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Switching osmolyte strategies: response of *Methanococcus thermolithotrophicus* to changes in external NaCl

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Abstract

Methanococcus thermolithotrophicus, a thermophilic methanogenic archaeon, produces and accumulates β-glutamate and L-α-glutamate as osmolytes when grown in media with <1 M NaCl. When the organism is adapted to grow in >1 M NaCl, a new zwitterionic solute, N^{ε}-acetyl-β-lysine, is synthesized and becomes the dominant osmolyte. Several techniques, including in vivo and in vitro NMR spectroscopy, HPLC analyses of ethanol extracts, and potassium atomic absorption, have been used to monitor the immediate response of *M. thermolithotrophicus* to osmotic stress. There is a temporal hierarchy in the response of intracellular osmolytes. Changes in intracellular K^+ occur within the first few minutes of altering the external NaCl. Upon hypoosmotic shock, K^+ is released from the cell; relatively small changes occur in the organic osmolyte pool on a longer time scale. Upon hyperosmotic shock, *M. thermolithotrophicus* immediately internalizes K^+, far more than would be needed stoichiometrically to balance the new salt concentration. This is followed by a decrease to a new K^+ concentration (over 10–15 min), at which point synthesis and accumulation of primarily L-α-glutamate occur. Once growth of the *M. thermolithotrophicus* culture begins, typically 30–100 min after the hyperosmotic shock, the intracellular levels of organic anions decrease and the zwitterion (N^{ε}-acetyl-β-lysine) begins to represent a larger fraction of the intracellular pool. The observation that N^{ε}-acetyl-β-lysine accumulation occurs in osmoadapted cells but not immediately after osmotic shock is consistent with the hypothesis that lysine 2,3-aminomutase, an enzyme involved in N^{ε}-acetyl-β-lysine synthesis, is either not present at high levels or has low activity in cells grown and adapted to lower NaCl. That lysine aminomutase specific activity is 8-fold lower in protein extracts from cells adapted to low NaCl compared to those adapted to 1.4 M NaCl supports this hypothesis.

Keywords: Osmoregulation; Methanogen; β-Amino acid; Potassium ion; Osmolyte; Nuclear magnetic resonance spectroscopy; High-performance liquid chromatography

1. Introduction

Most methanogens respond to changes in external NaCl by accumulating several of a relatively small number of organic solutes (including β-amino acids and derivatives [1–4] and unusual phosphate compounds [5,6]) and balancing the charges of the anionic ones with intracellular K^+ ions [1,3]. *Methanococcus thermolithotrophicus*, a thermophilic marine methanogen, has been extremely well-studied in this regard [3,4,7]. When grown on H_2/CO_2 in media with less than 1 M NaCl, these cells synthesize and accumulate α- and β-glutamate isomers [4]. However, when the NaCl in the growth medium is increased above the 1 M threshold, adapted cells synthesize and accumulate the zwitterionic solute, N^{ε}-acetyl-β-lysine, in addition to the two glutamate isomers [3]. At higher NaCl levels this novel zwitterion becomes the major solute in the cells. Both β-amino acids are slowly metabolized by *M. thermolithotrophicus* if the NaCl concentration is not changed [3]. Nothing is known about what regulates the switch from accumulation of the glutamate isomers to accumulation of the zwitterion at high external NaCl (i.e., what controls the osmoadapted response).

A first step in unraveling this regulatory process requires monitoring the intracellular osmolyte (α-glutamate, β-glutamate, N^{ε}-acetyl-β-lysine, and K^+) concentrations immediately after altering the external NaCl. Several approaches, including in vivo ^{13}C-edited ^1H and ^{15}N nuclear magnetic resonance (NMR) spectroscopy (to monitor solute turnover and biosynthesis), in vitro ^1H NMR and high-performance liquid chromatography (HPLC) analyses of ethanol extracts to quantify osmolyte levels, and atomic absorption (to estimate intracellular K^+ concentrations) have been used to this end. The results show

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that K^+ plays an important role in the immediate response of this organism to increased NaCl. Within the first few minutes after transfer to the higher NaCl-containing medium, *M. thermolithotrophicus* internalizes K^+; this is followed by a decrease to steady-state levels over the time course of 10–15 min. Once the K^+ reaches a new steady-state concentration, synthesis and accumulation of L-α-glutamate occur. The K^+-α-glutamate pair functions as a 'temporary' osmolyte. Since glutamate is a key intermediate in nitrogen metabolism as well as a component of proteins, its intracellular concentration is likely to be regulated within defined ranges for optimal cell growth. And indeed, once growth of the *M. thermolithotrophicus* culture begins, typically 30–100 min after the hyperosmotic shock, the levels of glutamate decrease and there is a redistribution of the organic osmolytes. However, N^{ε}-acetyl-β-lysine accumulation does not represent a large fraction of the solute pool in these stressed cells. That this zwitterion is accumulated at high concentrations only in osmoadapted cells is consistent with the hypothesis that activity of one of its biosynthetic enzymes is limiting in cells grown and adapted to lower NaCl.

2. Materials and methods

2.1. Chemicals

$^{13}CO_2$ (99% ^{13}C)/H_2 (1:4, v/v) and $^{15}NH_4Cl$ (99% ^{15}N) were obtained from Cambridge Isotope Laboratories and used without further purification; $^{12}CO_2/H_2$ (1:4, v/v) was obtained from Wesco. Isoleucine, α-glutamate, β-glutamate, and ^2H_2O (99.9% ^2H) were obtained from Sigma; *o*-phthalaldehyde (OPA) was obtained from Pierce. All other chemicals used were reagent grade.

2.2. Bacterial growth and culture conditions

The media components and methods for growth of *M. thermolithotrophicus* strain SN1 under a 4:1 (v/v) H_2/CO_2 gas atmosphere have been described previously [3]. Cell growth (at 62°C) was monitored by measuring the solution optical density at 660 nm (OD_{660}). The total protein content in the cell cultures was determined using a modified version of the Bradford assay [8]. For in vivo NMR experiments (carried out at 50°C), cells were also adapted to grow at various external NaCl concentrations with 200 mM (13.60 g/l) sodium formate as a soluble substrate for methanogenesis [7] under 5 psi N_2/CO_2 (4:1, v/v). Buffer, 25 mM Pipes (piperazine-*N*-*N*′-ethanesulfonic acid) and 27.2 mM MES (2-[*N*-morpholino]ethanesulfonic acid), pH 6.8, was present in the media to alleviate any pH changes caused by generation of bicarbonate from formate. To introduce ^{13}C label into the solute (and precursor) pools prior to the in vivo NMR experiments, cells adapted to growth on formate were grown to an OD_{660} of 0.4 to 0.6 on $^{13}CO_2/H_2$ in the absence of 200 mM formate. All subsequent operations were carried out anaerobically in a He-series Dri-Lab glovebox containing N_2/H_2 (13:1, v/v) using solutions that were anaerobic. Typically 1 or 2 bottles containing 150 ml of cells (OD_{600} = 0.4–0.6) were used for a single in vivo experiment. Cultures were centrifuged and the pellet washed (with equiosmolar media) and resuspended in buffer containing 50 mM imidazole adjusted to pH 7.0 and the desired NaCl concentration (as described in detail in [7]). A small aliquot of the cell suspension was removed for Bradford assay of total protein. The remaining sample was then anaerobically transferred to a 5 mm NMR tube with 0.1 ml ^2H_2O (15 to 20% D_2O in the final suspension) and 40 μl EDTA (16 mM). The sample was stoppered and vented for 10 to 15 min under 2 psi N_2/CO_2 (4:1, v/v), then stored at room temperature until use, usually within 0.5 h. After an initial ^{13}C-edited ^1H NMR spectrum, a sodium [^{12}C]formate solution was added via syringe to a final concentration of 100 mM; an anaerobic cysteine solution (40 μl of 40 mM stock) was also added.

2.3. Preparation of ethanol extracts

Cell pellets were extracted with 70% ethanol as described previously [9]. After removal of the ethanol and lyophilization of the remaining solution, the sample was resuspended in D_2O (0.5 ml). If the sample was cloudy, it was either centrifuged to remove any particulate material or filtered using a sterile Millex-GV 0.22 μm filter (obtained from Millipore). The pH of the sample was adjusted to be in the range 6.9–7.1 using NaOD or DCl.

2.4. HPLC analyses of intracellular and extracellular amino acids

Cells of *M. thermolithotrophicus* were grown on $^{12}CO_2/H_2$ (1:4, v/v) as the sole carbon source to an OD_{660} of 0.6 containing 0.68 or 1.37 M NaCl. Aliquots (1 ml) of cells were removed periodically during growth. Following centrifugation, the supernatant and an ethanol extract of the cell pellet were analyzed by HPLC to monitor α- and β-amino acids. In these assays, 20 μl of sample (10 μl ethanol extract or supernatant and 10 μl isoleucine standard, 0.001% w/v) was incubated with 20 μl OPA for 2 min. Amino acid analysis of each OPA derivatized sample was carried out in duplicate (and the values averaged) with a two pump Rainin system using a Microsorb 25 cm C18 reverse-phase column with a 100-min modified linear gradient containing a two-buffer system: buffer A (90% 0.1 M sodium acetate at pH 7.2, 9.5% methanol, and 0.5% tetrahydrofuran), and buffer B (HPLC grade methanol). Derivatized primary amines were detected by fluorescence at 440 nm (excitation at 340 nm). Amounts of solutes determined in duplicate analyses of a given sample agreed within 20%.

For experiments where the NaCl content of the medium was increased, cells of *M. thermolithotrophicus* were grown to an OD_{660} of 0.8 with 0.68 M external NaCl. Cultures were diluted with an equal volume of media containing 2.73 M NaCl, for a final NaCl concentration of 1.70 M, and incubated at 62°C for up to 150 min. Aliquots were removed as a function of time after altering the NaCl concentration and centrifuged. Both the supernatant and ethanol extracted cell pellet were analyzed for osmolyte distribution by HPLC. Values of osmolytes (μmol) were normalized to total cell protein for that sample.

2.5. Atomic absorption analyses of intracellular K^+

Cells of *M. thermolithotrophicus* were grown to an OD_{660} of 0.6–0.7 in medium with either 0.68 or 1.02 M NaCl, then mixed with an equal volume of medium containing a different concentration of NaCl to yield the final desired NaCl concentration. As a control, cells were grown in medium containing 0.68 M NaCl and then diluted 1:1 with the same medium causing a change in cell density but not a change in external NaCl. The hyperosmotic shock experiment involved growing cells in 0.68 M NaCl, and then increasing the external NaCl; the hypoosmotic shock (decreased external NaCl) involved the reverse of this, cells grown in 1.02 M NaCl were diluted to 0.68 M NaCl. Upon dilution, 10-ml aliquots were removed at specific time intervals for atomic absorption analysis, and 1-ml aliquots were removed and centrifuged for Bradford assays of total protein. The cell suspensions for atomic absorption were treated as described previously [3,6]. A Perkin–Elmer 3100 model EDS spectrometer equipped with a furnace (Perkin Elmer HGA 60) and autosampler was used to analyze K^+ content. The μmol of K^+ from each cell suspension was then normalized to the total protein in that sample.

2.6. Preparation of protein extracts

All manipulations of cell suspensions, centrifuged pellets, and extracts were carried out in a He-series Dri-Lab glove box unless otherwise noted. All solutions were made anaerobic by degassing overnight with stirring. A pellet of *M. thermolithotrophicus* (5 g) grown in 0.68 or 1.37 M NaCl was resuspended in breakage buffer (50 mM Tris–HCl, 0.1 mM disodium EDTA, 1 mM DTT), and a number of protease inhibitors (Boehringer Mannheim Biochemicals) including 50 μM PMSF (phenylmethylsulfonyl fluoride), 0.1 mM Pefabloc SC (4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride), 1 μg/ml pepstatin, and 1 μg/ml leupeptin. The cell suspension was lysed by sonication (Branson sonicator Model 200) in a glove bag under an argon atmosphere; treatment with bovine pancreas deoxyribonuclease I (final concentration of 10 μg/ml DNAse and 10 mM $MgCl_2$) was used to reduce solution viscosity. The mixture was gently stirred at 4°C for 20 min. The supernatant was collected and solid ammonium sulfate was added over a 20-min period at 4°C to a final concentration of 85%. The solution was stirred overnight then centrifuged at 10 000 rpm for 30 min. The pellet was resuspended in buffer (5 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). The sample was then dialyzed against 4 l of the same buffer with 0.1 mM DTT and protease inhibitors added (25 μM PMSF, 0.1 mM Pefabloc, 1 μg/ml pepstatin, 1 μg/ml leupeptin). Protein concentrations were estimated using the Bradford assay, with BSA as the standard [8]. The crude protein extract was concentrated using Centriprep protein concentrators with a 10 000 Da cut-off (Amicon). This crude protein extract was stored in 1.5 ml Eppendorf vials and frozen at −20°C until use.

2.7. Detection of lysine 2,3-aminomutase and glutamate oxaloacetate transaminase activities in vitro

Equivalent amounts of crude protein, prepared from cultures grown in 0.68 and 1.37 M NaCl, were reactivated by dithionite using the protocol developed for the lysine aminomutase from *Clostridium* SB4 [10]. The protein extract (200 μl) was incubated anaerobically at 37°C for 2–4 h in 38 mM Tris–HCl (pH 8.25) with 0.046 mM pyridoxal phosphate, 12 mM glutathione, 0.92 mM ferrous ammonium sulfate, and 1.85 mM sodium dithionite. Aliquots (100 μl) of the reactivated protein were incubated at 37°C for several hours (4–24) with 25 mM α-lysine in the following assay mixture: 13 mM Tris–HCl, 1.92 μM *S*-adenosylmethionine, and 1 mM dithionite. After incubation, the mixture was transferred to a Microcon microconcentrator (3000 Da cutoff) and centrifuged for 10 min at $10\,000 \times g$. This was done to remove the high molecular mass components from the solution before application to the HPLC column. Fifteen μl of the assay mixture was then incubated with 15 μl OPA and analyzed by HPLC for the production of β-lysine [11]. The β-lysine standard was generated by hydrolysis of ethanol extracts containing N^{ε}-acetyl-β-lysine in 6 N HCl at 113°C for 24 h followed by chromatography using QAE Sephadex resin.

Glutamate oxaloacetate transaminase (GOT) activity was also measured in the protein extracts from cells grown in low and high NaCl. The reaction mixture, consisting of 20 mM aspartate, 20 mM α-ketoglutarate, 0.1 mM pyridoxal phosphate, in 100 mM Tris–HCl (pH 8.4) was incubated with the crude protein extract at 37°C. Glutamate production was assayed with the same HPLC system used for β-lysine analysis.

2.8. NMR spectroscopy

^1H NMR (500 MHz) spectra were acquired on a Varian Unity 500 MHz spectrometer and used to monitor the content of osmolytes in ethanol extracts. Each lyophilized extract was solubilized in 0.5–0.6 ml ^2H_2O). Imidazole (5 μmol) was added to each sample as an internal standard.

Errors in measuring peak intensities compared to the imidazole were less than 15%. Amounts of solutes were then normalized to the total protein determined by Bradford assays. For in vivo NMR experiments, a one-dimensional $\{^1H\}^{13}C$-HMQC experiment using a Varian Unity 500 MHz spectrometer and a 5 mm inverse probe at 50°C (the optimum temperature for *M. thermolithotrophicus* growing on formate) was carried out using data acquisition and processing parameters described in detail previously [7]. ^1H WALTZ decoupled ^{15}N NMR (50.65 MHz) spectra of cell suspensions were acquired on the same spectrometer using the following parameters: 7600 Hz sweep width, 18 176 datum points, 23 μs pulse (90° flip angle), 5–8 s delay time, 50°C probe temperature, and 32 transients per time point (collected every 6.6 min). Each free induction decay was processed with a 4-Hz line-broadening factor. A capillary containing 4 M $^{15}NH_4Cl$ was used as an external chemical shift and intensity standard. The ^{15}N in vivo experiments were carried out in duplicate.

3. Results

3.1. Solutes secreted by *M. thermolithotrophicus* during growth

Critical to understanding how *M. thermolithotrophicus* responds to salt stress is determining if increased intracellular solutes arise only from intracellular pools or if they can be internalized from the extracellular solution. This was accomplished by HPLC analysis of supernatants obtained from centrifuged cell suspensions of *M. thermolithotrophicus* at different stages of growth on H_2/CO_2. Four solutes corresponding to α-glutamate, β-glutamate, N^{ε}-acetyl-β-lysine, and aspartate were identified in cell-free media as detected in the HPLC elution profiles. Organic solutes secreted by cells grown in 0.68 M NaCl (from OD_{660} of 0.13 to 0.68) increased with cell growth, but were present at relatively low concentrations. For example, L-α-glutamate levels reached 2.7 ± 0.6 μM, β-glutamate and aspartate levels were ~2.5 times lower (1.1 ± 0.3 μM), while N^{ε}-acetyl-β-lysine was not detected. Cells of *M. thermolithotrophicus* grown in medium with higher salt (1.37 M NaCl) showed an enhanced secretion of solutes into the medium, most notably N^{ε}-acetyl-β-lysine. L-α-glutamate, β-glutamate, and aspartate concentrations reached 6.5, 2.3, and 1.6 μM, respectively, while levels of N^{ε}-acetyl-β-lysine secreted in the media rose to approximately 20 μM in cultures at OD_{660} of 0.7. The N^{ε}-acetyl-β-lysine was considerably higher than any other solute detected. However, these levels of solutes are significantly below detection by NMR spectroscopy and will not contribute to the NMR analysis with washed, resuspended cells. If all the N^{ε}-acetyl-β-lysine were internalized by the cells (which might be a response to increased external NaCl), that would correspond to < 10 mM intracellular N^{ε}-acetyl-β-lysine – an amount inconsequential compared to the 100–200 mM of each solute present under steady-state growth conditions [1,3].

3.2. Solute turnover in nonstressed and stressed pseudostationary cells

NMR spectroscopy is an ideal technique for monitoring turnover of solute pools in cells if an appropriate NMR-active isotope can be introduced into the solutes, then 'chased' using an isotope with no nuclear spin [3]. This technique has been used to monitor amino acid turnover in intact cells of *M. thermolithotrophicus* adapted to growth in formate that have been labeled with ^{13}C-formate and chased with ^{12}C-formate under steady state and osmotic stress conditions [7]. Cells adapted to formate have a longer doubling time than those grown on H_2/CO_2 [7]; they also have a large intracellular formate pool that can complicate measurements of ^{13}C turnover in solutes under different conditions. As a variation on this $H^{13}COO^-$-pulse/$H^{12}COO^-$-chase experiment, cells adapted to growth on formate were instead grown on $H_2/^{13}CO_2$ prior to the NMR experiments. Upon the addition of ^{12}C-formate, ^{13}C intensity was clearly incorporated into methane. Since no ^{13}C-labeled substrate was added to the cell suspension, the $^{13}CH_4$ must originate from labeled formate generated from $^{13}CO_2$ that was 'fixed' or immobilized inside the cell. A careful look at ^1H spectra revealed a resonance at 8.5 ppm identified as arising from ^{13}C-formate (^1H–$^{13}COO^-$). This pool of labeled material was about one third that found in cells grown at the same NaCl concentration but using $H^{13}COO^-$ as the substrate for methanogenesis [7]. It was stable to washing the intact cells, and provided an intracellular pool for labeling other molecules. The ^{13}C intensity of label in each solute was monitored as a function of time after a hyperosmotic or hypoosmotic shock. One such example is shown in Fig. 1 where cells grown in 0.48 M NaCl were resuspended in 1.20 M NaCl (plus 0.10 M sodium formate). This large increase in extracellular Na^+ (from 0.68 M to 1.30 M) had a pronounced effect on ^{13}C levels in the three different osmolytes over a 30-min time scale: the ^{13}C content of α-glutamate increased significantly (at a rate of 0.034 μmol ^{13}C/min per mg); β-glutamate intensity also increased (at a rate of 0.018 μmol ^{13}C/min per mg). In contrast, N^{ε}-acetyl-β-lysine intensity showed only a small increase in intensity (corresponding to a rate of 0.004 μmol ^{13}C/min per mg). For comparison, if the same cell suspension was mixed with medium that kept the external NaCl at 0.48 M (with sodium formate also constant), all three osmolytes showed a decreasing ^{13}C intensity with turnover rates of −0.006, −0.003, and −0.002 μmol ^{13}C/min per mg protein for L-α-glutamate, β-glutamate, and N^{ε}-acetyl-β-lysine, respectively [7]. Since the extracellular pools of amino acids are extremely small, these cannot account for the

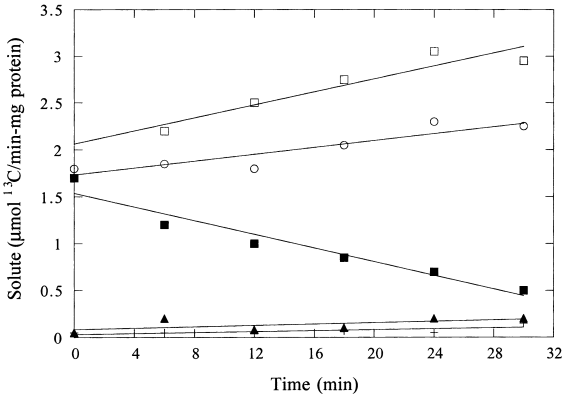

Fig. 1. Time dependence of the ^{13}C content of the endogenous intracellular osmolytes, formate, and methane in *Methanococcus thermolithotrophicus* initially grown on $^{13}CO_2/H_2$ at 0.48 M external NaCl and resuspended in medium with 1.2 M external NaCl. Solutes include α-glutamate (□), β-glutamate (○), N^{ε}-acetyl-β-lysine (▲), formate (■), and methane (+).

increased ^{13}C content in glutamate isomers upon hyperosmotic shock from 0.48 to 1.20 M NaCl (0.68–1.30 M Na^+). In contrast to cells adapted for growth in 1.20 M NaCl, these shocked cultures did not synthesize much N^{ε}-acetyl-β-lysine to balance the increased external Na^+. This behavior suggested that the cells were initially counteracting the osmotic stress by selectively synthesizing the anionic solutes (and L-α-glutamate in particular) and not N^{ε}-acetyl-β-lysine. The response was somewhat gradual, appearing linear for at least up to 30 min after the hyperosmotic shock.

A hypoosmotic shock caused more rapid turnover of all the osmolytes with perhaps a slight preference for turnover of both β-glutamate and N^{ε}-acetyl-β-lysine. For example, when the external NaCl was decreased from 0.83 to 0.48 M, the rate of ^{13}C turnover increased significantly in both β-amino acid pools (−0.029 and −0.030 μmol/min per mg total protein for N^{ε}-acetyl-β-lysine and β-glutamate, respectively); turnover in α-glutamate was roughly half those values. This suggests that in these pseudostationary cells, decreasing the external NaCl concentration causes some turnover (possibly efflux) of solute. However, in the 30 min of the experiment, 25–30% of anionic osmolytes (presumably balanced by a loss of intracellular K^+) were lost. This is smaller than the change in external Na^+ (a 38% decrease from 0.93 to 0.58 M Na^+). Cells grown in 1.20 M NaCl (1.3 M Na^+) and resuspended in 0.48 M NaCl (0.58 M Na^+) also showed relatively small changes ($<20\%$) in the ^{13}C label content in any of the organic osmolyte pools. Thus, the higher turgor pressure created by hypoosmotic shock has relatively minor effects on the overall osmolyte pool in *M. thermolithotrophicus*, at least for the first 30 min after osmotic shock.

The results from hypoosmotic shocks suggested that the β-amino acids were preferentially removed from the solute pool. All of the solutes used by *M. thermolithotrophicus* contain amino groups and these may be recycled more rapidly than the carbon framework because of the action of transaminases [3]. Changes in the rate of amino group scrambling may also provide insight into this response. Therefore, cells were prelabeled with $^{15}NH_4^+$, the washed cell pellet resuspended in medium with $^{14}NH_4^+$ at the same or a reduced external NaCl, and monitored for ^{15}N content after the addition of formate. As shown in Table 1, L-α-glutamate nitrogen turnover was more rapid ($t_{1/2}$ (^{15}N) = 5 ± 0.5 min) than either β-glutamate or N^{ε}-acetyl-β-lysine under nonstress conditions (for both β-amino acids $t_{1/2}$ (^{15}N) ≥ 50 min). However, after hypoosmotic shock, all three solute pools exhibited dramatically increased turnover of amino groups. The observation that ^{13}C half-lives, even under hypoosmotic stress conditions, were much longer than the ^{15}N $t_{1/2}$ values (see Table 1) suggests that transaminase activities increased when the cells were osmotically shocked. The nitrogen fixed in the large solute pool is apparently accessible to the cell under hypoosmotic stress conditions.

3.3. Intracellular osmolyte levels following osmotic shock of exponentially growing cultures

The ^{1}H NMR in vivo experiments used cell populations at extremely high cell densities with limited biosynthetic capabilities. Whether the same responses to hyperosmotic shock are exhibited by a mid-log phase culture similarly challenged with external Na^+ is an important question. As an alternate method of confirming that cells of *M. thermolithotrophicus* increased levels of glutamate isomers rather than N^{ε}-acetyl-β-lysine immediately after NaCl hyperosmotic shock, cells grown to mid-log phase on H_2/CO_2 at 0.68 M NaCl were osmotically shocked by increasing the external salt concentration to 1.02 M. Aliquots of the culture were removed as a function of time, centrifuged, washed and ethanol extracted. ^{1}H NMR spectra of the extracts were used to quantify the concentration of solutes after the hyperosmotic shock. As can be seen in Fig. 2A, 30–40 min after increasing the NaCl, the α-glutamate levels increased to a maximum (roughly two-fold the initial value) and then decreased; β-glutamate and aspartate also increased to a maximum, then decreased. N^{ε}-acetyl-β-lysine levels increased (initially this solute was barely detectable) but did not make up a significant fraction of the osmolyte pool even after an hour ($<15\%$

Table 1
Half-lives of ^{15}N- and ^{13}C-labeled solute under nonstress and hypoosmotic stress conditions in *M. thermolithotrophicus* as determined by in vivo NMR spectroscopy

Solute	^{13}C $t_{1/2}$ (min)		^{15}N $t_{1/2}$ (min)	
	Steady state	Decreased NaCl	Steady state	Decreased NaCl
L-α-Glutamate	>70	~50	5	2
β-Glutamate	>70	~50	48	6
N^{ε}-Acetyl-β-lysine	125	~50	>100	10

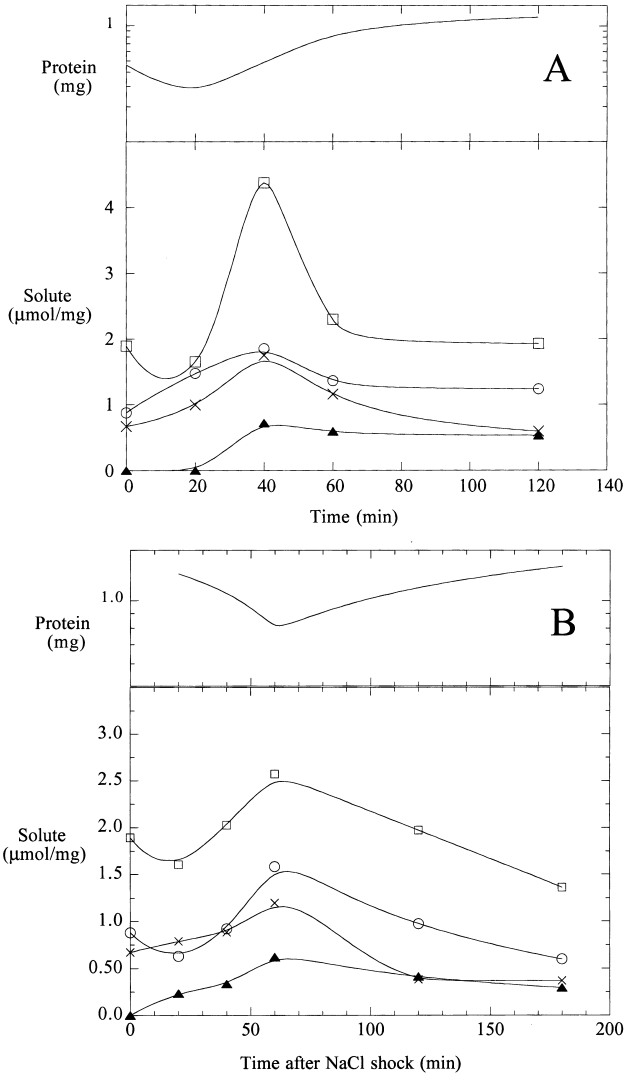

Fig. 2. Organic osmolyte distribution in *M. thermolithotrophicus* as a function of time after increasing external NaCl from 0.68 to (A) 1.02 M and (B) 1.4 M: α-glutamate (□), β-glutamate (○), aspartate (×), and N^{ε}-acetyl-β-lysine (▲). The insets above each plot indicate cell growth as reflected in total protein after hyperosmotic shock.

of the total solute pool). Changes in the organic solute pool were not immediate but occurred over a 1-h time scale. For comparison to the change in intracellular solutes, the total protein of the culture is shown as the insert in Fig. 2A. The maximum in the intracellular solute pool (which is roughly half α-glutamate) coincides with resumed growth of the cells.

The same approach but transferring cells from 0.68 to 1.37 M external NaCl (a larger hyperosmotic shock), again showed an initial (after a 20-min lag) increase in α-glutamate to a maximum value at 60 min along with smaller increases in β-glutamate, aspartate, and N^{ε}-acetyl-β-lysine on the same time scale (Fig. 2B). At this solute maximum, α-glutamate represented 0.43 mole fraction of the total solute pool; N^{ε}-acetyl-β-lysine represented only 0.10 mole fraction. At 60 min after this hyperosmotic shock, the cells began to grow again. As the cells grew, the levels of solutes decreased. At 180 min after the hyperosmotic shock, N^{ε}-acetyl-β-lysine represented 0.12 of the total osmolyte pool. For comparison, when cells are adapted to growth in 1.37 M external NaCl, N^{ε}-acetyl-β-lysine represents 0.4–0.5 mole fraction of the total solutes [3,7].

Another experiment increasing external NaCl from 0.68 to 1.71 M was carried out to compare solute levels and cell growth after hyperosmotic shock. HPLC was used to analyze the solutes rather than ^{1}H NMR so that solute concentrations in both extracts from intact cells and in the extracellular medium could be measured. Consistent with the NMR analyses, HPLC experiments monitored little intracellular N^{ε}-acetyl-β-lysine accumulation following the increase in external osmotic pressure (Fig. 3A). Thus, *M. thermolithotrophicus* requires an extended period to induce accumulation of N^{ε}-acetyl-β-lysine to high levels when transferred into high salt medium. After an initial lag of about 15–20 min (which was observed in all hyperosmotic shock experiments (Table 2)), the total intracellular organic solute pool, made up of L-α-glutamate, β-glutamate, and aspartate, increased over a rather long time scale (~100 min) in this experiment. The maximum in the

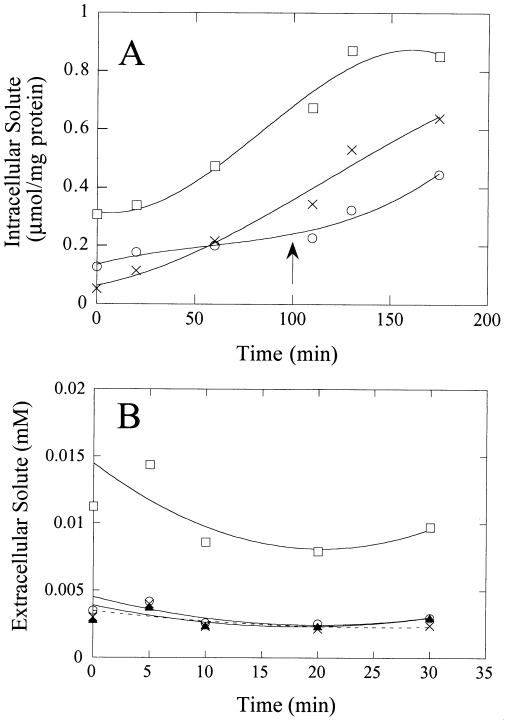

Fig. 3. (A) HPLC analysis of intracellular organic solutes (μmol/mg protein) as a function of time after *M. thermolithotrophicus* cells in medium with 0.68 M external NaCl were diluted 1:1 with high salt medium to a final concentration of 1.7 M NaCl. (B) Extracellular solutes from the same cultures after hyperosmotic shock. Solutes include α-glutamate (□), β-glutamate (○), N^{ε}-acetyl-β-lysine (▲), and aspartate (×). Significant amounts of intracellular N^{ε}-acetyl-β-lysine were not detected on this time scale by HPLC analyses. The arrow in A indicates the time after hyperosmotic shock at which the culture began to grow.

intracellular solute pool coincided with resumption of cell growth. There were also modest decreases in the small extracellular solute pool when the external NaCl was increased (Fig. 3B). Significant intracellular levels of N^{ε}-acetyl-β-lysine were not detected in this HPLC experiment.

In all three of these examples, there was an increase in osmolytes in response to the osmotic shock that did not correspond to the distribution of osmolytes observed in cells adapted to the higher NaCl conditions. That the solute increase did not occur rapidly (within seconds or a few minutes of the change in external NaCl) but over a time scale (30–100 min) that increased with the severity of the hyperosmotic shock strongly suggested that the first response of the cells to maintain turgor pressure must involve accumulation of a different solute with the most likely candidate potassium ions. The time at which a maximum in intracellular organic solutes (predominantly α-glutamate) occurs after osmotic stress coincides with the point at which cells resumed growth (see Table 2). After this, organic solute levels decreased approaching what is detected in exponentially growing cells. However, the distribution of anions and zwitterions is quite different in these stressed cultures from what is observed in adapted cultures. This difference appears to persist for at least one generation.

3.4. Changes in intracellular K^+ following osmotic shock

M. thermolithotrophicus maintains a high intracellular concentration of K^+ as the counterion for the glutamate isomers [3]. Since the response of the cells to a hyperosmotic shock exhibited a lag before the accumulation of glutamate, it was of interest to examine the changes in K^+ under similar conditions – would they parallel the change in organic anions or show more immediate changes as has been seen for several bacteria, notably *E. coli* [12,13]. Atomic absorption was used to monitor the intracellular K^+ concentration following increased or decreased external NaCl. The experiments involved diluting a culture grown in 0.68 M NaCl with an equal volume of medium to a final concentration of 1.02 M NaCl, or diluting a culture adapted to growth at 1.02 M NaCl to 0.68 M NaCl. The changes were smaller than those used in the analysis of the response of organic osmolytes to external NaCl in order to avoid cell lysis which would artificially decrease intracellular K^+ levels. In each case the control consisted of the cells at 0.68 M NaCl diluted with medium of the same composition. As can be seen in Fig. 4, upon hyperosmotic shock, there was a rapid increase in intracellular K^+ within the first few minutes, followed by a decrease to steady-state levels (within 15 min or so). The steady-state level for K^+ in the hyperosmotic shock experiment was roughly 1.5 times that of the control cells, consistent with a response that involved accumulation of glutamate rather than a zwitterionic solute (N^{ε}-acetyl-β-lysine). After the hypoosmotic shock (Fig. 4), there was a rapid reduction in intracellular K^+ to a value expected for the new steady-state level. In both cases, the response of the cell K^+ pool was on a much more rapid time scale (< 15 min) than changes in organic osmolytes.

3.5. Lysine aminomutase activity in cell extracts

One possible explanation for the lack of accumulation of N^{ε}-acetyl-β-lysine and preferential accumulation of glutamate in cells grown in 0.68 M NaCl and challenged with NaCl is that the activity of a key enzyme in the biosynthesis of the zwitterionic solute is limiting when the cells are adapted to growth in lower NaCl-containing medium. α-Lysine is synthesized by the diaminopimelate pathway in this organism. The α-lysine is converted to β-lysine by an aminomutase; the β-amino acid is then acetylated to form N^{ε}-acetyl-β-lysine [3]. Free β-lysine is not present at NMR-detectable concentrations in ethanol extracts of *M. thermolithotrophicus*. Since α-lysine is needed for protein synthesis, it is unlikely that any of the enzymes required for its synthesis would be regulated either directly or indirectly by external Na^+. Thus, a likely candidate for control of the accumulation of N^{ε}-acetyl-β-lysine is lysine 2,3-aminomutase. To test this hypothesis, lysine aminomutase activity was assayed in dialyzed crude protein extracts from cells grown in 0.68 and 1.37 M NaCl. If the same amount of active enzyme is present under both conditions, specific activities should be similar. If adaptation to high external Na^+ induces synthesis of the lysine aminomutase or is coupled to activation of inhibited lysine aminomu-

Table 2
Times required for *M. thermolithotrophicus* cultures grown in 0.68 M NaCl that have been hyperosmotically shocked to begin synthesis of L-α-glutamate, accumulate maximum organic solutes, and resume growth

Final NaCl (M)	τ_{lag} (min)[a] (α-Glu)	t_{max} (min)[b]	α-Glu[c] (mole fraction)	NAβLys[c] (mole fraction)	t_{growth} (min)[d]
1.0	20	40	0.52	0.08	30–40
1.4	20	60	0.43	0.10	60–70
1.7	15–20	100	0.50	< 0.05	100

The fraction of α-glutamate and N^{ε}-acetyl-β-lysine at the point of maximum solute accumulation is also shown.
[a]Time after increasing NaCl before α-glutamate is accumulated by the cells.
[b]Time after hyperosmotic shock when the accumulation of intracellular solutes has reached a maximum.
[c]Mole fraction of the maximum total solutes accumulated.
[d]Time at which growth of hyperosmotically shocked culture resumes.

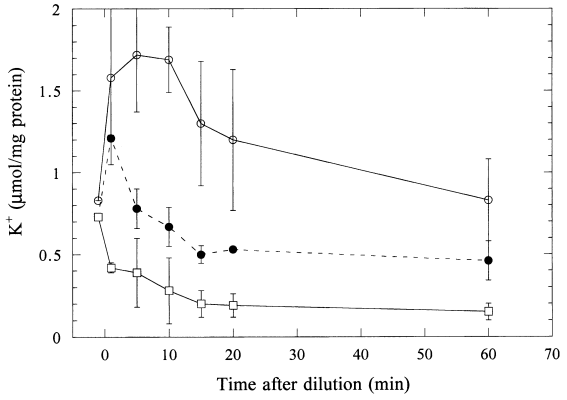

Fig. 4. Intracellular K^+ concentrations, measured by atomic absorption, in *M. thermolithotrophicus*. Cells initially grown in 0.68 M NaCl diluted with medium to 1.02 M NaCl (○), or cells initially grown in 1.02 M NaCl and diluted to 0.68 M NaCl (□) are compared to control cells (2-fold dilution with 0.68 M NaCl) (●).

tase, then there should be considerably more activity in the protein extract from cells grown in 1.37 M NaCl.

Lysine aminomutase activity could be detected in protein extracts from cells grown in 1.37 M NaCl (Fig. 5A). The assays were carried out at 37°C because at 62°C, the growth temperature of the organism, several of the assay and reactivation components (notably *S*-adenosylmethionine and pyridoxal phosphate) were unstable over the time courses required. Incubation times of 4–8 h were initially used to examine assay requirements of the activity in *M. thermolithotrophicus*. Pyridoxal phosphate and dithionite but not ferrous ammonium sulfate were needed for optimal reactivation of this activity, and generation of β-lysine from α-lysine was dependent on the addition of *S*-adenosylmethionine. These characteristics indicate the archaeal enzyme behaves much like the clostridial lysine aminomutase [10,14,15]. The specific activity estimated for lysine aminomutase in protein extracts from cells grown in 1.37 M NaCl was 4 ± 2 nmol/min per mg. An activation energy for the enzyme of 50 kJ/mol (assuming an average value) would increase the rate 4-fold, and at 62°C *M. thermolithotrophicus* cells could generate 0.017 μmol/min per mg β-lysine. A typical culture adapted to 1.37 M NaCl has about 1 μmol N^{ε}-acetyl-β-lysine per mg protein [3]; this amount of N^{ε}-acetyl-β-lysine could be synthesized in 60 min, roughly the doubling time of the organism. N^{ε}-acetyl-β-lysine is not degraded by the cells or used as anything other than a compatible solute [3] so that this specific activity, though low, is sufficient. In comparison, lysine aminomutase activity in the protein extract from cells grown in 0.68 M NaCl was quite low and not detectable above the blank after a 4 h incubation. Therefore, a considerably longer incubation time (24 h) and larger amount of total protein (1.4 mg) was used for a more direct comparison of lysine aminomutase specific activity in crude protein extracts from cells grown in 1.37 versus 0.68 M NaCl. Towards a substrate concentration of 25 mM α-lysine (well above the K_m for α-lysine exhibited by the clostridial enzyme), lysine aminomutase had a specific activity of ~3 nmol/min per mg at 37°C (Fig. 5A, note the arrow at 43 min indicating the β-lysine). The same reactivation and incubation conditions for protein from cells grown in 0.68 M NaCl yielded much less β-lysine in the same 24 h period (Fig. 5B) and corresponded to a specific activity ~0.4 nmol/min per mg. Clearly, lysine aminomutase activity was at least 8-fold lower than for cells grown in 1.37 M NaCl.

For comparison to the changes observed in lysine aminomutase activities in the two protein extracts, the specific activity of glutamate oxaloacetate transaminase (GOT), one of the enzymes involved in glutamate production, was also measured in the extracts from cells adapted to 0.68 and 1.37 M NaCl. GOT specific activity was 10.5 nmol/min per mg in the extract from cells grown in 0.68 M NaCl and 6.5 nmol/min per mg in cells grown in 1.37 M NaCl. While there was a change in enzyme activity between the two cultures, this change was relatively small (60% decrease in specific activity in the protein extract from higher NaCl conditions) compared to the change for lysine aminomutase (750% increase in activity in the protein extract grown in higher NaCl). Thus, the lower lysine aminomutase activity under low NaCl conditions could be responsible for why the cells synthesize and accumulate glutamate isomers (and mostly L-α-glutamate) when such a culture is challenged with increased NaCl. Adaptation to high NaCl appears to increase the amount of active lysine aminomutase, and such a response is likely to require resources within the cell.

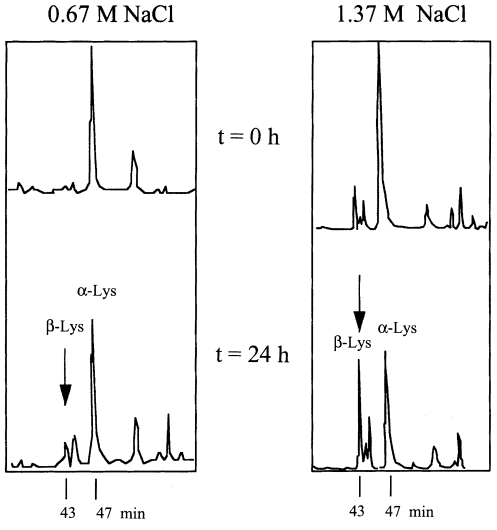

Fig. 5. HPLC analyses of lysine aminomutase activity in protein extracts from *M. thermolithotrophicus* cells grown on H_2/CO_2 in medium containing (A) 0.68 and (B) 1.4 M NaCl. Only the portion of the elution profile around the lysine substrate (5 mM) is shown; isoleucine served as an internal standard. The top chromatogram shows the $t = 0$ control; the bottom in each panel shows the amount of β-lysine generated after incubation of the extract with substrate for 24 h. The arrow indicates the β-lysine peak.

4. Discussion

Methanococcus thermolithotrophicus, a thermophilic marine methanogen, synthesizes several organic solutes, including β-amino acids, in response to different external NaCl concentrations as determined by both NMR spectroscopy and HPLC. When growing at salt concentrations <1 M, these cells synthesize and accumulate α- and β-glutamate; at salt concentrations >1 M, these cells synthesize and accumulate the zwitterionic solute, N^{ε}-acetyl-β-lysine, in addition to the two glutamate isomers [3]. The anionic solutes are balanced by intracellular K^+ ions. Although β-amino acids are rare in nature, they have a specific role as compatible solutes in several marine methanogens [1–4,16]. Once synthesized, they are only slowly metabolized if the NaCl concentration is not changed [3]. However, hypoosmotic shock appeared to enhance degradation of the β-amino acids and more specifically exchange of the nitrogen moiety (as detected by the ^{13}C and ^{15}N turnover rates or half-lives).

The immediate response of cultures of *M. thermolithotrophicus* to changes in external NaCl is quite different from the adapted osmotic response and has distinct stages that involve different intracellular solutes. Intracellular K^+ levels respond almost immediately to decreasing or increasing external NaCl in a fashion similar to that observed in *E. coli* [12,13]. Rearrangement of the organic osmolyte pool occurs on a slower time scale. For hypoosmotic shock, loss of ^{15}N precedes loss of ^{13}C suggesting that the large concentration of nitrogen stored in the osmolytes is available to the cell under stress situations. However, the overall rather small decreases in osmolyte pools when external NaCl is decreased suggest that *M. thermolithotrophicus* cells can withstand the higher turgor pressure that is the immediate result of decreasing external NaCl. For hyperosmotic stress, the immediate response of the cells is quite different from what is observed in cultures adapted to grow at specific external NaCl concentrations. The results show that N^{ε}-acetyl-β-lysine is not synthesized and accumulated immediately after cells are transferred to media with high NaCl. Instead, cells rapidly accumulate primarily α-glutamate with some β-glutamate and aspartate to achieve osmotic balance; N^{ε}-acetyl-β-lysine is accumulated but to only ≤ 0.10 mole fraction of the total osmolyte pool. Most of the accumulated species are anions (as opposed to N^{ε}-acetyl-β-lysine) and are probably intertwined with K^+ uptake and accumulation. The HPLC analysis of medium solutes showed that the external osmolyte concentration (<4 μM upon dilution) was too low to supply α-glutamate for internalization. For a culture of 150 ml cells $OD_{660} \sim 0.4$, roughly 10 μmol of α-glutamate would be necessary to balance the increased NaCl. The external medium could supply at most 0.6 μmol α-glutamate for reinternalization by the cells. Therefore, the increased α-glutamate is generated by biosynthesis. For the NMR experiments, the external solute pool has been removed by washing and is not available, therefore, the increased α-glutamate must be synthesized by the cells.

The observation that glutamate isomers are accumulated in preference to N^{ε}-acetyl-β-lysine suggests that adaptation of *M. thermolithotrophicus* to high NaCl involves the synthesis or modification of protein(s) involved in either synthesis or net accumulation of N^{ε}-acetyl-β-lysine. A possible candidate for a key activity regulated in this fashion is lysine 2,3-aminomutase, the enzyme that generates β-lysine from α-lysine. Assays of crude cell extracts of *M. thermolithotrophicus* grown in medium with 0.68 versus 1.37 M NaCl clearly show a significant difference in the specific activity of lysine 2,3-aminomutase activity consistent with this enzyme as the probable target for regulating accumulation of the zwitterionic osmolyte. Further delineation of the regulatory mechanism for N^{ε}-acetyl-β-lysine should focus on isolation and characterization of this enzyme. In contrast to the lysine aminomutase activity, glutamate oxaloacetate transaminase specific activity did not vary dramatically in protein extracts prepared from cells adapted to grow in low and high NaCl.

The hierarchy of the response of *M. thermolithotrophicus* to osmotic stress can be summarized as follows. Immediately after decreasing the external NaCl, it is presumed that water begins to rush into the cells. This is counteracted by decreasing intracellular K^+ to a new lower steady-state level (within 10–15 min). Cell growth resumes soon after the new intracellular K^+ concentration is stabilized. Over a longer time period, intracellular concentrations of organic solutes are reduced with a slight preference for reducing both β-amino acids. The response to increased NaCl is more complex, but reminiscent of what has been observed for several well studied bacteria and more specifically for *E. coli* [12,13]. Immediately after hyperosmotic shock, water is presumed to rush out of the cells. The first response of the cells is to internalize more K^+. The amount internalized overshoots what would be needed to balance the external Na^+ if an appropriate counterion were also internalized. After 10–20 min, the K^+ 'overshoot' is reduced and a new K^+ steady state is produced. Organic osmolyte accumulation (most notably L-α-glutamate) begins once the intracellular K^+ is stabilized. L-α-glutamate is the major solute accumulated under these conditions and its intracellular levels reach a maximum that coincides with the point where growth resumes. N^{ε}-acetyl-β-lysine levels increase, but this osmolyte is not accumulated to the levels observed in cells adapted to growth in high NaCl. Longer periods (several cell divisions) are need for the adapted distribution of organic osmolytes to be detected. Thus, K^+ and the glutamate isomers (L-α-glutamate in particular) act as temporary osmolytes in the hyperosmotic response of *M. thermolithotrophicus*. While this provides a start in understanding how this methanogen responds to changes in external NaCl, it is clear that work is needed to characterize the proteins responsible for this temporal response.

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