PPase.** The PP_i solution was injected (*arrows*) into the reaction mixture (Tris-HCl at pH 8.0) containing the *H. japonica* extract (*A*) or *E. coli* PPase (*B*) at various concentrations of NaCl. The resulting calorimetric outputs are displayed as traces of the heat absorption/unit time *versus* time, which are labeled with the NaCl concentrations (M). The *dotted lines* represent the base level.

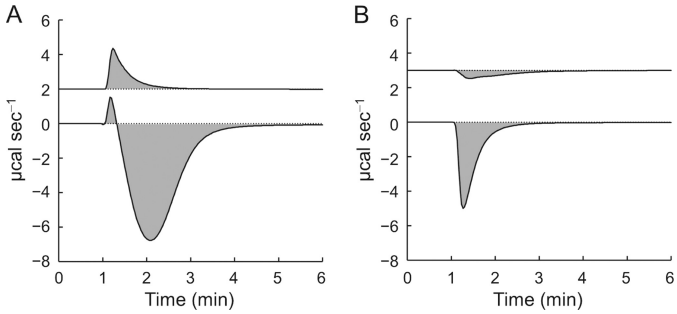

FIGURE 3. **ITC measurement for estimation of ΔH_{Exp} and ΔH_{Ion}.** Calorimetric output is displayed as traces similar to those in Fig. 2. These traces were obtained with 3.0 M NaCl in Tris-HCl (pH 8.0). *A*, ITC measurement for estimation of ΔH_{Exp}. The *upper trace* represents the output on the second of six sequential PP_i injections in the absence of the *H. japonica* extract, and the *dotted line* represents the base level. The *lower trace* represents the output on the second of six sequential PP_i injections in the presence of the *H. japonica* extract, and the *dotted line* represents the base level. The areas used for ΔH_{Exp} estimation are *shaded*. *B*, ITC measurement for estimation of ΔH_{Ion}. The *upper trace* represents the output on the second of six sequential HCl injections in the absence of the buffer, and the *dotted line* represents the base level. The *lower trace* represents the output on the second of six sequential HCl injections in the presence of the buffer, and the *dotted line* represents the base level. The areas used for ΔH_{Ion} estimation are *shaded*.

ITC of PPases—The PPase activities detected at the wide NaCl concentration range (0.1–4.0 M) examined were further verified by biophysical means using ITC. The traces in Fig. 2*A* show the calorimetric output due to the *H. japonica* PPase activity determined after the second of sequential injections of a constant amount of PP_i at various NaCl concentrations. In these traces, just after the PP_i injection (*arrow*), positive spikes appeared, representing the dilution heat of the PP_i solution. After the positive spikes, negative displacement was immediately observed, representing an exothermic feature due to the enzymatic reaction.

The steepest negative displacement was observed at 2.0–3.0 M NaCl (Fig. 2*A*). In addition, the time interval until the negative displacement returned to the base level (*dotted line*) at 2.0–3.0 M NaCl was shorter than those at lower and higher NaCl concentrations, indicating the faster reaction with 2.0–3.0 M NaCl. It was obvious that the levels of negative displacement at all of the NaCl concentrations tested correlated well with the levels of PPase activity determined in the conventional biochemical assay shown in Fig. 1.

Similar to the case with the *H. japonica* extract, the calorimetric output due to the purified *E. coli* PPase determined after the second of sequential injections of a constant amount of PP_i at various NaCl concentrations was measured (Fig. 2*B*). Again, the levels of negative displacement at all of the NaCl concentrations tested correlated well with the levels of PPase activity determined in the conventional biochemical assay shown in Fig. 1. Essentially similar results were obtained with the *S. cerevisiae* PPase (data not shown).

Evaluation of the PP_i Hydrolysis Enthalpy Change Value—The calorimetric output (Fig. 2*A*) was further analyzed to quantitatively compare the effects of NaCl on the hydrolysis enthalpy of PPase activity as shown in Fig. 3. The integrated area of the positive spike obtained upon PP_i injection into the calorimeter cell without the *H. japonica* extract (Fig. 3*A*, *upper*) was deducted from the sum of the areas of the positive spike and the negative displacement driven by the PPase activity (*solid trace*) with regard to the base level (*dotted line*) (Fig. 3*A*, *lower*). The resulting calculated value was defined as the experimentally determined molar enthalpy change (ΔH_{Exp}) for the PP_i hydrolysis reaction.

It has been postulated that the ΔH_{Exp} value can depend on the ionization heat of the buffer (ΔH_{Ion}) (13). Therefore, an enthalpy change value that does not depend on ΔH_{Ion} must be obtained to be quantitatively compared regardless of the reaction conditions used in the study. Such a ΔH_{Ion}-independent value, *i.e.* the whole catalytic enthalpy change denoted as ΔH_{Whole}, cannot be directly measured. To obtain ΔH_{Whole}, we measured ΔH_{Exp} as described above in three different buffers (Tris-HCl, HEPES-NaOH, and imidazole HCl) at pH 8.0, with different ΔH_{Exp} values obtained depending on the buffer used. At the same time, ΔH_{Ion} was measured using the same buffers (14). A HCl solution (4 mM) was injected sequentially six times, with 12 nmol of HCl constantly introduced into the solutions with or without buffer each time, and calorimetric displacement was exhibited (Fig. 3*B*). Deduction of the integrated area obtained without buffer (Fig. 3*B*, *upper*) from that with buffer (Fig. 3*B*, *lower*) gave ΔH_{Ion}.

Following the scheme described above, the correlation between the resulting ΔH_{Exp} and ΔH_{Ion} values was determined (Fig. 4). Linear regression of plots gave ΔH_{Whole} through extrapolation of the line to zero heat of buffer, as defined previously (15–17), with the following equation: $\Delta H_{Exp} = n\,\Delta H_{Ion} + \Delta H_{Whole}$, where n represents the slope of the line (Fig. 4) giving the experimentally determined number of protons released due to the PP_i hydrolysis reaction (and absorbed by the buffer). The same experiments and data analyses as those represented in Figs. 3 and 4 for the *H. japonica* extract were also carried out in the presence of various NaCl concentrations for the purified *E. coli* and *S. cerevisiae* PPases (data not shown).

NaCl Dependence of the PP_i Hydrolysis Enthalpy Change Value and Water Activity—Through the studies described above, we finally determined the PP_i hydrolysis enthalpy change (ΔH_{Whole}) values for the three enzyme sources in the presence of various concentrations of NaCl (Fig. 5). At 2.0 M

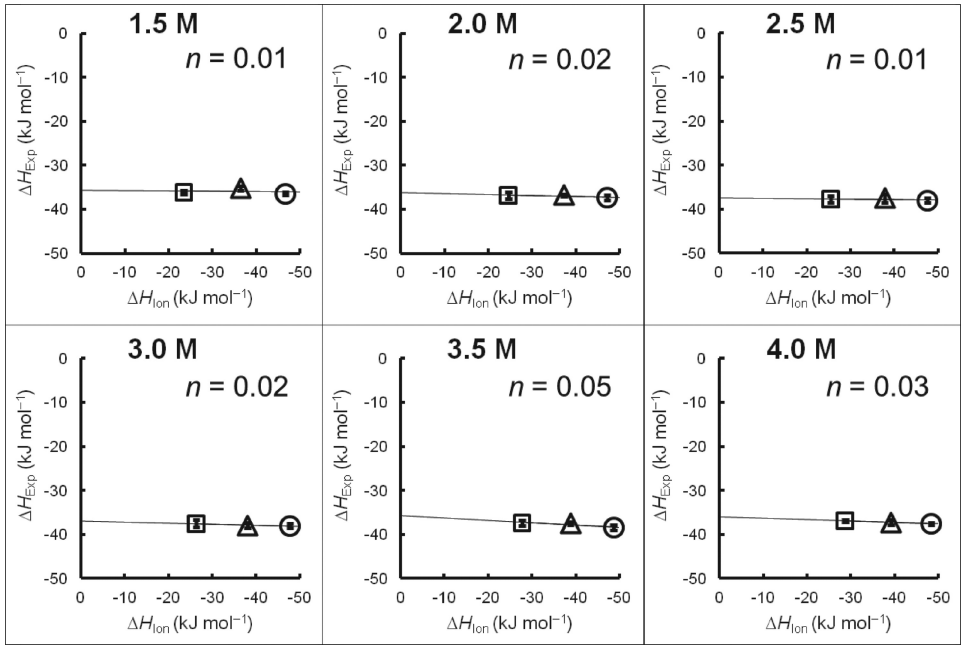

FIGURE 4. **ΔH_{Exp} *versus* ΔH_{Ion} plots.** ΔH_{Exp} for PP_i hydrolysis and ΔH_{Ion} for buffer protonation (Tris-HCl (*circles*), imidazole HCl (*triangles*), and HEPES-NaOH (*squares*)) were measured with the *H. japonica* extract by ITC at the various NaCl concentrations (1.5–4.0 M) indicated. The numbers of protons released (*n*) are also shown. Each *symbol* represents the mean value for at least three measurements performed as described in the legend to Fig. 3 with the error. The linear regression of plots is also shown by *solid lines*.

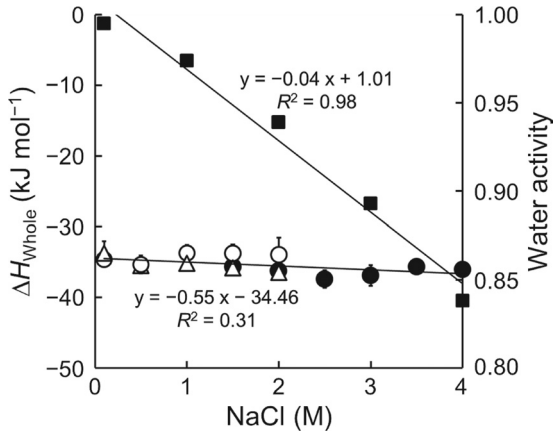

FIGURE 5. **NaCl dependence of the ΔH_{Whole} value and water activity.** The ΔH_{Whole} values for the *H. japonica* extract (●), purified *E. coli* PPase (○), and purified *S. cerevisiae* PPase (△) are shown. The water activity reduction over the NaCl concentration range is shown (■). Each *symbol* represents the mean value for at least three measurements with the error. Linear least-square fitting curves for the ΔH_{Whole} and water activity values are also shown with the equations.

NaCl, which gave the maximal PPase activity for the halophilic *H. japonica* extract (Fig. 1), the exothermic ΔH_{Whole} value was −36.2 ± 1.0 kJ/mol. Similarly, at 0.1 M NaCl, which gave the maximal non-halophilic PPase activities (Fig. 1), the exothermic ΔH_{Whole} values obtained were −34.6 ± 0.3 kJ/mol for the *E. coli* enzyme and −33.7 ± 1.7 kJ/mol for the *S. cerevisiae* enzyme. From the scatter of only these three experimental points, it was difficult to determine whether the values were significantly the same or not. The ΔH_{Whole} values obtained for the *H. japonica* extract at 1.5–4.0 M NaCl and those for the non-halophilic PPases at 0.1–2.0 M NaCl were plotted, and linear least-square fitting against all of the data points was carried out (Fig. 5).

As a result, the average ΔH_{Whole} value at 0.1–4.0 M NaCl obtained for the *H. japonica* extract and the purified PPases from *E. coli* and *S. cerevisiae* was calculated to be −35.4 ± 1.5 kJ/mol, and this value did not correlate with the NaCl concentration with the yielded R^2 value of 0.31. Therefore, we concluded that the ΔH_{Whole} values at 0.1–4.0 M NaCl were significantly the same. This constant enthalpy change value is close to that experimentally determined in an aqueous solution (water activity of 1.0): −32 kJ/mol (1).

We also measured the water activities of the NaCl solutions used in the PPase activity assays by ITC. The solutions containing 0.1 and 4.0 M NaCl exhibited water activities of 1.00 and 0.84, respectively (Fig. 5); therefore, NaCl under these experimental conditions was regarded as a water activity-reducing solute. In contrast to the correlation between the ΔH_{Whole} values and NaCl concentrations, the water activity values were linearly correlated with the NaCl concentration with the yielded R^2 value of 0.97.

In this study, dealing with halophilic and non-halophilic enzyme activities, we have revealed the relationship between water activity and PP_i hydrolysis enthalpy change under conditions in which life forms occur. Although the water activity is dependent on the physiological NaCl concentration, the enthalpy change in PP_i hydrolysis is not. Therefore, changes in water activity and solvation within the physiological limits of NaCl do not affect the PP_i hydrolysis enthalpy change.

Extended Views—Our results for PP_i used as a substrate can be extended to ATP. Because PP_i and ATP contain the same high energy phosphate bond and the hydrolysis of both compounds is enthalpically driven (7), their energy origins appear to be the same (18). Therefore, our results indicate that hydrolysis of high energy phosphate compounds, which are responsible for biological energy conversion, is enthalpically driven within

the physiological limits of NaCl. Extremely halophilic archaea such as *H. japonica* can thrive in high NaCl (up to 4 M) environments by accumulating salts inside their cells without thermodynamic problems.

The significance of the results of this study can also be extended in terms of environmental water activity. There is a microbe that can survive at 1.8 M $MgCl_2$ (water activity of ~0.8) (19). Although the salt concentrations inside such $MgCl_2$-surviving cells are unknown, $MgCl_2$-rich environments will be of interest because this salt causes further reduced water activity compared with NaCl (20, 21). In the future, the microbiology of extreme halophiles in further lower water activity environments thus offers a chance for research on both the biochemistry and biophysics of biological energy metabolism. The findings and methods presented in this study will facilitate future studies.

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