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Effects of Osmolytes on the SLN1-YPD1-SSK1 Phosphorelay system from *Saccharomyces cerevisiae*[†]

Alla O. Kaserer, Babak Andi, Paul F. Cook, and Ann H. West*

Department of Chemistry and Biochemistry, University of Oklahoma, 620 Parrington Oval, Norman, Oklahoma 73019

Abstract

The multi-step His-Asp phosphorelay system in *Saccharomyces cerevisiae* allows cells to adapt to osmotic, oxidative and other environmental stresses. The pathway consists of a hybrid histidine kinase SLN1, a histidine-containing phosphotransfer (Hpt) protein YPD1 and two response regulator proteins, SSK1 and SKN7. Under non-osmotic stress conditions, the SLN1 sensor kinase is active and phosphoryl groups are shuttled through YPD1 to SSK1, therefore maintaining the response regulator protein in a constitutively phosphorylated state. The cellular response to hyperosmotic stress involves rapid efflux of water and changes in intracellular ion and osmolyte concentration. In this study, we examined the individual and combined effects of NaCl and glycerol on phosphotransfer rates within the SLN1-YPD1-SSK1 phosphorelay. The results show that the combined effects of glycerol and NaCl on the phosphotransfer reaction rates are different from the individual effects of glycerol and NaCl. The combinatory effect is likely more representative of the *in vivo* changes that occur during hyperosmotic stress. In addition, the effect of osmolyte concentration on the half-life of the phosphorylated SSK1 receiver domain in the presence/absence of YPD1 was evaluated. Our findings demonstrate that increasing osmolyte concentrations negatively affects the YPD1•SSK1~P interaction thereby facilitating dephosphorylation of SSK1 and activating the HOG1 MAP kinase cascade. In contrast, at the highest osmolyte concentrations, reflective of the osmoadaptation phase of the signaling pathway, the kinetics of the phosphorelay favor production of SSK1~P and inhibition of the HOG1 pathway.

Two-component signal transduction systems in prokaryotes and eukaryotes regulate cellular responses to a variety of environmental stresses (2). Canonical two-component systems contain a membrane-bound sensor histidine kinase (HK)¹ and a cytoplasmic response regulator (RR). Upon stimulation, the HK autophosphorylates a conserved histidine residue located within the cytoplasmic kinase domain. The phosphoryl group is then transferred to a conserved aspartate residue within the receiver domain of the RR protein. Typically, phosphorylated RRs modulate downstream gene expression (*e.g.* many bacterial response regulators are transcription factors). Multi-step phosphorelay systems are more complex and employ additional signaling components, such as a histidine-containing phosphotransfer (Hpt) protein and multiple HKs or RRs (3-6).

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*Corresponding author: awest@ou.edu Tel: 405-325-1529 Fax: 405-325-6111.

¹Abbreviations: ATP, adenosine-5'-triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GST, glutathione-S-transferase; HK, histidine kinase; Hpt, histidine-containing phosphotransfer; IPTG, isopropyl β -D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; RR, response regulator; MAP, mitogen-activated protein; MAPK, MAP kinase; MAPKK, MAP kinase kinase; MAPKKK, MAP kinase kinase kinase; SDS, sodium dodecyl sulfate.

In *Saccharomyces cerevisiae*, a multi-step phosphorelay system is responsible for adaptation to osmotic, oxidative and other environmental stresses (7,8). The branched pathway consists of a sensor histidine kinase SLN1, a histidine-containing phosphotransfer (HPt) protein YPD1, and two independent response regulator proteins, SSK1 and SKN7. The *SLN1* gene encodes a hybrid histidine kinase with two membrane spanning regions and an extracellular sensing domain (9). The HPt protein, YPD1, serves as a non-enzymatic but essential mediator between SLN1 and two downstream RR proteins SSK1 and SKN7 (10-12). *In vitro* data suggest that YPD1 serves a regulatory function as well by forming a stable complex with the phosphorylated SSK1 response regulator domain under non-osmotic stress conditions thereby shielding the phosphoryl group from hydrolysis (13). Phosphorylation and dephosphorylation of SSK1 therefore functions as an on/off switch in controlling the activity of the downstream HOG1 mitogen-activated protein (MAP) kinase cascade (14,15). Under hyperosmotic conditions, we propose that dissociation of the YPD1•SSK1~P complex must occur, followed by dephosphorylation of SSK1. The unphosphorylated form of SSK1 directly interacts with and activates SSK2/SSK22 (MAPKKK), which leads to the activation of PBS2 (MAPKK) and the HOG1 MAP kinase (14,15).

In a previous study, we used rapid-quench kinetics to characterize the individual phosphotransfer reactions between YPD1 and the response regulator domains associated with SLN1, SSK1 and SKN7 (referred to as SLN1-R1, SSK1-R2, and SKN7-R3, respectively) (16). For the SLN1-R1~P to YPD1 reaction, a maximum forward rate constant of 29 s^{-1} was determined with a K_d of $1.4\text{ }\mu\text{M}$ for the SLN1-R1~P-YPD1 complex. A very rapid phosphotransfer rate of 160 s^{-1} was measured for the subsequent reaction between YPD1~P to SSK1-R2 and the reaction is strongly favored over phosphotransfer to SKN7-R3. Phosphotransfer reactions between YPD1 and SLN1-R1 or SKN7-R3 were reversible; while reverse transfer from SSK1-R2~P to YPD1 was not observed under the conditions tested. These parameters are in good agreement with the concept that SSK1 is constitutively phosphorylated under normal osmotic conditions (14-16).

When *S. cerevisiae* is exposed to hyperosmotic stress and water leaves the cell by passive diffusion, SLN1 kinase activity diminishes (8,17-21). This allows for accumulation of unphosphorylated SSK1 and its activation of the downstream HOG1 MAP kinase cascade. Activated phospho-HOG1 translocates into the nucleus (22) and activates gene expression of multiple genes including the *GPD1* (glycerol-phosphate dehydrogenase) and *GPP2* (glycerol-3-phosphate phosphatase) genes which leads to increased glycerol synthesis (8, 23-26). Accumulation of intracellular glycerol, a chemically inert osmolyte, allows yeast cells to adapt to hyperosmotic stress conditions.

A number of different osmolytes are found inside yeast and other fungi including monovalent salts, amino acids, polyols and carbohydrates (8,18,27). *S. cerevisiae*, *S. pombe*, *C. albicans*, and *D. hansenii* almost exclusively employ glycerol as an osmolyte in osmoregulation (8,17, 28-30). Osmoadaptation is achieved through a series of cellular responses that are temporally regulated. For instance, closure of the osmotically sensitive glycerol channel (Fps1) upon hyperosmotic shock provides an additional route to increase the glycerol concentration inside the cell almost immediately to compensate for water efflux (31,32). HOG1 phosphorylation occurs in the cell during the first 1-3 min after exposure to osmotic shock (33,34). Both GPD1 and glycerol concentrations are at half-maximal level within 20 min (35). An increase in intracellular glycerol concentration increases the turgor pressure which is mediated by the elasticity of the plasma membrane and cell wall upon water exchange (35).

Elucidating the molecular events that trigger the cellular responses to hyperosmotic stress including the rapid efflux of water, changes in intracellular ion and osmolyte concentration, phosphorylation/dephosphorylation of SSK1-R2 and the kinetics of the SLN1-YPD1-SSK1

phosphorelay is important for fully understanding the overall regulation of this signaling pathway. Previous results indicated that YPD1 can form a stable complex with SSK1-R2~P and significantly extend the phosphorylated lifetime of the RR domain under non-osmotic stress conditions (13). Upon hyperosmotic stress, however, we hypothesize that changes in intracellular ion/solute concentrations disrupts the YPD1•SSK1-R2~P complex, thus facilitating dephosphorylation of SSK1 and subsequent HOG1 MAP kinase cascade activation. We therefore examined the effect of osmolyte concentrations on the half-life of the phosphorylated SSK1-R2 in the presence and absence of YPD1 and the kinetics of the individual phosphorelay reactions. This study provides new insights into the mechanisms that underlie the osmoregulatory pathway in *S. cerevisiae* and the specific effects of osmolytes in regulating the pathway.

Materials and Methods

Materials

All chemicals and biochemicals were of ultrapure grade. Glutathione-Sepharose 4B resin was purchased from Amersham. [γ - 32 P] ATP (3000 Ci/mmol) was purchased from Perkin-Elmer. The SLN1-R1, YPD1, SSK1-R2, GST-HK and GST-HK-R1 proteins were purified as described previously (10,29,30). Ficoll 400, D-(+)-trehalose, proline and betaine were purchased from Sigma-Aldrich. NaCl and glycerol were from Mallinckrodt Chemicals and Pharmco-Aaper, respectively.

SLN1-R1 Phosphorylation

The SLN1-R1 domain was phosphorylated via incubation with the SLN1-HK domain as follows. GST-tagged SLN1-HK (7 μ M) bound to glutathione-Sepharose 4B resin was incubated with 7 μ M [γ - 32 P] ATP for 30 min. Unincorporated [γ - 32 P] ATP was washed from phospho-SLN1-HK with 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 15 mM MgCl₂, 2 mM DTT, and 20% glycerol by 3 consecutive centrifugations (1 min at 1000 \times g). The SLN1-R1 protein (18.6 μ M) was then added in the same buffer and incubated for 10 min at room temperature in a total volume of 300 μ L. Phospho-SLN1-R1 was recovered in the supernatant after gently pelleting the GST-SLN1-HK bound to the resin. EDTA was added to the supernatant to a final concentration of 30 mM to prevent autodephosphorylation (16).

YPD1 Phosphorylation

The YPD1 protein was phosphorylated similarly to that of SLN1-R1 with the following modifications. Incubation of GST-tagged SLN1-HK-R1 (7 μ M) and [γ - 32 P] ATP (7 μ M) was for 60 min (16). YPD1 protein (18.6 μ M) then was added in the reaction mixture for phosphorylation.

Rapid-Quench Experiments

Experiments were carried out to obtain rate data for the individual phosphotransfer steps using a rapid-quench kinetics instrument SFM-Q/4 Quench-Flow instrument (BioLogic). Phosphotransfer reactions were monitored in the millisecond timescale for acquisition of pre-steady state experimental data. The phosphodonor protein was diluted to 0.45 μ M in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT and glycerol (0.3-1.2 M), or NaCl (0.2-1.0 M). The diluted phosphorylated protein (60 μ L) was then mixed with 60 μ L of phospho-accepting protein partner (0.45 μ M-20 μ M) in 50 mM Tris-HCl, pH 8.0, 20 mM MgCl₂, 1 mM DTT and 0.3-1.2 M glycerol, or 0.2-1 M NaCl, or glycerol/NaCl combinations (0.3/0.2 M, 0.55/0.4 M and 0.75/0.6 M). The reactions were quenched with 60 μ L of the stop buffer (8% SDS, 80 mM EDTA) after a specified time. To analyze the results, 30 μ L of the quenched reaction was mixed with 10 μ L of 4 \times SDS-PAGE loading buffer (200 mM Tris pH 6.8, 400 mM DTT or β -

mercaptoethanol, 8% SDS, 0.4 % bromophenol blue, and 40% glycerol) , and then 30 μ L samples were loaded onto 15% SDS-PAGE gels. After gel electrophoresis, wet gels were wrapped in plastic wrap and analyzed using a phosphorimager (Molecular Dynamics, Storm 840). The phosphotransfer reaction kinetic parameters were quantified based on the disappearance of the 32 P-label from the phospho-donor protein or the appearance of 32 P-label in the phospho-accepting protein. The data were analyzed using the least squares fitting of Excel (Microsoft Office v. 10.1.12) and Enzfitter (version 2.04, Biosoft, Cambridge, U.K.). Individual datasets were analyzed using eq 1 for experiments with NaCl, glycerol, Ficoll 400 and NaCl-glycerol combinations,

$$k_{\text{obs}} = k_{\text{rev}} + k_{\text{fwd}} \left(\frac{[S]}{K_d + [S]} \right) \quad (1)$$

where [S] is the concentration of the phospho-accepting protein, k_{obs} is the observed first-order rate constant for the phosphotransfer reaction at a particular [S], k_{fwd} is the maximal forward net rate constant for phosphoryl transfer from the phosphorylated protein to the phospho-acceptor protein, k_{rev} is the corresponding maximum reverse net rate constant for the reaction between phospho-donor and phospho-acceptor proteins, and K_d is the dissociation constant of the phospho-donor-acceptor complex. The concentration of Ficoll 400 used in the control experiment was 0.75 M.

Phosphorylated life-time experiments of SSK1-R2 in the presence of osmolytes and presence/absence of YPD1

Phosphorylation of the response regulator domain SSK1-R2 was achieved similarly to that of SLN1-R1 with the following modifications. SSK1-R2 was added to the reaction mixture (40 μ L) with GST-HK (7 μ M) and [γ - 32 P] ATP (7 μ M) and incubated for 30 min. Phospho-SSK1-R2 was recovered in the supernatant (30 μ L) and was then added to a reaction mixture containing 3 μ M YPD1 and indicated osmolyte concentrations (NaCl, trehalose, glycerol, proline, or betaine) in a total reaction volume of 120 μ L. For the reaction in the absence of YPD1, the isolated SSK1-R2 (12 μ M) in 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM MgCl_2 , 2 mM DTT was added to the indicated osmolyte concentrations in a total reaction volume of 75 μ L. Aliquots (15 μ L) were removed at indicated time points for both reactions, mixed with 5 μ L of 4X stop buffer (0.25 M Tris-HCl pH 8.0, 8% SDS, 60 mM EDTA, 40% glycerol, 0.008% bromophenol blue) to terminate the reaction, and kept at 20 $^{\circ}\text{C}$ until gel analysis. Dephosphorylation of phospho-SSK1-R2 followed first-order rate kinetics and the half-life of phospho-SSK1-R2 was determined according to the formula $t_{1/2} = \ln 2/k$, where k is the rate constant for the dephosphorylation reaction.

Results

The phosphorylated life-time of SSK1-R2 in the presence of osmolytes

A value of 13 ± 3 min was previously measured for the half-life of phosphorylated SSK1-R2 in the absence of YPD1 (13). However, in the presence of YPD1, the phosphorylated SSK1-R2 half-life increased to 38 ± 4 hrs (13). Thus, it was proposed that the Hpt protein YPD1 forms a complex with SSK1 and sterically shields the phosphorylated aspartate residue from hydrolysis. However, the mechanism of rapid dephosphorylation of SSK1-R2~P under conditions of hyperosmotic stress remains unclear.

In order to assess the stability of the phosphorylated form of SSK1-R2 under conditions of hyperosmotic stress, the half-life of SSK1-R2~P was measured as a function of osmolyte concentration in the presence and absence of YPD1. Osmolytes used were glycerol, betaine,

proline, sodium chloride, and trehalose. Ficoll 400 was also tested as a control for viscosity. Through the course of the experiment, no trace of phosphorylated YPD1 was detectable, suggesting that there is no observable reverse phosphotransfer reaction between the two proteins. Our results demonstrate an approximately 2-fold decrease in the life-time of phosphorylated SSK1-R2 in the presence of YPD1 and osmolytes (with the exception of proline) (Fig. 1). In contrast, experiments conducted in the absence of YPD1 or the presence of Ficoll 400 showed no effect of osmolytes on the intrinsic stability of SSK1-R2~P (data not shown).

Effect of osmolytes on the phosphotransfer reaction between SLN1-R1 and YPD1

Rapid mixing of SLN1-R1~P and YPD1 followed by rapid quench allowed measurement of first order rate constants (Scheme 1) by concurrently monitoring the disappearance of SLN1-R1~P and the appearance of YPD1~P.

All data were obtained in triplicate and were analyzed using eq 1 (in the methods section) to calculate the values shown in Tables 1-3. In the absence of osmolytes, the maximum forward rate constant, k_{fwd} , was $27.0 \pm 2.6 \text{ s}^{-1}$, while the K_d for the SLN1-R1~P:YPD1 complex was $2.8 \pm 0.8 \text{ }\mu\text{M}$, in good agreement with previously obtained data in the absence of osmolytes (16).

Subsequent phosphotransfer reactions between SLN1-R1~P and YPD1 were performed in the presence of different NaCl concentrations (as shown in Table 1). The value of k_{fwd} decreases from $27.0 \pm 2.6 \text{ s}^{-1}$ with no NaCl present to a value of $14.7 \pm 0.8 \text{ s}^{-1}$ at 1 M NaCl, while k_{rev} increases from $9.2 \pm 3.3 \text{ s}^{-1}$ to $20.7 \pm 0.4 \text{ s}^{-1}$ at 1 M NaCl. No change in the dissociation constant was observed up to 0.6 M NaCl. A slight increase was observed at 1 M salt. For the second order rate constant (k_{fwd}/K_d), the limit of eq 1 when $[S]$ tends to zero and $k_{\text{rev}} = 0$, remained unchanged from 0 to 0.6 M NaCl. The highest concentration of NaCl (1 M) was not considered physiologically relevant (see discussion).

The phosphotransfer reactions between SLN1-R1~P and YPD1 in the presence of glycerol were done in a manner analogous to those in the presence of NaCl with concentrations of glycerol as shown in Fig. 2A, Table 2. The concentrations of glycerol chosen reflect the range of intracellular levels observed during hyperosmotic stress (35,36). The value of k_{fwd} increases from $27.0 \pm 2.6 \text{ s}^{-1}$ with no glycerol present to a value of $62.3 \pm 1.0 \text{ s}^{-1}$ at 1.2 M glycerol and the value of k_{rev} increases from $9.2 \pm 3.3 \text{ s}^{-1}$ to $14.5 \pm 0.1 \text{ s}^{-1}$, at 1.2 M glycerol. The dissociation constant increases approximately 6-fold (Fig. 2B) and the second order rate constant decreases approximately 3-fold in the presence of 1.2 M glycerol.

Rate constants were also measured in the presence of both NaCl and glycerol (Table 3). The maximum forward rate constant increased by about 2-fold at the highest concentration of both osmolytes (0.75 M glycerol, 0.6 M NaCl). The reverse rate constant and the K_d values remained unchanged. The combinatory effect of both osmolytes at the highest concentrations tested caused about a 3-fold increase in the second-order rate constant.

Effect of osmolytes on the phosphotransfer reaction between YPD1 and SSK1-R2

To observe the effect of sodium chloride and glycerol on the phosphotransfer rates between YPD1~P and SSK1-R2 (Scheme 2), experiments similar to those described above were carried out. In this case, YPD1~P was mixed with SSK1-R2 and the data were collected by following the appearance of SSK1-R2~P and disappearance of YPD1~P.

In the absence of osmolytes, the maximum forward rate constant, k_{fwd} , was $110 \pm 8.0 \text{ s}^{-1}$. The reverse rate constant, k_{rev} , and K_d for the reaction were $4.1 \pm 1.4 \text{ s}^{-1}$ and $3.8 \pm 1.5 \text{ }\mu\text{M}$, respectively, in good agreement with previously obtained data (16). The maximum forward

(k_{fwd}) and reverse rate constants (k_{rev}) in the presence of NaCl are shown in Table 1. The value of k_{fwd} decreases from $110 \pm 8.0 \text{ s}^{-1}$ with no NaCl present to a value of $32.0 \pm 3.2 \text{ s}^{-1}$ at 1 M NaCl, while k_{rev} remained essentially the same. The dissociation constant in these experiments did not change significantly. However, there is a deviation on k_{rev} and K_{d} in going from 0 to 0.2 M, which might be due to systematic error or some unknown phenomenon. The second order rate constant remained unchanged from 0 to 0.6 M NaCl. The highest concentration of NaCl (1 M) was not considered physiologically relevant (see discussion).

In contrast to the effect of glycerol on the phosphotransfer reaction between SLN1-R1~P and YPD1, the effect of glycerol on the phosphotransfer reactions between YPD1~P and SSK1-R2 was different. All experiments were conducted in a manner analogous to those obtained for SLN1-R1~P/YPD1. The forward (k_{fwd}) and the reverse rate constants (k_{rev}) change in the same direction; that is, their values increase with increasing glycerol concentration. Specifically, the value of k_{fwd} increases from $110 \pm 8.0 \text{ s}^{-1}$ with no glycerol present to a value of $185.0 \pm 4.0 \text{ s}^{-1}$ at 1.2 M glycerol and the value of k_{rev} increases from $4.1 \pm 1.4 \text{ s}^{-1}$ to $9.5 \pm 1.4 \text{ s}^{-1}$, at 1.2 M glycerol. The dissociation constant increases approximately 3-fold and the second order rate constant remains approximately the same in the presence of 1.2 M glycerol. The data are summarized in Table 2.

Experiments carried out to determine the combined effect of NaCl and glycerol on the YPD1~P and SSK1-R2 phosphotransfer reaction were conducted in the presence of varying concentrations of both NaCl and glycerol. As osmolyte concentrations increased, the maximum forward and reverse rate constants increased, while no change was observed in the second order rate constant or K_{d} .

To differentiate between a true glycerol effect and an effect of viscosity on the phosphotransfer reactions, a control experiment was performed in the presence of Ficoll 400. Ficoll 400, as a high molecular weight polymer (macroviscosogen) is able to vary viscosity of the reaction mixture; however it has no effect on the rates of diffusion of the phosphotransfer reactions (37). Glycerol (a microviscosogen), on the other hand, is capable of affecting both parameters. Kinetic data obtained in the presence of Ficoll 400 were similar to the data obtained in the absence of osmolytes (data not shown) suggesting that the effects on the phosphotransfer reactions observed in the presence of glycerol are viscosity-independent.

Discussion

The nature of the interaction between SSK1-R2 and YPD1 is important due to the regulatory function of SSK1-R2 and its phosphorylation state that leads to activation or inactivation of the HOG1 MAP kinase cascade. Previous *in vitro* studies have suggested a protective role for YPD1 in shielding the phosphoryl group of SSK1-R2 from hydrolysis (13,38). A protein-protein interaction such as this would help prevent activation of HOG1 under non-osmotic stress conditions. Increasing osmolyte concentrations caused a reduction in the half-life of SSK1-R2~P in the presence of YPD1 by approximately 2-fold (Fig. 1). In contrast, in the absence of YPD1, osmolytes had no effect on the dephosphorylation rate of SSK1-R2. Our data suggest that osmolytes negatively affect the stability of the SSK1-R2~P-YPD1 complex in a concentration-dependent manner (Fig. 1) and, as a consequence, the rate of phosphate hydrolysis increases. The underlying mechanism for complex destabilization can be explained as the ability of osmolytes to create extensive hydrogen bonding and/or ionic interactions with surface residues of the proteins involved in the complex as well as conformational changes. Proline was an exception in this case, and we suggest that its structural rigidity and higher hydrophobicity compared to other osmolytes, renders it incapable of influencing complex stability. We suggest that this modest 1.5- to 2-fold decrease observed for the SSK1-R2~P half-life in the presence of osmolytes is only one of potentially multiple contributing factors

that lead to dephosphorylation of SSK1 and subsequent activation of the HOG1 MAPK cascade. The existence of an SSK1-specific aspartyl phosphatase, for example, cannot be ruled out.

Osmotic shock can dramatically alter the intracellular concentrations of osmolytes. However, cells can adapt by modulating intracellular osmolyte concentrations in order to balance the external osmolarity of the cell. When homeostasis is restored, the osmotic pressure and the production of osmolytes are reduced and the activity of the osmoregulation pathway components returns to its default or prestimulus state.

In order to better understand the overall physiology of the cell response to hyperosmotic shock, detailed kinetic studies are important. Although many two-component systems have been identified in prokaryotes, only a small number of bacterial and eukaryotic phosphotransfer systems have been kinetically characterized (16,39-42). As shown in Scheme 1, for phosphotransfer between SLN1-R1 and YPD1, the ratio k_1/k_{-1} is defined as the K_d (the dissociation constant of the SLN1-R1~P•YPD1 complex). The forward and reverse net rate constants for the phosphotransfer reaction are k_{fwd} and k_{rev} , respectively. Transfer of phosphoryl groups from YPD1~P to SSK1-R2, as depicted in Scheme 2, involves formation of the YPD1~P•SSK1-R2 complex; the ratio k_4/k_{-4} is defined as the K_d (the dissociation constant of the YPD1~P•SSK1-R2 complex). k_7 is the rate constant for the formation of the dead-end complex. In eq. 1 above, the observed rate constant is given in terms of a forward and reverse rate constant, k_{fwd} and k_{rev} . However, these rate constants are net rate constants, not microscopic rate constants. k_{fwd} is defined as in eq. 2.

$$k_{fwd} = \frac{k_2 k_3}{k_2 + k_{-2} + k_3} \quad (2)$$

and if k_{-1} is not greater than k_2 , k_{rev} will be defined as in eq. 3

$$k_{rev} = \frac{k_{-1} k_{-2}}{k_2 + k_{-2} + k_{-1}} \quad (3)$$

This is likewise true for Scheme 2 where k_{fwd} and k_{rev} are given by eqs. 4 and 5

$$k_{fwd} = \frac{k_5 k_6}{k_{-5} + k_6 + k_5} \quad (4)$$

$$k_{rev} = \frac{k_{-4} k_{-5}}{k_{-4} + k_5 + k_{-5}} \quad (5)$$

The treatment of eq. 2-5 assumes a rapid pre-equilibrium (reflected in the K_d) of the initial encounter complex compared to the chemical steps and k_{-3} and k_{-6} are negligible under the conditions tested. If dissociation of the product complex, SLN1-R1•YPD1~P, is fast compared to the phosphotransfer step, $k_3 > k_2$, then $k_{fwd} = k_2$. This is the case for the kinetics of the reverse reaction, transfer of phosphate from YPD1 to SLN1-R1 in the absence of osmolytes (16).

The kinetic data obtained in this study showed that in the presence of osmolytes, the rates of phosphoryl transfer were saturable at high YPD1 concentration suggesting the formation of a YPD1~P•SSK1-R2 complex as observed previously (16). Phosphoryl transfer between SLN1-

R1~P and YPD1 gives a significant k_{rev} , thus $k_3 \leq k_2$. This is also true for phosphoryl transfer between YPD1~P and SSK1-R2; k_{rev} is finite and $k_6 \leq k_5$. However, phosphoryl transfer from SSK1-R2~P to YPD1 is not observed, and thus either the YPD1·SSK1-R2 complex is not formed or is non-productive. However, attempts to demonstrate phosphoryl transfer from SSK1-R2~P to YPD1 were unsuccessful and thus a dead-end complex must form between SSK1-R2~P and YPD1. Scheme 2 shows the formation and dissociation of the dead-end complex with rate constant k_7 and k_{-7} . Thus, SSK1-R2~P and YPD1 can form either a [SSK1-R2~P·YPD1] or [SSK1-R2~P·YPD1]* complex, but the latter is more stable. The measurement of the observed rate constants (k_{fwd} and k_{rev}) and binding affinity (K_d) on the basis of eq 1 provides quantitative data for comparison of the phosphotransfer reactions between SLN1-R1 and YPD1, and YPD1 and SSK1-R2 in the absence and presence of osmolytes.

The maximum rate, k_{fwd} , will only be observed if the concentrations of the response regulator molecules (SLN1-R1 or SSK1-R2) are significantly greater than the K_d for the YPD1•RR complex *in vivo*. However, the physiologic concentrations of these molecules are much lower than the K_d . As reported in the *Saccharomyces* Genome Database (www.yeastgenome.org), the *in vivo* concentrations of SLN1-R1, YPD1, and SSK1-R2 are estimated to be 0.03, 0.29, and 0.05 μM , respectively². Given a K_d of 2.8 μM for the SLN1-R1~P·YPD1 complex, the concentration of the complex formed is estimated to be 0.005 μM , 16 % of the limiting component, SLN1-R1. As a result, it is the second order rate constant, k_{fwd}/K_d that will be physiologically important. This is equally true of the YPD1~P·SSK1-R2 complex. At limiting concentrations of the components in the reaction, the second order rate constant applies and includes all processes from the initial binding of the components to give a productive complex through the dissociation of the product complex. For Schemes 1 and 2, rate constants k_1 through k_3 and k_4 through k_6 will be included in k_{fwd}/K_d for the SLN1-R1~P to YPD1 and YPD1~P to SSK1-R2 reactions, respectively. The rate constant for reformation of the SLN1-R1·YPD1~P complex from SLN1-R1 and YPD1~P (k_{-3}) or the YPD1·SSK1-R2~P complex from YPD1~P and SSK1-R2 (k_{-6}) are not included in the second order rate constant. Thus, any changes in the K_d values of the reactant complex, *i.e.*, the SLN1-R1~P·YPD1 and YPD1~P·SLN1-R2 complexes, are accounted for in the second order rate constant. This would include a change in the half-life for the reactant complexes in the presence of viscosogen.

For both phosphotransfer reactions, SLN1-R1~P to YPD1 and YPD1~P to SSK1-R2 in the presence of NaCl, no significant change in k_{fwd}/K_d or K_d is observed up to 0.6 M NaCl. Thus, NaCl exhibits no significant effect on the SLN1-R1~P and YPD1 or YPD1~P and SSK1-R2 rates of phosphotransfer.

The concentration of NaCl at 1 M was not considered physiologically relevant for these experiments. Upon osmotic shock, the yeast cell volume can decrease by as much as 50-60%, which accordingly would increase the internal osmolyte concentration by only two- to three-fold (43). Under normal conditions, a basal internal concentration of NaCl and KCl within *S. cerevisiae* is approximately 0.2 M (44), consequently upon hyperosmotic shock this concentration will raise to approximately 0.6 M.

The effect of glycerol on phosphotransfer kinetics is in general different than the effect of NaCl. For the SLN1-R1~P·YPD1 complex, the K_d increases by about 6-fold as the glycerol concentration increases to 1.2 M (Table 2), suggesting less initial complex is present at the same SLN1-R1~P and YPD1 concentrations, compared to the absence of glycerol. In addition, the second-order rate constant for the forward reaction (k_{fwd}/K_d), Fig. 2A, decreases with an

²An average cell volume of 37 μm^3 (1) was used to calculate the protein concentrations inside the cell using number of molecules/cell given in the *Saccharomyces* Genome Database website (www.yeastgenome.org).

increase in glycerol concentration, and thus glycerol alone cannot contribute to an increase in phosphorylation of SSK1-R2 in the osmoadaptation phase, once osmotic pressure has been re-established.

In the case of the YPD1~P-SSK1-R2 complex, the K_d does not change as glycerol concentration increases to 1.2 M. Although, k_{fwd} and k_{rev} increase slightly, the second-order rate constant (k_{fwd}/K_d) remains relatively constant. Therefore, the net effect of glycerol on the YPD1~P-SSK1-R2 complex parallels the effect of glycerol on SLN1-R1~P-YPD1 complex, and glycerol alone does not give a net increase in phospho-SSK1-R2.

The combinatory effects of NaCl and glycerol on the pathway, however, are more revealing. As shown in Table 3, in the case of SLN1-R1~P-YPD1 complex, the second-order rate constant increases three-fold, as the concentration of glycerol and NaCl increase to 0.75 M and 0.6 M, respectively. A synergistic effect is observed that is different from the effect of glycerol or NaCl alone. For the YPD1~P to SSK1-R2 phosphotransfer reaction, k_{fwd}/K_d also increases, although by only 30%. The combined effect of NaCl and glycerol is thus to increase the level of phosphorylated SSK1-R2 and return the pathway to its prestimulus state, *i.e.* the effect of osmotic stress has been attenuated.

The subtle effects of NaCl and glycerol on the SLN1-YPD1-SSK1 phosphorelay system reveal some important regulatory aspects of the cell response to osmotic shock. In the early stages of the cell's response to hyperosmotic stress, immediate water loss can lead to a modest increase in intracellular ion/solute concentrations. The half-life studies in the presence of osmolytes suggest a reduction in SSK1-R2~P-YPD1 complex stability; thereby leading to a higher rate of SSK1-R2 dephosphorylation. This likely represents one contributing factor that leads to subsequent HOG1 pathway activation. However, in the osmoadaptation phase, after HOG1-dependent transcriptional targets such as *GPD1* have been upregulated and intracellular glycerol levels approach molar concentrations, our results indicate that the combinatory effect of high levels of NaCl and glycerol on rates of phosphotransfer favors phosphorylation of SSK1 and signal attenuation. Thus, the studies on the phosphorelay kinetics in the presence of osmolytes provide insight into the post-hyperosmotic shock events, restoration of intracellular homeostasis and cessation of glycerol production. These studies explain for the first time the effects of osmolytes on the SLN1-YPD1-SSK1 phosphorelay and elucidate some basic aspects of the osmoregulation pathway including the presence of a feedback-like control mechanism and the combinatory effect of NaCl and glycerol.

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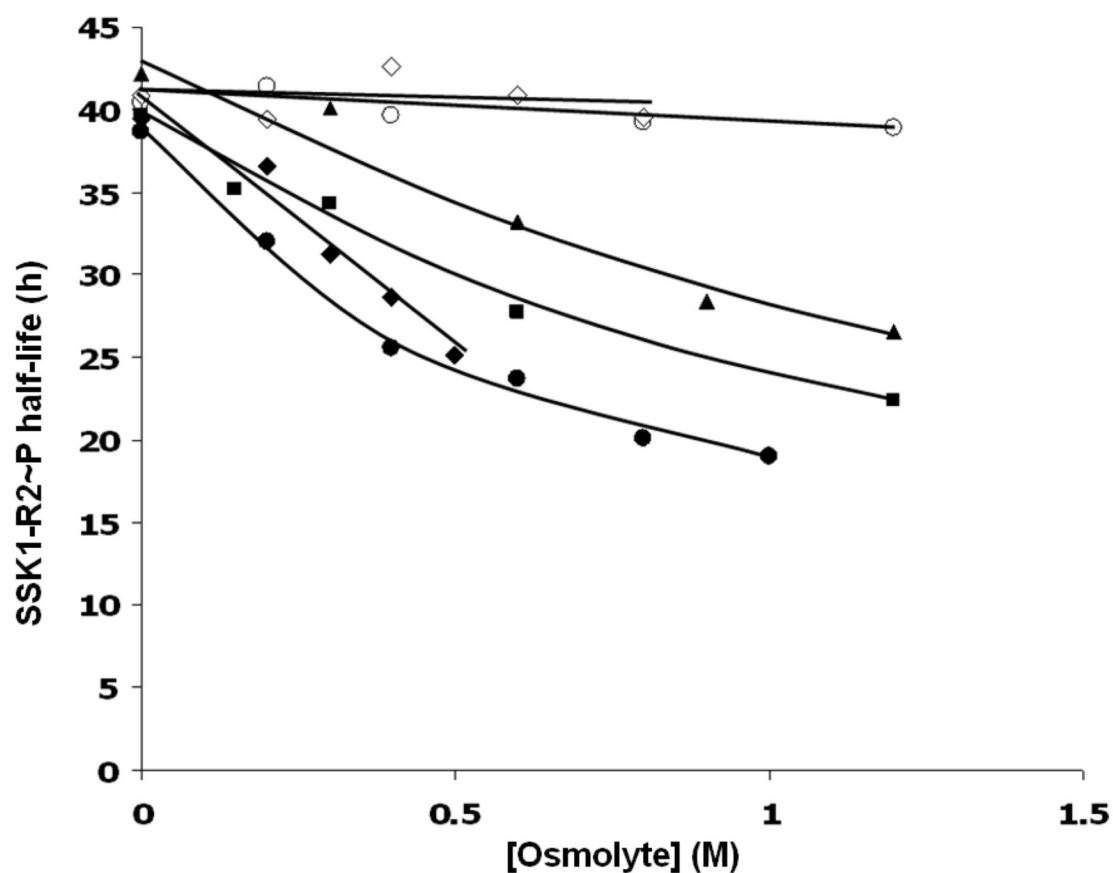


Fig. 1. Effects of osmolytes on the half-life of SSK1-R2~P in the presence of YPD1. Curves are drawn by hand for (○) proline, (◇) Ficoll 400, (▲) glycerol, (■) betaine, (◆) trehalose, and (●) NaCl.

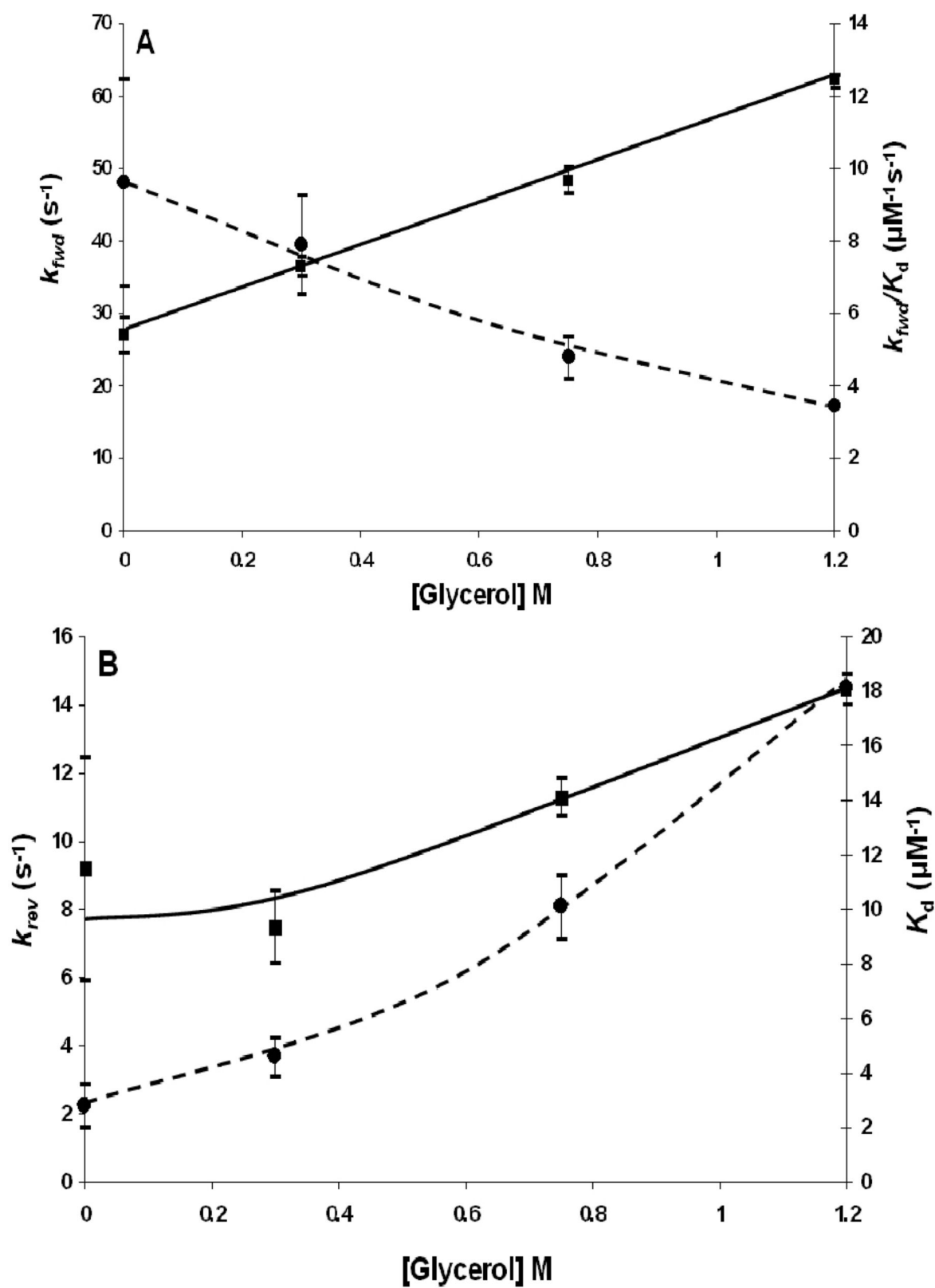
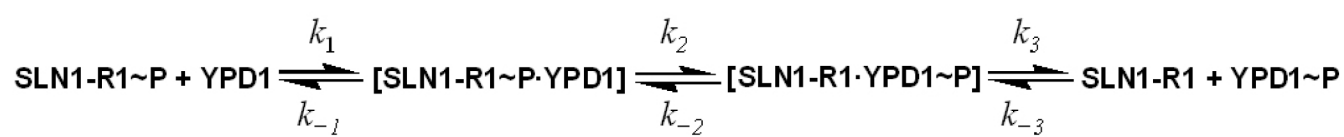
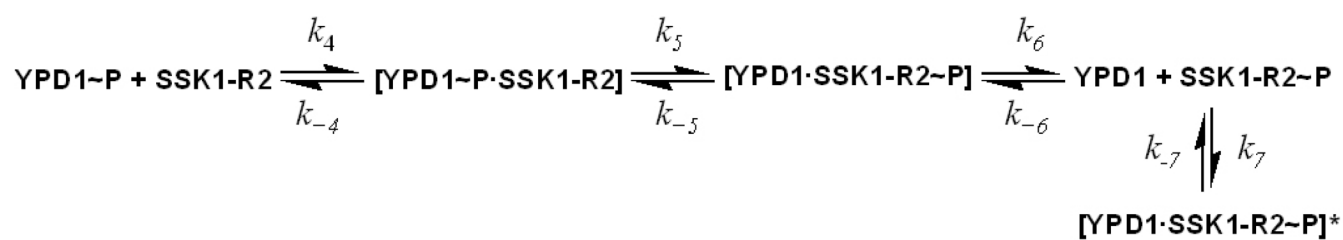


Fig. 2.

A) k_{fwd} and $k_{\text{fwd}}/K_{\text{d}}$ for the SLN-R1~P·YPD1 phosphotransfer reaction at different concentrations of glycerol. Curves are drawn by hand for k_{fwd} (■) and $k_{\text{fwd}}/K_{\text{d}}$ (●). B) k_{rev} and K_{d} for the SLN-R1~P·YPD1 phosphotransfer reaction at different concentrations of glycerol. Lines are drawn by hand for k_{rev} (■) and K_{d} (●).

**Scheme 1.**



Scheme 2.

Table 1
Kinetic constants ^a for phosphotransfer reactions in the absence and presence of NaCl.

Reaction	SLN1-R1~P + YPD1				YPD1~P + SSK1-R2			
NaCl	0 M	0.2 M	0.6 M	1 M ^d	0 M	0.2 M	0.6 M	1 M ^b
k_{fwd} (s ⁻¹)	27.0 ± 2.6	32.9 ± 1.8	24.6 ± 0.8	14.7 ± 0.8	110 ± 8	77.0 ± 0.2	41.0 ± 0.5	32.0 ± 3.2
k_{rev} (s ⁻¹)	9.2 ± 3.3	0.21 ± 2.1 ^c	10.0 ± 0.9	20.7 ± 0.4	4.1 ± 1.4	0.8 ± 0.3	2.2 ± 0.7	6.1 ± 2.9
K_d (μM)	2.8 ± 0.8	2.3 ± 0.6	2.6 ± 0.4	6.2 ± 1.6	3.8 ± 1.5	2.2 ± 0.3	1.4 ± 0.1	3.0 ± 1.6
k_{fwd}/K_d (μM ⁻¹ s ⁻¹)	9.6 ± 2.9	14.3 ± 3.8	9.5 ± 1.5	2.4 ± 0.6	29 ± 4	35.0 ± 4.8	28.6 ± 2.0	10.6 ± 5.8

^aThe data used to calculate the values in the Tables 1-3 obtained in triplicates and the errors represent standard errors of the mean.

^b 1 M NaCl considered not physiologically relevant.

^c Undefined

Table 2
Kinetic constants^a for phosphotransfer reactions in the absence and presence of glycerol.

Reaction	SLN1-R1~P + YPD1			YPD1~P + SSK1-R2		
	0 M	0.3 M	1.2 M	0 M	0.3 M	1.2 M
Glycerol						
k_{fwd} (s ⁻¹)	27.0 ± 2.6	36.5 ± 1.4	62.3 ± 1.0	110 ± 8.0	107 ± 2	185 ± 4.0
k_{rev} (s ⁻¹)	9.2 ± 3.3	7.5 ± 1.1	14.5 ± 0.1	4.1 ± 1.4	3.8 ± 0.9	9.5 ± 1.4
K_d (μM)	2.8 ± 0.8	4.6 ± 0.8	18 ± 1	3.8 ± 1.5	2.1 ± 0.3	11.5 ± 2.7
k_{fwd}/K_d (μM ⁻¹ s ⁻¹)	9.6 ± 2.9	7.9 ± 1.4	3.4 ± 0.1	28.6 ± 4.3	28.2 ± 7.2	35.2 ± 3.2

^aThe data used to calculate the values in the Tables 1-3 obtained in triplicates and the errors represent standard errors of the mean.

Table 3
Kinetic constants^a for phosphotransfer reactions in the absence and presence of glycerol and NaCl.

Reaction	SLN1-R1~P + YPD1		YPD1~P + SSK1-R2			
[Glycerol]/[NaCl]/M	0/0	0.3/0.2	0.55/0.4	0.75/0.6	0/0	0.3/0.2
$k_{\text{wd}} \text{ (s}^{-1}\text{)}$	27.0 ± 2.6	53.8 ± 1.3	57.5 ± 0.4	66.0 ± 0.8	110 ± 8.0	136 ± 1.0
$k_{\text{rev}} \text{ (s}^{-1}\text{)}$	9.2 ± 3.3	5.4 ± 1.4	6.4 ± 0.5	7.2 ± 0.9	4.1 ± 1.4	3.6 ± 0.5
$K_{\text{d}} \text{ (}\mu\text{M)}$	2.8 ± 0.8	3.2 ± 0.4	2.7 ± 0.1	2.3 ± 0.1	3.8 ± 1.5	4.4 ± 0.1
$k_{\text{wd}}/K_{\text{d}} \text{ (}\mu\text{M}^{-1} \text{ s}^{-1}\text{)}$	9.6 ± 2.9	16.8 ± 2.1	21.3 ± 0.8	28.7 ± 1.3	28.6 ± 4.3	30.9 ± 0.7
					32.8 ± 1.6	37.2 ± 2.2

^aThe data used to calculate the values in the Tables 1-3 obtained in triplicates and the errors represent standard errors of the mean.