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Evidence for Sulfite Proton Symport in *Saccharomyces cerevisiae*

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Abstract The kinetics of sulfite uptake were examined in a wild-type laboratory strain of *Saccharomyces cerevisiae* to determine if carrier-mediated sulfite uptake involved a proton symport, as previous studies on sulfite uptake have suggested both an active process and facilitated diffusion. Accumulation of intracellular sulfite was initially rapid and linear up to 50 sec. Uptake was saturable at final concentrations equal to or greater than 3 mM sulfite, and increased 2-fold in the presence of 2% glucose. Uptake was significantly reduced in cells pretreated with 100–500 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) or 2,4-dinitrophenol (DNP), both of which dissipate proton gradients. Uptake was also significantly inhibited in the presence of 1 mM arsenate, an inhibitor of ATP synthesis. Extracellular alkalization was observed in cells incubated with 1–2 mM sulfite in a weak tartrate buffer at pH 3.5 and 4.5. These findings suggest that the bisulfite ion, HSO_3^-, an anionic form of sulfite, is taken up by a carrier-mediated proton symport. A *met16 sul1 sul2* mutant, impaired in both sulfite formation and sulfate uptake, was found able to grow on a medium with sulfite as the sole sulfur source, indicating that the sulfate transporters Sul1p and Sul2p are not required for sulfite uptake.

Key words: *Saccharomyces cerevisiae*, sulfite, uptake, transport, sulfate, proton symport

Sulfite is widely used as a food preservative in foods, beverages, and pharmaceuticals, as it has both antimicrobial and antioxidant activities [25]. Sulfite is also a potentially toxic but normal yeast metabolite which occurs as an intermediate in the reductive sulfate assimilation pathway. In aqueous solution, sulfite exists in three forms, dependent on pH: sulfurous acid (H_2SO_3), bisulfite ion (HSO_3^-), and sulfite ion (SO_3^{-2}). In this paper, the generic term "sulfite" is used to refer to all species of sulfurous acid. The dissociation equilibria for sulfurous acid are given below [18].

$SO_2+H_2O=H_2SO_3$	Hydration of sulfur dioxide
$H_2SO_3=HSO_3^-+H^+$	$pKa_1=1.77$
$HSO_3^-=SO_3^{-2}+H^+$	$pKa_2=7.2$

Because sulfite is used as a preservative in wine making, sulfite tolerance is an important characteristic in wine strains of *S. cerevisiae*. Previously, we identified genes conferring sulfite resistance and their roles in sulfite efflux [20, 21]. *SSU1* was identified in a screen for sulfite-sensitive mutants [27], and was found to encode a plasma membrane protein with nine predicted membrane-spanning domains [3]. Overexpression of *SSU1* was found to confer heightened resistance by mediating efflux of the free form of sulfite, consistent with the decreased intracellular sulfite accumulation observed in cells expressing multicopy *SSU1* [20, 21]. However, the mechanisms that account for sulfite uptake in yeast are still unclear. In *Candida utilis*, a common transport system for sulfite, sulfate, and thiosulfate has been suggested by the observation that mutants defective in sulfate uptake were found to grow poorly on sulfite or thiosulfate as sole sulfur sources [15]. Significant inhibition of sulfate uptake by sulfite or thiosulfate has also been reported [1, 5]. In *S. cerevisiae*, previous studies on sulfite uptake suggest an active carrier-mediated process [19, 22] or facilitated diffusion [25]. The present study provides evidence that carrier-mediated sulfite uptake occurs via a proton symport in *S. cerevisiae*.

Materials and Methods

Yeast Strains, Media, and Reagents

YEPD is 2% Difco Bacto peptone (Difco Laboratories, Detroit, MI, U.S.A.), 1% Difco Bacto yeast extract, and 2% dextrose [17]. SM is glucose-based synthetic complete medium (SD plus required amino acids and bases at the prescribed conditions), and drop-out media are SM lacking

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Table 1. Yeast strains

Strains	Genotype	Source
2407-1a	*a gal2 mal mel CUP1*	Bakalinsky and Snow (1990)
CP154-7D	*a ade2 his3 trp1 ura3 leu2 sul1::LEU2 sul2::URA3*	Y. Surdin-Kerjan
C132	*a ura3 leu2 his3 trp1 met16::URA3*	Y. Surdin-Kerjan
3167-12	*a his3 trp1 ura3 leu2 met16::URA3 sul1::LEU2 sul2::URA3*	This study
3167-15	*a his3 trp1 ura3 leu2 met16::URA3 sul1::LEU2*	This study
3167-41	*a his3 trp1 ura3 leu2 met16::URA3 sul2::URA3*	This study
3167-60	*a his3 trp1 ura3 leu2 met16::URA3*	This study

the indicated amino acid or base [16]. YEPD+TA is YEPD containing 75 mM L-tartaric acid buffered at pH 3.5. Sulfur-free B medium was prepared as described [11]. Yeast strains are listed in Table 1. All chemicals were of reagent grade.

Measurement of Sulfite Accumulation and Initial Uptake Rates

Cells were grown to an OD_{600}~1.0 in 200 ml of YEPD+TA, washed twice with 75 mM L-tartaric acid, pH 3.5, containing 2% glucose, suspended in the same buffer to give a final cell concentration of about 20 mg dry weight ml^{-1}, and allowed to equilibrate for 10 min at 25°C. To test the effect of inhibitors, final concentrations of 100–500 μM CCCP (carbonyl cyanide m-chlorophenylhydrazone) or DNP (2,4-dinitrophenol), or 1 mM arsenate were added to 20 ml of cell suspension and reacted for 5 min at 25°C with stirring. Cell viability was not reduced at these concentrations. Sulfite uptake was then initiated by adding final concentrations of 0.5–6 mM Na_2SO_3 to 20 ml of cell suspension in 75 mM tartrate buffer (pH 3.5) containing 2% glucose at 25°C with stirring. In certain experiments, cells grown in YEPD+TA (pH 3.5) were washed with glucose-free tartrate buffer (pH 3.5), and uptake was then determined in cells suspended in the same glucose-free buffer. At appropriate time intervals, 1-ml aliquots were taken and rapidly vacuum filtered through a 0.45-μm membrane (Gelman Science, MI, U.S.A.) and washed with five volumes of 75 mM cold tartaric acid (pH 3.5). The cell pellet was recovered by centrifugation and resuspended in 50 μl of 0.25 M phosphate buffer containing 5 mM EDTA (pH 7.3). A volume of acid-washed glass beads equal to that of the cell suspension was added and the mixture was then vortexed six times at high speed in 30 sec bursts on ice. The liquid fraction was centrifuged at 12,000 ×*g* for 20 min at 4°C to remove cell debris. Total sulfite was determined in the supernatant by the pararosaniline method as described [2]. Endogenous sulfite was not detected in control cells incubated without sodium sulfite. The limit of detection of the assay was found to be 0.07 nmol sulfite/mg dry weight cells. Although no loss of sulfite was observed in samples stored in -80°C for a week, sulfite was always assayed in freshly prepared samples in all experiments.

Proton Uptake Coupled to Transport of Sulfite

Proton flux was measured by following the change in pH of the external medium during incubation of cells with sulfite. Cells were grown to an OD_{600}~1.0 in 400 ml of YEPD+TA, washed twice with 1 mM tartrate buffer at pH 3.5 or 4.5, suspended in the same buffer to give a final cell concentration of about 30 mg dry weight ml^{-1}, and the pH of the cell suspension was adjusted to pH 3.5 or 4.5. Sulfite stock solution (pH 3.5 or 4.5) was prepared as 50 mM of potassium metabisulfite in 1 mM tartrate buffer. Proton flux was initiated by adding final concentrations of 0–2 mM sulfite to 20 ml of cell suspension at 25°C, and was recorded with a Beckman Φ44 pH meter (Irvine, CA, U.S.A.).

Genetic Analysis

Genetic crosses, complementation analysis, and related procedures were performed by standard methods [16]. Random spores were generated by ether treatment [12], essentially as described [4]. Because the *sul1 met16* and *sul1 sul2 met16* mutants were indistinguishable on the basis of auxotrophic requirements, an additional phenotype was used. The *sul1 sul2 met16* mutant was identified by its ability to grow in a liquid sulfur-free B medium containing 0.1 mM chromate, in which the *sul1 met16* mutant was unable to grow.

Results and Discussion

Sulfite Uptake is Saturable

In order to confirm that sulfite transport is a carrier-mediated process, sulfite uptake rates of strain 2407-1a were determined as a function of sulfite concentration. Sulfite accumulation was initially rapid and linear up to 50 sec. Uptake rates of sulfite were saturable at final concentrations ≥3 mM (Fig. 1), suggesting that transport is mediated by a saturable carrier. Cell viability was not reduced by the concentrations of sulfite used during the assay. Sulfite consumption by sulfite reductase was not observed during the accumulation assay, as more than 95% of the initial sulfite added was recovered as the sum of intracellular and extracellular sulfite at the end of the experiment (data not shown).

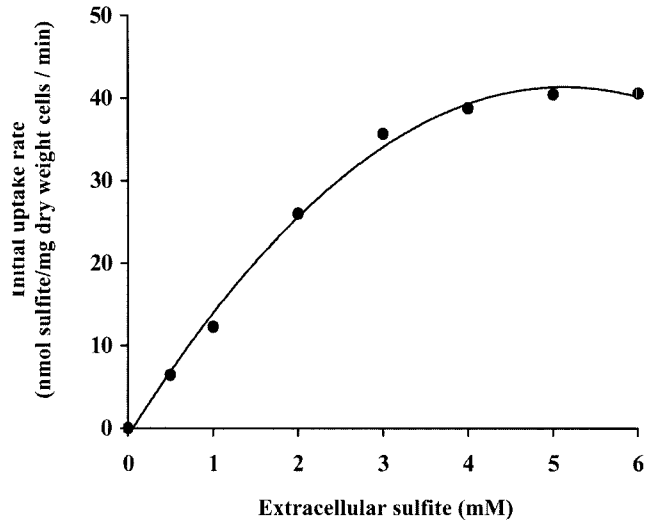

Fig. 1. Kinetics of sulfite uptake.
Intracellular sulfite was measured as a function of sulfite concentration (0.5–6 mM) in strain 2407-1a suspended in 75 mM tartrate buffer (pH 3.5) containing 2% glucose. The initial uptake rates were based on measurements of total intracellular sulfite at 5, 10, and 20 sec, where rates were found to be linear. Data are means of duplicates. Standard deviations were less than 10% of the means.

Effects of CCCP, DNP, Arsenate, and Glucose

To determine if sulfite uptake is facilitated by a proton symport, accumulation and initial uptake rates were determined in cells pre-incubated with CCCP, DNP, or arsenate. Addition of CCCP or DNP caused a large decrease in sulfite accumulation (Fig. 2). Accumulation was also significantly inhibited by 1 mM arsenate. The uncouplers, CCCP or DNP, have been shown to make the lipid bilayer permeable to protons, thereby collapsing the transmembrane electrochemical proton gradient and inhibiting proton-dependent processes [13]. Arsenate is a phosphate analog, which depletes cells of ATP, thereby indirectly interfering with maintenance of the proton gradient [6]. Table 2 shows that initial uptake rates were significantly inhibited by CCCP, DNP, and arsenate. While the approximately 50% inhibition of uptake caused by 100–500 μM CCCP or DNP is comparable to that reported at similar inhibitor concentrations for other energy-dependent transport processes in *S. cerevisiae* [7, 14, 23], Sousa *et al.* [24] reported a greater sensitivity of acetic acid uptake to CCCP. Glucose was found to stimulate sulfite transport as initial uptake was about 2-fold higher in the presence of 2% glucose than in its absence, consistent with uptake being an energy-dependent process (Table 2).

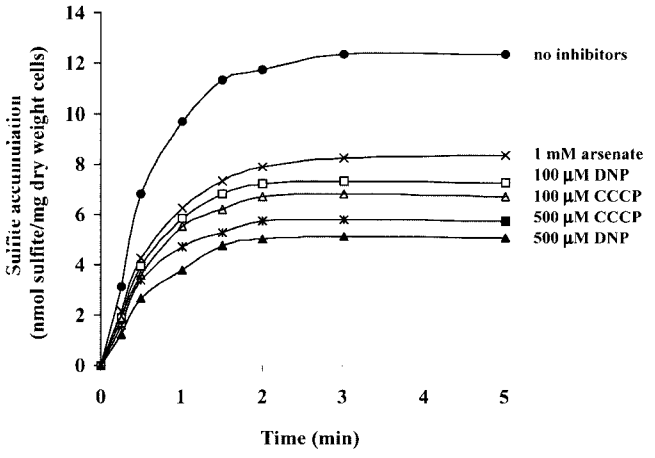

Fig. 2. CCCP, DNP, and arsenate inhibit sulfite uptake in *S. cerevisiae*.
Following pre-incubation of strain 2407-1a in 100-500 μM CCCP or DNP, or 1 mM arsenate in 75 mM tartrate buffer (pH 3.5) containing 2% glucose, accumulation of intracellular total sulfite was determined after adding sulfite to a final external concentration of 1 mM. Data are means of duplicates. Standard deviations were less than 10% of the means.

Table 2. Initial rates of sulfite uptake in the presence of DNP, CCCP, and arsenate.

Inhibitor	Concentration (mM)	Glucose supplementation	
		2% glucose	None
None	-	12.5[a]±0.9 (100)[b]	6.3±0.3 (100)
DNP	0.5	5.0±0.4 (60)	2.7±0.1 (58)
CCCP	0.5	6.4±0.5 (48)	3.6±0.2 (44)
Arsenate	1.0	8.5±0.3 (32)	4.8±0.2 (25)

Initial uptake rates are based on three measurements of total sulfite at 5, 10, and 20 sec as described in Materials and Methods.
[a]Rates are expressed as nmol/mg dry weight cells/min. Data are means of duplicates±S.D.
[b]The percentage of reduction of initial uptake rates by inhibitors is indicated in parentheses.

Extracellular Alkalization Accompanies Sulfite Uptake

If sulfite uptake is facilitated by a proton symport, the process should result in alkalization of the medium. Therefore, medium pH was monitored during incubation of cells with 0–2 mM sulfite. Addition of sulfite to the cell suspension resulted in significant extracellular alkalization (initial pH 3.5, Fig. 3A). At initial pH 4.5, where the concentration of undissociated sulfurous acid was 0.2% of the total sulfite added, extracellular alkalization was also observed in cells incubated with 1 and 2 mM sulfite (Fig. 3B). Alkalization is consistent with at least two possibilities: 1) uptake of HSO_3^- with a proton, and 2) uptake of undissociated sulfurous acid and subsequent re-establishment of the acid-base equilibrium via protonation of bisulfite ($H^+ + HSO_3^- \rightarrow H_2SO_3$). If alkalization occurs by uptake of undissociated sulfurous acid, alkalization at pH 3.5 would be expected to be 10-fold greater than that at 4.5 at equilibrium (2 min reaction with 1 mM sulfite) because the concentration of this species at pH 3.5 is 10-fold higher than that at 4.5. However, the alkalization observed at pH 3.5 was found to be only 2.6-fold greater than that at 4.5. Thus, it appears unlikely that undissociated sulfurous acid is taken up. On the other hand, the 2.6-fold increase in

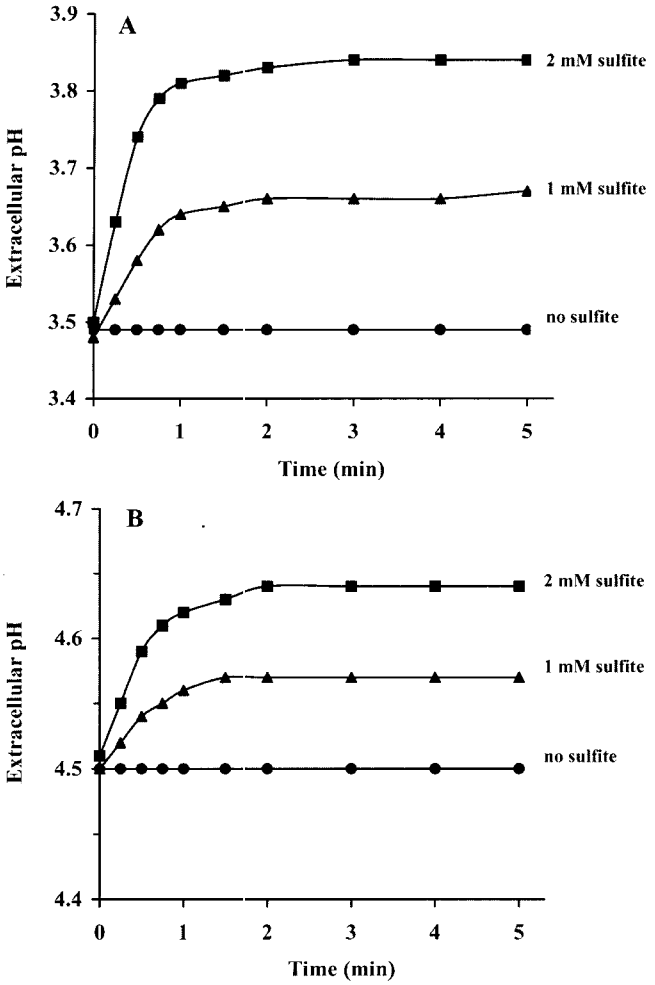

Fig. 3. Alkalization of incubation medium during sulfite uptake in *S. cerevisiae*.
pH was measured during incubation of strain 2407-1a with 0–2 mM sulfite in 1 mM tartrate buffer at pH 3.5 (A) or 4.5 (B) for 5 min as described in Materials and Methods. Data are means of duplicates. Standard deviations were less than 5% of the means.

alkalization is consistent with the bisulfite ion being taken up by a proton symport. While the concentration of the bisulfite ion does not change significantly at pH 3.5 vs 4.5 (98.2% vs. 99.8% of total sulfite, respectively), the 10-fold increase in the transmembrane proton gradient would increase the overall electrochemical gradient driving uptake.

Figure 4 relates measured sulfite uptake to calculated proton uptake in cells incubated with 1 mM sulfite in 1 mM tartrate buffer at pH 3.5 and 4.5. Intracellular sulfite was assayed directly in cell extracts. The intracellular proton concentration was calculated based on monitoring medium pH, and assuming that the rise was due to proton uptake. Proton uptake paralleled that of sulfite, as accumulation of both was initially rapid, reaching a plateau after approximately 2 min. The ratio of $[sulfite]_{in}/[proton]_{in}$ over time was 0.9–0.93 at both pH values, suggesting an electroneutral process with a

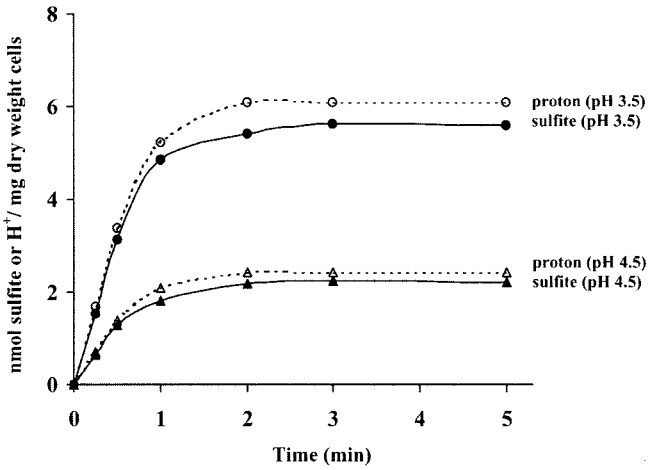

Fig. 4. Proton and sulfite accumulation in *S. cerevisiae*.
Intracellular total sulfite was measured and accumulation of protons was calculated in strain 2407-1a incubated with 1 mM sulfite as described in Materials and Methods and in the text. Data are means of duplicates. Standard deviations were less than 5% of the means.

proton: sulfite stoichiometry of one, analogous to the proton symporters described for other weak acids in yeast [7, 8, 9, 24].

The Sulfate Permeases Ssu1p and Ssu2p Are Not Required for Sulfite Uptake

We tested whether *SUL1* and *SUL2*, encoding high affinity sulfate/proton cotransporters [10], are required for sulfite transport. A *met16* mutant, defective in 3'-phosphoadenosine 5'-phosphosulfate reductase (PAPS), can use sulfite, but not sulfate as a sole sulfur source. If *SUL1* or *SUL2* is required for sulfite transport, a *sul1 sul2 met16* mutant (3167-12) will not grow, and a *sul1 met16* mutant (3167-15) and a *sul2 met16* mutant (3167-41) may not grow, or may grow poorly, on a minimal medium containing sulfite as the sole sulfur source. Qualitatively, all mutants including a *met16* control (3167-60) grew equally well on sulfur-free B medium plates supplemented with 0.2 or 0.5 mM sulfite, indicating that sulfite uptake does not require the sulfate transporters Ssu1p and Ssu2p.

Sulfite Uptake Occurs via a Proton Symport

In summary, our data suggest that the bisulfite ion HSO_3^- enters cells by a carrier-mediated proton symport as uptake was found to be saturable and sensitive to inhibition by CCCP and DNP, and because alkalization of the medium was observed during uptake, consistent with an electroneutral process and a proton: sulfite stoichiometry of one.

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