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Phosphate and Succinate Use Different Mechanisms to Inhibit Sugar-induced Cell Death in Yeast

*INSIGHT INTO THE CRABTREE EFFECT**

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Yong Joo Lee[‡], Elodie Burlet[‡], Floyd Galiano[§], Magdalena L. Circu[¶], Tak Yee Aw[¶], B. Jill Williams[§‖], and Stephan N. Witt[‡1]

From the Departments of [‡]Biochemistry and Molecular Biology, [¶]Molecular and Cellular Physiology, and [‖]Urology and the [§]Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71130-3932

Stationary-phase *Saccharomyces cerevisiae* cells transferred from spent rich media into water live for weeks, whereas the same cells die within hours if transferred into water with 2% glucose in a process called sugar-induced cell death (SICD). Our hypothesis is that SICD is due to a dysregulated Crabtree effect, which is the phenomenon whereby glucose transiently inhibits respiration and ATP synthesis. We found that stationary-phase cells in glucose/water consume 21 times more O_2 per cell than exponential-phase cells in rich media, and such excessive O_2 consumption causes reactive oxygen species to accumulate. We also found that inorganic phosphate and succinate protect against SICD but by different mechanisms. Phosphate protects by triggering the synthesis of Fru-1,6-P_2, which inhibits respiration in isolated mitochondria. Succinate protects in wild-type cells but fails to protect in *dic1*Δ cells. *DIC1* codes for a mitochondrial inner membrane protein that exchanges cytosolic succinate for matrix phosphate. We propose that succinate depletes matrix phosphate, which in turn inhibits respiration and ATP synthesis. In sum, restoring the Crabtree effect, whether with phosphate or succinate, protects cells from SICD.

Saccharomyces cerevisiae cells undergo glucose-induced inhibition of respiration and oxidative phosphorylation and a parallel up-regulation of both glycolysis and glucose uptake by a short-term mechanism called the "Crabtree effect" (1–5). The Crabtree effect is a reversible process, and the precise mechanism of this phenomenon is controversial (6–8). In addition to the down-regulation of genes involved in respiration and oxidative phosphorylation by glucose (9, 10), the Crabtree effect may involve competition between mitochondrial respiratory enzymes and glycolytic enzymes for ADP and inorganic phosphate (11, 12), changes in the permeability of the outer mitochondrial membrane (8), and the accumulation of certain metabolic intermediates, especially Fru-1,6-P_2 (6).

The possibility that Fru-1,6-P_2 mediates the Crabtree effect was shown in a recent study that used mitochondria isolated from Crabtree-positive and Crabtree-negative yeast (6). Notably, Fru-1,6-P_2 decreased the rate of O_2 consumption in mitochondria isolated from the Crabtree-positive yeast (*S. cerevisiae*), but not that in mitochondria isolated from the Crabtree-negative yeast (*Candida utilis*). Such a result indicates that Fru-1,6-P_2 mediates the Crabtree effect.

Although glucose triggers the Crabtree effect when yeast cells are cultured in rich media, glucose in water is very toxic to cells, especially stationary-phase (G_0) cells. When stationary-phase cells are shifted into 2% glucose or fructose in water, the cells begin to bud but then rapidly lose viability within a few hours (13, 14). The cells undergo an apoptotic death triggered by reactive oxygen species (ROS)[2] accumulation (15). ROS accumulation suggests that the respiratory pathway in mitochondria is turned on rather than repressed. The sugar-induced cell death (SICD) is independent of the adenylate cyclase pathway (13) and requires glucose or fructose phosphorylation (16), which suggests that SICD is due to an abnormal catabolic reaction of glucose rather than improper signaling. Evidence is presented here that SICD is a failure of the Crabtree effect: stationary-phase cells in glucose/water cannot inhibit mitochondrial respiration and oxidative phosphorylation, which leads to cell death.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Reagents—The primary yeast strain used in this study was BY4741 (MAT**a**, *his3*Δ*1*, *leu2*Δ*0*, *met15*Δ*0*, *ura3*Δ*0*; American Type Culture Collection, Manassas, VA). Deletion mutants from the Yeast Knock-out Collection were purchased from Open Biosystems. Strain W303-1a (MAT**a**, *ade2-1*, *his3-11*, *leu2-3*,*112*, *trp1-1*, *ura3-1*, *can1-100*; provided by Peter Walter, University of California, San Francisco, CA) was used to confirm the results obtained with BY4741. BY4741 *rho*^{0} cells were constructed using ethidium bromide treatment as described (17). Liquid rich medium consisted of 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, and 2% (w/v) dextrose (Sigma) (YPD medium). Liquid rich medium containing acetate consisted of 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, and 2% (w/v) sodium acetate (YPA medium). 2% (w/v) Bacto-agar was added for plates. For the SICD experiments, cells were incubated in 2% glucose/water (referred to hereafter as "glucose/water"). The water for all experiments was

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[1] To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130-3932. Tel.: 318-675-7891; Fax: 318-675-5180; E-mail: switt1@lsuhsc.edu.

[2] The abbreviations used are: ROS, reactive oxygen species; SICD, sugar-induced cell death; 2,5-AM, 2,5-anhydro-D-mannitol; cfu, colony-forming unit; DCFH-DA, 2′,7′-dichlorofluorescein diacetate.

purified by a Milli-Q reagent system (Millipore) and sterilized by autoclave, and the pH was 5.8. 2,5-Anhydro-D-mannitol (2,5-AM) was purchased from Santa Cruz Biotechnology.

SICD, Viability, and Growth Assays—The SICD assay was performed as described (13). Strains were pre-grown in 4 ml of YPD medium in glass tubes with shaking for 2 days at 30 °C to a density of 2–6 $\times$ 10^8 cells/ml. Cells were washed and resuspended in 2 ml of water, and aliquots were transferred to 3 ml of water, glucose/water, or glucose/water with various reagents to yield 2.0 $\times$ 10^7 cells/ml (15). For every sample, the initial pH was adjusted to pH 5.8, and cultures were incubated with shaking at 37 °C. For the viability assay, aliquots were taken at the indicated times, diluted, and plated on YPD plates. The plates were incubated for 3 days at 30 °C, and then colony-forming units (cfu) were counted. The cfu value at the end of day 2 is the zero time point and represents 100% survival. A survival curve is the percent of viable cells *versus* time. For the growth assay, cells were pre-grown in YPD medium for 2 days at 30 °C and then washed and resuspended in water to a concentration of 1.0 $\times$ 10^8 cells/ml. Cells were serially diluted in 10-fold steps, and 5 μl of each dilution was spotted onto the indicated plates. Plates were incubated for several days at 30 °C.

ROS Detection—A BioTek Synergy 4 multi-detection microplate reader was used to measure ROS accumulation. At the indicated times, the dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was added to each sample of cells to yield a final concentration of 5 μg/ml, and the samples were then incubated for 1 h at room temperature. Samples were washed and resuspended to the original volume in water and then aliquoted into the individual wells of the plate. The excitation and emission wavelengths were 485 and 535 nm, respectively. The absorbance of each well was also measured at 560 nm. The "ROS signal" is $S = F_{535\ nm}/A_{560\ nm}$. Experiments were conducted in three independent experiments, each in triplicate.

Oxygen Consumption—Oxygen consumption was monitored using a YSI Model 5300 biological oxygen monitor equipped with a Clark-type oxygen electrode (YSI Inc.). The samples were prepared as described above. Briefly, cells were pre-grown in YPD medium for 2 days, washed and resuspended in the indicated media, and incubated at 37 °C. Before determining the rate of oxygen consumption, the absorbance ($A_{600\ nm}$) of each sample was measured. Samples (5 ml) were then transferred to an airtight chamber maintained at 37 °C, and the oxygen content was monitored for at least 5 min. To ensure that the oxygen consumption was due to mitochondrial activity, sodium azide was routinely added to the cultures (final concentration, 0.05% (w/v)), and the rates with and without azide were compared. Each experiment was repeated three times.

Detection of Intracellular Metabolites—Cells were pre-grown in YPD medium for 2 days and then washed and resuspended in various media to a final concentration of 2.0 $\times$ 10^7 cells/ml. Cells were incubated with shaking for 3–6 h at 37 °C. The cultures were centrifuged at 7000 $\times$ *g* for 5 min at 4 °C, and the pellet was resuspended in 0.6 ml of water. 100 μl of 35% (v/v) perchloric acid was added per sample, and the samples were incubated on ice for 1 h. Samples were neutralized with 145 μl of 2 M K_2CO_3. The ATP content of the cells was measured using an ATP bioluminescence assay kit (Sigma), which is based on the luciferin-luciferase reaction (18). Glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-bisphosphate were determined using NAD(P)H-coupled enzymatic reactions according to Bergmeyer (19). Protein concentrations were determined by the Lowry method (20).

Statistical Analysis—*p* values were determined using unpaired, two-tailed Student's *t* tests. The mean survival time ($t_{1/2}$) is when 50% of the cells were dead.

RESULTS

The SICD experiments described herein were performed in pre-grown cells (see "Experimental Procedures") that were transferred to and incubated in glucose/water or water at 37 °C, the temperature at which the SICD phenotype is strongest (13, 14).

To illuminate the biochemical pathway that controls SICD in *S. cerevisiae*, we initially screened the Prestwick Chemical Library for drugs that block this mode of cell death and discovered that antimycin A, which inhibits mitochondrial respiratory enzyme complex III, partially inhibits SICD. This finding led us to discover even more potent inhibitors.

Small Molecule Inhibitors of Respiration Partially Protect against SICD

Antimycin A partially protects stationary-phase cells (BY4741 and W303-1a) in glucose/water from SICD (Fig. 1*A*). The mean survival time ($t_{1/2}$) increased from, on average, 0.3 days (without antimycin A) to 1.6 days (with antimycin A) for each WT strain. Respiratory-deficient *rho*^{0} cells, generated by treatment with EtBr, were also partially resistant to SICD (Fig. 1*B*). Note that antimycin A and EtBr were toxic to starved cells (water) (Fig. 1*C*). Specifically, the $t_{1/2}$ values of stationary-phase cells in water and water/antimycin A were 12.2 $\pm$ 0.8 and 3.2 $\pm$ 0.5 days, respectively. This latter value is similar to the $t_{1/2}$ for *rho*^{0} cells in water.

The effect of antimycin A and EtBr on ROS accumulation in stationary-phase cells was also examined (Fig. 1*D*). These compounds decreased ROS accumulation by 57% (12 h) for cells in glucose/water. In contrast, these compounds increased ROS accumulation by 489% (12 h) for cells in water. Ascorbic acid, an antioxidant, increased the survival time of WT cells in glucose/water by 568% (Fig. 1*E*), indicating that ROS accumulation contributes to cell death. The results show that small molecule inhibitors of respiration partially protect cells from SICD (cells in glucose/water) but are toxic to starved cells (cells in water).

Mutants Lacking Acetate Utilization Protect against SICD

The Krebs and glyoxylate cycles share intermediates and are coordinately regulated; deletion mutants of each cycle cannot use acetate as a sole carbon source (21, 22) and often have growth defects on non-fermentable substrates (23). We hypothesized that such mutants would protect cells from SICD. To test this hypothesis, three Krebs cycle mutants (*sdh1*Δ, *sdh2*Δ, and *fum1*Δ) and one glyoxylate cycle mutant (*icl1*Δ) were evaluated. Stationary-phase mutants in glucose/water had dramatically reduced O_2 consumption compared with WT cells (Table 1), and it was verified that the mutants fail to grow on acetate (Fig. 2*A*).

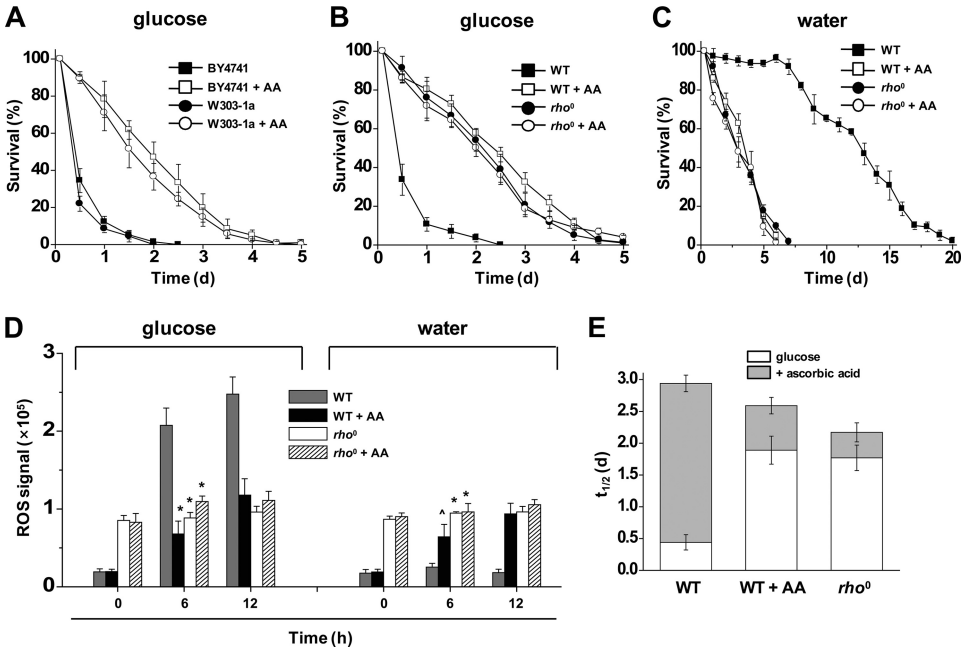

FIGURE 1. **Inhibiting respiration partially protects against SICD.** *A–C*, survival curves. Survival curves show the effect of antimycin A (*AA*) or respiratory deficiency (*rho*^{0}) on the viability of stationary-phase cells in glucose/water (*A* and *B*) or water (*C*) at 37 °C. The BY4741 and W303-1a wild-type strains are compared in *A*; otherwise, the BY4741 strain was used (*B* and *C*). *rho*^{0} cells were generated using EtBr. Survival curves are the average of three to four independent experiments. 1 μM antimycin A or drug vehicle (ethanol) was used. *D*, ROS accumulation. Stationary-phase cells with or without drug were stained with DCFH-DA (5 μg/ml) at the indicated times after transfer into glucose/water or water and analyzed for fluorescence. Values are the mean ± S.E. of four independent experiments. *, $p < 0.001$; ∧, $p < 0.05$ (two-tailed Student's *t* test, *versus* WT at 6 h). *E*, ascorbic acid protects against SICD. The time to 50% survival ($t_{1/2}$) was determined from the survival curves. Values are the mean ± S.E. of three independent experiments. *d*, days.

TABLE 1

O_2 consumption of cells incubated under various conditions

The rate of oxygen consumption of yeast cultures was monitored using a YSI Model 5300 biological oxygen monitor equipped with a Clark-type oxygen electrode at 37 °C.

Condition	O_2 consumption
	O_2 %/min/A
YPD medium (6 h)	
WT	0.04 ± 0.02
Stationary-phase cells in water (6 h)	
WT	0.26 ± 0.03
+Sodium azide	0.04 ± 0.02
+Succinate (10 mM)	0.11 ± 0.03
+Phosphate (1 mM)	0.24 ± 0.02
+Phosphate (10 mM)	0.25 ± 0.04
rho^0	0.01 ± 0.01
*sdh1*Δ	0.03 ± 0.01
*sdh2*Δ	0.03 ± 0.01
*fum1*Δ	0.02 ± 0.01
*icl1*Δ	0.05 ± 0.02
Stationary-phase cells in glucose/water (6 h)	
WT	0.84 ± 0.08[a]
+Sodium azide	0.05 ± 0.03
+Succinate (10 mM)	0.22 ± 0.10
+Phosphate (1 mM)	0.28 ± 0.06
+Phosphate (10 mM)	0.14 ± 0.03
rho^0	0.08 ± 0.05
*sdh1*Δ	0.06 ± 0.01
*sdh2*Δ	0.08 ± 0.02
*fum1*Δ	0.04 ± 0.01
*icl1*Δ	0.11 ± 0.03

[a] From a cfu assay, we estimated that, at 6 h of incubation, 20% of the cells were dead in this experiment. The adjusted value that takes into account this cell death would give 1.1 (O_2 %/min/*A*). Because no other values required this adjustment, we kept the minimum value of 0.84 in the table.

The four mutants clearly protected against SICD, as evidenced by a 7–13.7-fold increase in survival compared with WT cells (Fig. 2*B*). Although these mutants displayed increased fitness against SICD, they displayed decreased fitness against starvation (water) (Fig. 2*C*), *i.e.* the survival time for WT cells of 11.6 ± 1.2 days decreased to 4.0–4.7 days for the mutants.

ROS accumulation was measured at various times after shifting stationary-phase cells into glucose/water or water and incubating at 37 °C. In glucose/water, ROS accumulation was, on average, 62% (12 h) less in the Krebs and glyoxylate cycle mutants than in WT cells (Fig. 2*D*). In contrast, in water, ROS levels increased by 310% (12 h) in the mutants compared with WT cells. Similar ROS levels occurred in these mutants grown in glucose/water *versus* water. Because of down-regulated respiration, Krebs and glyoxylate cycle deletion mutants protect cells from SICD to the same extent as or even better than small molecule inhibitors of respiration.

Glucose Increases the Rate of O_2 Consumption

We hypothesized that stationary-phase yeast cells in glucose/water fail to down-regulate O_2 consumption. To test this hypothesis, a Clark-type electrode was used to measure O_2 consumption under a variety of conditions. For stationary-phase cells transferred into glucose/water and incubated for 6 h at 37 °C, the O_2 consumption rate was 21-fold faster than for exponential-phase cells in YPD medium and 3.2-fold faster than for stationary-phase cells in water (Table 1). Such results indicate that glucose in the absence of other nutrients fails to inhibit respiration.

Molecules That Protect against SICD

Succinate—Because succinate accumulates in the succinate dehydrogenase mutant *sdh2*Δ (24), we questioned whether succinate, independent of the deletion of *SDH2*, can rescue cells from SICD. Wild-type cells were pre-grown to stationary phase

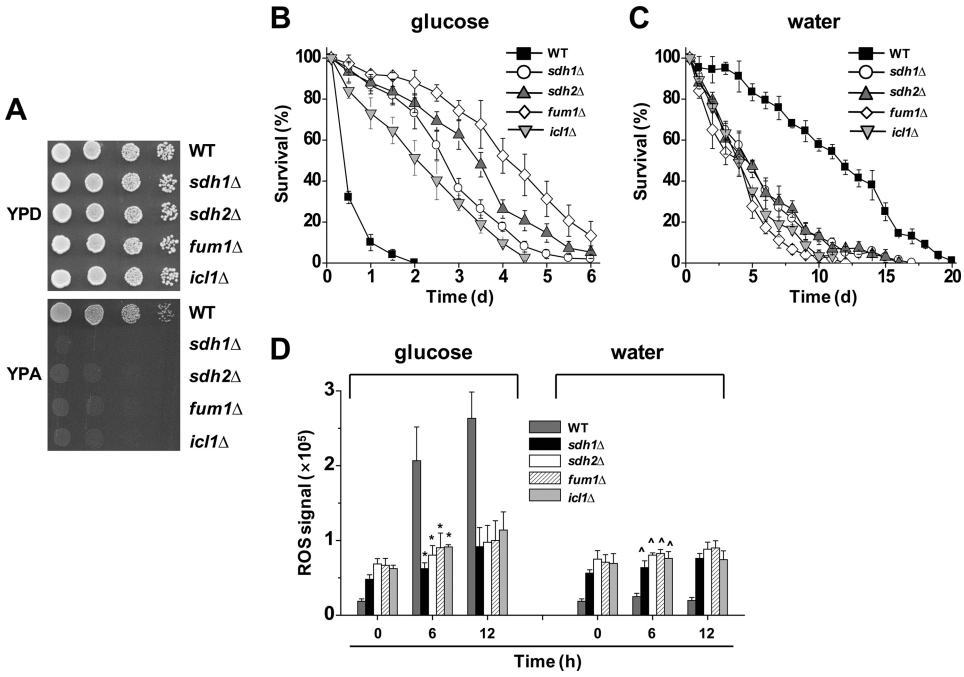

FIGURE 2. **Krebs and glyoxylate cycle mutants protect against SICD.** *A*, growth assay. The indicated mutants in liquid media were serially diluted and spotted onto YPD and YPA plates, which were incubated at 30 °C for 3 days. *B* and *C*, survival curves. Survival curves show the viability of the indicated stationary-phase cells in glucose/water (*B*) or water (*C*) at 37 °C. Curves are the average of three to four independent experiments. *d*, days. *D*, ROS accumulation. Stationary-phase cells cultured at 37 °C were stained with DCFH-DA at the indicated times after transfer into glucose/water or water and analyzed for fluorescence. Values are the mean ± S.E. of three independent experiments. The mutants used were *sdh1*Δ, *sdh2*Δ, *fum1*Δ, and *icl1*Δ. *, $p < 0.001$; ∧, $p < 0.05$.

and then transferred to and incubated in glucose/water or water with 10 mM succinate (or acetate or glycerol). For cells in glucose/water, succinate and acetate were prosurvival, increasing survival by 10- and 3.3-fold, respectively, whereas glycerol had no effect compared with cells without additives (Fig. 3*A*). For cells in water, succinate and acetate decreased survival by 73 and 44%, respectively, whereas glycerol had no effect (Fig. 3*B*).

Succinate and acetate, but not glycerol, decreased ROS accumulation in stationary-phase cells in glucose/water but increased ROS accumulation in stationary-phase cells in water (Fig. 3*C*). Succinate also decreased the rate of O_2 consumption of stationary-phase cells in glucose/water and water (Table 1). The combined results show that succinate (and to a lesser extent, acetate) protects cells from SICD and is toxic to starved cells.

Phosphate—Phosphate has been suggested to regulate the Crabtree effect (11, 12). Phosphate (1 mM) increased the survival of stationary-phase cells in glucose/water by 16.7-fold compared with cells without phosphate (Fig. 4*A*) but had no effect on cells in water (Fig. 4*B*). Phosphate also decreased ROS accumulation and O_2 consumption of stationary-phase cells in glucose/water. Specifically, phosphate (but not $NaSO_4$) decreased ROS accumulation by 88% at 12 h compared with control cells without phosphate (Fig. 4*C*), and 1 and 10 mM phosphate decreased the rate of O_2 consumption by 66 and 83% (Table 1), respectively. Overall, phosphate exhibits a powerful protective effect against SICD but has no effect on starved cells.

Fructose 1,6-Bisphosphate—Recent work indicates that the glycolytic intermediate Fru-1,6-P_2 regulates the Crabtree effect (6). Fru-1,6-P_2 is synthesized by phosphofructokinase, which is

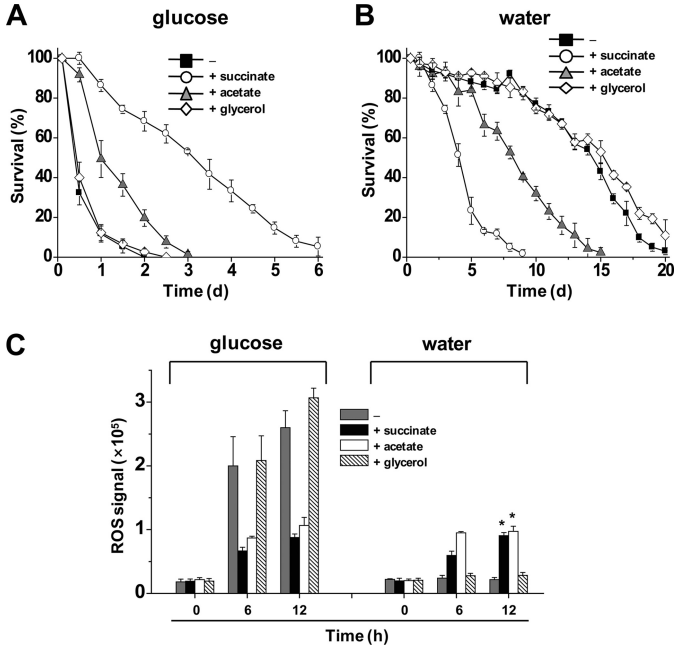

FIGURE 3. **Succinate protects against SICD.** *A* and *B*, survival curves. Survival curves show the effect of 10 mM succinate, acetate, or glycerol on the viability of stationary-phase cells in glucose/water (*A*) or water (*B*) at 37 °C. Curves are the average of three to four independent experiments. *d*, days. *C*, ROS accumulation. Stationary-phase cells were stained with DCFH-DA at the indicated times after transfer into glucose/water or water with the indicated additive and analyzed for fluorescence. Values are the mean ± S.E. of three independent experiments. *, $p < 0.005$ (two-tailed Student's *t* test, *versus* water at 12 h).

allosterically activated by phosphate (25). Late-stage stationary-phase cells can have a suboptimal concentration of cytosolic phosphate (26, 27), even before transfer into glucose/water, and we suggest that this deficit decreases the activity of phos-

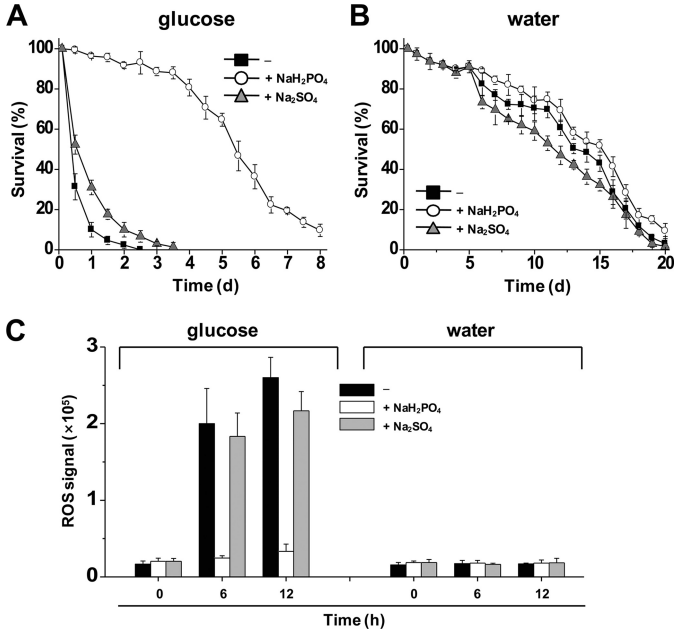

FIGURE 4. **Phosphate protects against SICD.** *A* and *B*, survival curves. Survival curves show stationary-phase cells incubated in glucose/water (*A*) or water (*B*) at 37 °C (with 1 mM NaH_2PO_4 or 1 mM Na_2SO_4). Curves are the mean ± S.E. of three independent experiments. *d*, days. *C*, ROS accumulation. Stationary-phase cells were stained with DCFH-DA at the indicated times after transfer into glucose/water or water with the indicated additive and analyzed for fluorescence. Values are the mean ± S.E. of three independent experiments.

phofructokinase. We tested whether added Fru-1,6-P_2 protects cells from SICD and whether added phosphate triggers the synthesis of Fru-1,6-P_2.

Fru-1,6-P_2 has been reported to enter myocytes and large unilamellar vesicles (28, 29), although the mechanism is controversial. Instead of using Fru-1,6-P_2, we used the cell-permeable analog 2,5-AM, which enters cells and is phosphorylated to yield 2,5-AM-ol bisphosphate (30), which accumulates in the cytosol because it is not metabolized. 2,5-AM increased the survival (5.5-fold) of stationary-phase cells in glucose/water and inhibited ROS accumulation and O_2 consumption (Fig. 5, *A–C*). This Fru-1,6-P_2 analog partially protects cells from SICD.

Phosphate Triggers Fru-1,6-P_2 Synthesis and Inhibits ATP Synthesis

The effect of phosphate, succinate, and 2,5-AM on the level of glycolytic intermediates (Fru-1,6-P_2, Glc-6-P, and Fru-6-P) and ATP in stationary-phase cells in glucose/water was determined (Table 2). The phosphate concentration was 1 mM, and the 2,5-AM and succinate concentrations were both 10 mM. After 3 h, phosphate increased the concentrations of Fru-1,6-P_2 (2.6-fold) and Fru-6-P (1.9-fold) but had no effect on Glc-6-P relative to cells without phosphate in glucose/water; phosphate also decreased ATP by 47%. 2,5-AM increased Fru-1,6-P_2 (1.6-fold) and decreased ATP, but not as much as phosphate. In contrast, succinate had no effect on the three glycolytic intermediates, but it decreased ATP by 76% compared with cells without succinate in glucose/water. The results show that (i) phosphate, but not succinate, triggers the synthesis of Fru-1,6-P_2; and (ii) phosphate and succinate each inhibit ATP synthesis,

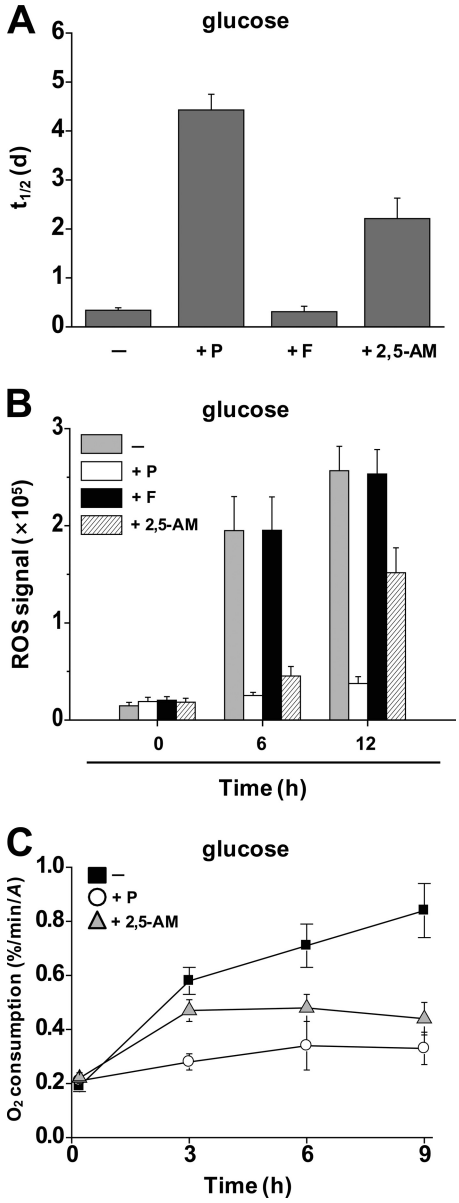

FIGURE 5. **2,5-AM partially protects against SICD.** *A*, effect of phosphate and 2,5-AM on survival ($t_{1/2}$). WT cells were pre-grown and then transferred into glucose/water containing 1 mM NaH_2PO_4 (*P*), 10 mM fructose (*F*), or 10 mM 2,5-AM and incubated at 37 °C, and viability was measured by the cfu assay. $t_{1/2}$ is the time when 50% of the cells were dead. Values are the mean ± S.E. of three independent experiments. *d*, days. *B*, ROS accumulation. Stationary-phase cells (prepared as described for *A*) were stained with DCFH-DA at the indicated times after transfer into glucose/water or water with the indicated additive and analyzed for fluorescence. Values are the mean ± S.E. of three independent experiments. *C*, O_2 consumption. Stationary-phase cells in glucose/water with the indicated additive were incubated at 37 °C, and then oxygen consumption was measured at the indicated times. Values are the mean ± S.E. of three independent experiments.

but only phosphate alters the levels of the glycolytic intermediates.

Phosphate Protection against SICD Does Not Involve Phosphate Signaling

S. cerevisiae cells have five phosphate transporters. Pho84 is the high affinity transporter, and this "transceptor" also mediates phosphate signaling. Phosphate signaling through Pho84 requires phosphate binding, not transport (31, 32), and results

TABLE 2
Analysis of intracellular metabolites in stationary-phase cells

Intracellular metabolites[a]	Additives			
	Glucose/ water	+Phosphate (1 mM)	+2,5-AM (10 mM)	+Succinate (10 mM)
ATP (3 h)[b]	6.6 ± 0.8	3.5 ± 1.4	3.8 ± 1.1	1.6 ± 0.3
Glc-6-P (3 h)	21 ± 2.2	23.3 ± 2.1	18.4 ± 1.5	21.3 ± 3.1
Fru-6-P (3 h)	5.4 ± 0.8	10.3 ± 1.6	4.6 ± 1.2	6.7 ± 0.4
Fru-1,6-P_2 (3 h)	5.5 ± 1.3	14.5 ± 0.9	8.8 ± 0.8	6.6 ± 0.5
Fru-1,6-P_2 (6 h)	5.8 ± 1.1	18.2 ± 0.7	11 ± 1.5	7.2 ± 0.8

[a] Intracellular levels of metabolites (nanomoles/mg of protein) were analyzed by the enzymatic assay method carried out in triplicate.
[b] Incubation times at 37 °C with the indicated additives.

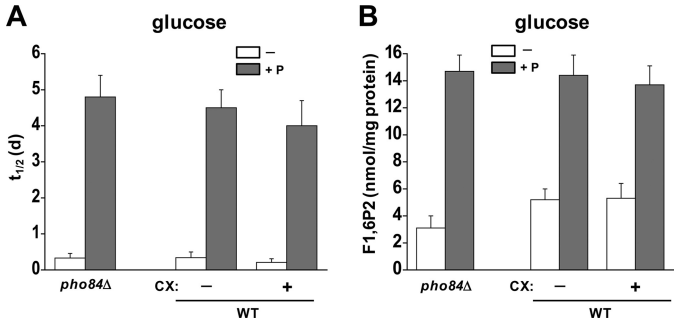

FIGURE 6. **Phosphate signaling and protein synthesis are not involved in protection against SICD.** *A*, plot of survival time ($t_{1/2}$). WT and *pho84*Δ cells were pre-grown and then transferred into glucose/water with or without 1 mM phosphate (*P*) or 100 μg/ml cycloheximide (*CX*). Stationary-phase cells were then incubated at 37 °C, viability was measured by the cfu assay, and $t_{1/2}$ values were determined from the survival curves. Values are the mean ± S.E. of three independent experiments. *d*, days. *B*, determination of intracellular Fru-1,6-P_2 (*F1,6P2*). Pre-grown cells were transferred to and incubated in glucose/water with the indicated additive at 37 °C for 3 h, and then the metabolite level was measured. Values are the mean ± S.E. of three independent experiments.

in activation of the protein kinase A pathway, which in turn up-regulates the activity of various glycolytic enzymes. Phosphate signaling is abolished in *pho84*Δ cells. We tested whether phosphate would protect stationary-phase *pho84*Δ cells from SICD. Cells with added phosphate had increased Fru-1,6-P_2 levels (4.7-fold) and increased survival (15-fold) compared with the same cells without phosphate (Fig. 6, *A* and *B*). Additionally, the protein synthesis inhibitor cycloheximide had no effect on the ability of phosphate to trigger Fru-1,6-P_2 synthesis and to protect against SICD in this mutant (Fig. 6, *A* and *B*). The results show that protection against SICD does not involve phosphate signaling or protein synthesis.

Succinate Uses the Dic1 Transporter to Protect against SICD

Succinate is synthesized in the mitochondrial matrix in the Krebs cycle and in the cytosol in the glyoxylate cycle. Cytosolic succinate is transported into mitochondria by two transporters. Dic1 transports cytosolic succinate into the mitochondrial matrix in exchange for matrix phosphate. Sfc1 transports cytosolic succinate into the mitochondrial matrix in exchange for matrix fumarate (33).

If added succinate enters mitochondria through the Dic1 transporter, the mitochondrial matrix phosphate should be depleted, resulting in the inhibition of respiration and ATP synthesis, which protects cells from SICD. Thus, if Dic1 is deleted, succinate should fail to protect against SICD. To test this hypothesis, the effects of succinate and phosphate on the survival of stationary-phase *dic1*Δ, *sdh1*Δ, *sfc1*Δ, and wild-type cells in glucose/water were determined. Succinate increased the survival of *sdh1*Δ (1.7-fold), *sfc1*Δ (2.3-fold), and wild-type (9.8-fold) cells, but not that of *dic1*Δ cells, whereas phosphate increased the survival of all strains (Fig. 7*A*). In parallel experiments conducted in water, succinate was toxic to *sdh1*Δ, *sfc1*Δ, and wild-type cells but less so to *dic1*Δ cells (Fig. 7*B*). Succinate also failed to decrease O_2 consumption of *dic1*Δ cells in glucose/water and water (Fig. 7, *C* and *D*). The results indicate that the Dic1 phosphate-succinate exchanger mediates succinate protection against SICD.

Acetate Fails to Protect against SICD in the dic1Δ, icl1Δ, and sdh1Δ Mutants

We hypothesized that acetate protects against SICD because it is converted to succinate via the glyoxylate cycle. Succinate derived from added acetate then inhibits respiration and oxidative phosphorylation through the Dic1 transporter. On this basis, we expected that acetate would fail to protect stationary-phase *icl1*Δ and *dic1*Δ cells in glucose/water because the former mutant cannot make succinate and the latter cannot exchange cytosolic succinate for matrix phosphate, and this is what we found (Fig. 8). Acetate also failed to protect *sdh1*Δ cells. For comparison, the effect of succinate on the various mutants is shown. The results show that protection by acetate is mediated by the glyoxylate and Krebs cycles.

DISCUSSION

Stationary-phase yeast cells in glucose/water consume O_2 at a much higher rate compared with cells treated with glucose in other media (Table 1). Our interpretation of this phenomenon is that the Crabtree effect, which normally occurs upon exposure to glucose, fails when cells are grown in glucose/water, and this is the underlying basis for SICD. More specifically, the Crabtree effect fails because of a low level of Fru-1,6-P_2. Phosphate and succinate each can restore the Crabtree effect, but they do so by different mechanisms.

ROS accumulate and trigger rapid cell death when stationary-phase cells are grown in glucose/water (15). Consistent with this previous work, we found that ascorbic acid abolished ROS accumulation and increased the survival of stationary-phase cells in glucose/water (Fig. 1*E*), although phosphate protected even better. The ROS accumulation due to the failure of the Crabtree effect is likely the result of both excess O_2 consumption and ROS production. Although antioxidants neutralize most of the ROS, the most effective way to increase survival is to inhibit O_2 consumption by adding phosphate.

Phosphate Triggers the Synthesis of Fru-1,6-P_2—The results in this study are consistent with added phosphate entering cells and triggering the synthesis of Fru-1,6-P_2 (Table 2). No involvement of the phosphate signaling pathway was uncovered (Fig. 6). We propose that stationary-phase cells in glucose/water have a suboptimal level of cytosolic phosphate, and because phosphate allosterically activates phosphofructokinase to synthesize Fru-1,6-P_2 (25), a suboptimal level of cytosolic phosphate results in a suboptimal level of Fru-1,6-P_2. Adding phosphate corrects the cytosolic phosphate deficit, which then enables phosphofructokinase to synthesize more Fru-1,6-P_2.

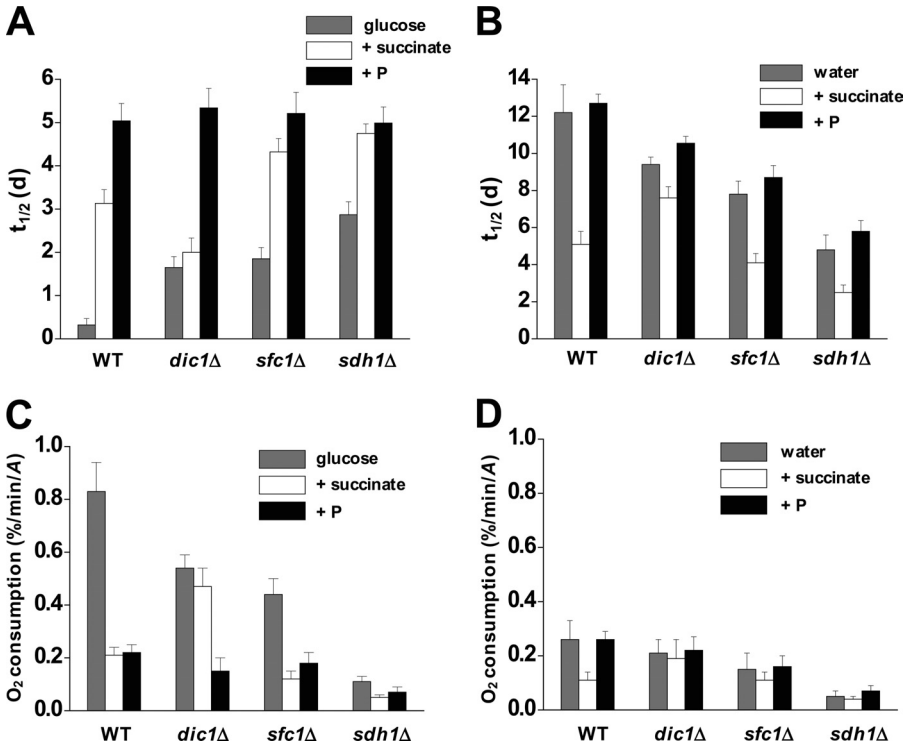

FIGURE 7. **Succinate uses the Dic1 transporter to protect against SICD.** *A* and *B*, plots of the effect of succinate on the survival ($t_{1/2}$) of different mitochondrial mutants. Stationary-phase cells in glucose/water (*A*) or water (*B*) with 10 mM succinate or phosphate (*P*) were incubated at 37 °C, and viability was measured by the cfu assay. $t_{1/2}$ is the time when 50% of the cells were dead. Values are the mean ± S.E. of three independent experiments. *d*, days. *C*, *D*, O_2 consumption. Stationary-phase cells were incubated in glucose/water (*C*) or water (*D*) at 37 °C for 6 h, and then the rate of oxygen consumption was determined. Values are the mean ± S.E. of three independent experiments.

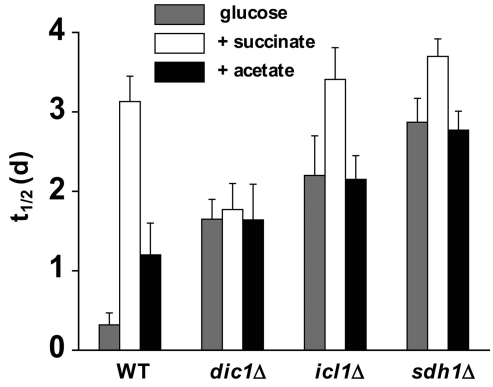

FIGURE 8. **Acetate fails to increase the survival of *dic1*Δ, *icl1*Δ, and *sdh1*Δ mutants.** The plot shows the effect of acetate and succinate on the survival ($t_{1/2}$) of different mutants. Stationary-phase cells in glucose/water with 10 mM succinate or acetate were incubated at 37 °C, and viability was measured by the cfu assay. $t_{1/2}$ values are the mean ± S.E. of three independent experiments. *d*, days.

Fru-1,6-P_2 suppresses ATP synthesis and respiration (6), which protects cells from SICD. Consistent with this model, 2,5-AM also protects against SICD (Fig. 5).

Our model stipulates that late-stage stationary-phase cells transferred into glucose/water have a suboptimal level of cytosolic phosphate. Numerous studies, using quite different methods, have analyzed the changes in phosphate and polyphosphate levels in *S. cerevisiae* cells as a function of growth at 30 °C (26, 27, 34, 35). In general, the polyphosphate pool peaks at late exponential phase (~15 h in culture; $A = 3–4$) and then decreases by as much as 80% in the post-diauxic phase (~24 h in culture; $A = 8–10$) (27). Cytosolic phosphate also decreases over the same time frame (26, 35). One study detected a 50% decrease in cytosolic phosphate as cells grew from a density of 1.0×10^7 to 1.2×10^8 cells/ml (26). Another study showed that a 20% decrease in cytosolic phosphate levels occurs after transferring exponential-phase cells in rich media into phosphate-depleted media (35); these authors concluded that the vacuolar polyphosphate pool can sustain cell growth during phosphate starvation for a "couple of rounds of the cell cycle." We suggest that the vacuolar polyphosphate pool and cytosolic phosphate should be depleted even faster at 37 °C, which is probably why the SICD phenotype is stronger at 37 °C than at 30 °C. Overall, it seems reasonable that late stationary-phase cells transferred into glucose/water can have a reduced level of cytosolic phosphate, perhaps by as much as 50%, compared with exponential-phase cells in rich media.

Succinate—This study identified the Dic1 phosphate-succinate exchanger as mediating succinate protection against SICD. We propose that added succinate enters mitochondria through Dic1 in exchange for matrix phosphate and that decreasing the mitochondrial matrix phosphate level inhibits respiration and ATP synthesis (Tables 1 and 2), which in turn protects from SICD. Consistent with this model, succinate fails to protect against SICD in the *dic1*Δ mutant (Fig. 7*A*).

Succinate is harmful to stationary-phase cells in water (Fig. 7*B*). Mechanistically, succinate is harmful to stationary-phase cells in water because it inhibits respiration and oxidative phosphorylation, as described above. Because starved cells strictly rely on mitochondria for viability (36, 37), any compound that inhibits mitochondrial function will harm starved cells.

Acetate—Acetate partially protects stationary-phase WT cells from SICD and is slightly toxic to starved cells (Fig. 3, *A* and *B*). We propose that this acetate effect is due to the conversion of acetate to succinate via the glyoxylate cycle (33). This hypothesis was tested by deleting the glyoxylate cycle gene that codes for isocitrate lyase (*icl1*Δ). In the cytosol, added acetate is converted to acetyl-CoA. Acetyl-CoA condenses with oxaloacetate to yield citrate, which then converts to isocitrate. Icl1 catalyzes the cleavage of isocitrate into succinate and glyoxylate. Succinate then enters mitochondria through the Dic1 and Sfc1 transporters. We found that the *icl1*Δ mutant, like the Krebs cycle mutants, failed to grow on acetate, increased survival, decreased O_2 consumption, and failed to accumulate ROS (Fig. 2 and Table 1). Because these two metabolic cycles share common intermediates, disruption of one of these cycles perturbs the other. Thus, functionally, *icl1*Δ is similar to the Krebs cycle mutants: the down-regulated respiration and oxidative phosphorylation activity protect cells from SICD.

The failure of acetate to protect stationary-phase *dic1*Δ and *icl1*Δ cells from SICD (Fig. 8) supports our hypothesis that acetate is converted to succinate in the glyoxylate cycle. First, acetate fails to protect *dic1*Δ cells because cytosolic succinate cannot enter mitochondria through the Dic1 phosphate-succinate exchanger in this mutant. Second, acetate fails to protect *icl1*Δ cells because the glyoxylate cycle conversion of acetate to cytosolic succinate is blocked in this mutant. Note that *sdh1*Δ cells cannot utilize acetate, and acetate indeed fails to protect this mutant from SICD.

In sum, we have shown that phosphate and succinate are potent prosurvival Crabtree inducers that inhibit SICD by different mechanisms. Our results show that down-regulating respiration and oxidative phosphorylation protects cells from SICD but is very harmful to starved cells, which strictly rely on mitochondria for energy production.

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