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Sulfate Transport in *Penicillium chrysogenum* Plasma Membranes

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Transport studies with *Penicillium chrysogenum* plasma membranes fused with cytochrome *c* oxidase liposomes demonstrate that sulfate uptake is driven by the transmembrane pH gradient and not by the transmembrane electrical potential. Ca^{2+} and other divalent cations are not required. It is concluded that the sulfate transport system catalyzes the symport of two protons with one sulfate anion.

One of the transport processes which plays an important role in the biosynthesis of penicillins by filamentous fungi is the uptake of sulfate. Current penicillin production processes yield final penicillin titers which are more than 500 times higher than those obtained with the parental *Penicillium chrysogenum* strain NRRL 1951 (9). Sulfate is the primary sulfur source in industrial production of penicillin and is the precursor of cysteine that together with valine forms the backbone of the penicillin molecule (9, 15). Therefore, the strongly increased demand for cysteine during penicillin production requires an elevated rate of sulfate uptake from the environment. Studies of the mechanism of sulfate uptake by *P. chrysogenum*, related filamentous fungi, and yeasts have led to the conclusion that this process occurs through active transport (1, 6, 8, 12–14, 16, 17). It has been suggested that in *Penicillium notatum*, sulfate is taken up in symport with one proton and one calcium ion (2). According to such a mechanism, the driving forces for sulfate uptake would be the transmembrane electrical potential ($\Delta\Psi$), the transmembrane pH gradient (ΔpH), and the chemical gradient of calcium ions ($\Delta\bar{\mu}_{Ca^{2+}}/F$). Although sulfate transport was shown to promote uptake of Ca^{2+}, a stoichiometric coupling could not be demonstrated. It has been suggested that the sulfate transport system exchanges $SO_4^{2-}/H^+/Ca^{2+}$ against $Ca^{2+}/2OH^-$ (or HPO_4^{2-} instead of $2OH^-$) (2). Since these studies have been performed with metabolically active mycelium, the possibility of involvement of plasma membrane-localized Ca^{2+} transport systems and Ca^{2+} ATPases cannot be excluded. Moreover, sulfate appears to be sequestered in two distinct intracellular pools, one of which only slowly exchanges with external sulfate (7). The complex mycelial morphology and the presence of intracellular compartments make it impossible to relate the uptake of sulfate in a quantitative manner to the driving forces. We therefore reinvestigated the mechanism of sulfate uptake in *P. chrysogenum* using a well-defined model system, i.e., isolated plasma membranes fused with cytochrome *c* oxidase-containing liposomes (5).

Sulfate transport in fused plasma membranes. *P. chrysogenum* shows a high level of sulfate uptake activity when cells are deprived of sulfur (19). *P. chrysogenum* Wisconsin 54-1255 (kindly supplied by Gist-brocades NV) was first grown for 70 h at 25°C on production medium (pH 6.3) (10) supplemented with 10 mM glutamate and 10% (mass/vol) glucose and subsequently incubated for 16 h in production medium in which all sulfate salts were replaced by equivalent chloride salts. Plasma membranes were isolated by a method previously described (5) and fused with cytochrome *c* oxidase-containing liposomes (3, 4) composed of 61% (by mass) acetone-ether-washed *Escherichia coli* lipid, 20% (by mass) egg yolk L-phosphatidylcholine, and 19% (by mass) ergosterol (3, 5). Fusion was induced by a repeated freeze-thawing step, and the membranes were sized by extrusion through polycarbonate filters (Avestin Inc., Ottawa, Canada) with pore diameters of 400 and 200 nm (5). The internal volume of the hybrid membranes was 2.75 μl/mg of protein. For uptake experiments, the membranes (1.2 mg of protein per ml) in 50 mM potassium phosphate (pH 6.5) containing 5 mM $MgCl_2$ were preincubated for 1 min with the electron donor system ascorbate (30 mM), TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine) (150 μM), and horse heart cytochrome *c* (7.5 μM); [^{35}S]sulfate (20 mCi/μmol; Amersham) was added to a final concentration of 10 μM; and uptake was analyzed by filtration on 0.45-μm-pore-size cellulose-nitrate filters (Schleicher & Schuell) (5). Hybrid membranes derived from sulfur-starved cells rapidly accumulated the sulfate (Fig. 1A), with a V_{max} of 0.125 nmol/min · mg of protein and a K_m of 10 μM (Fig. 1B). Hybrid membranes derived from cells grown with sufficient sulfur showed a reduced uptake (Fig. 1A), with K_m and V_{max} values of 12 μM and 0.026 nmol/min · mg of protein, respectively (Fig. 1B). These data demonstrate that the enhanced sulfate uptake rate that has been reported previously for sulfur-starved mycelia (19) is due to an increase in the transport activity rather than an elevated metabolic conversion of sulfate. The kinetic analysis suggests that the same transport system is functional under both sulfur-sufficient and -deprived conditions. In further experiments, membranes derived from sulfur-starved cells were used.

Driving forces in sulfate accumulation. The mechanism of sulfate transport was analyzed by the use of iono- and protonophores. The $\Delta\Psi$ (interior negative) was calculated from the distribution of the tetraphenylphosphonium ion by using an ion-selective electrode as described elsewhere (3, 5, 11). The pH gradient across the membrane (ΔpH, interior alkaline) was estimated from the fluorescence of membrane-entrapped pyranine (4, 5). In the presence of reduced cytochrome *c*, a high proton motive force (Δp) consisting of a $\Delta\Psi$ of −105 mV

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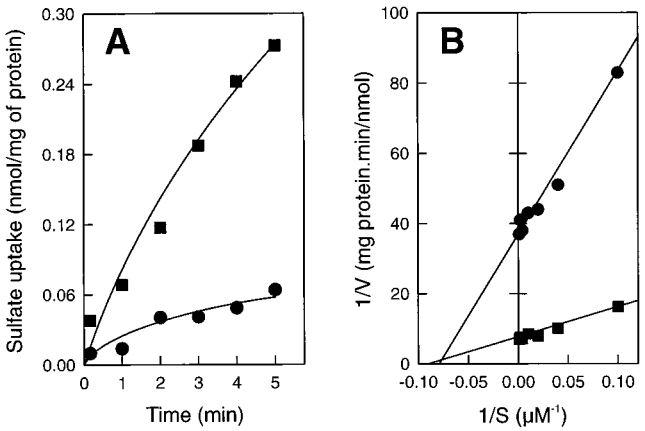

FIG. 1. Induction of sulfate uptake by sulfur starvation. Shown are net uptake (energized minus nonenergized) of sulfate (A) and kinetics of sulfate uptake (B) by hybrid membranes obtained from mycelium grown with sufficient sulfur (●) and sulfur-starved mycelium (■). S, sulfate concentration; V, initial rate of uptake.

and a $Z\Delta pH$ of 45 mV at pH 6.0 was formed (see also reference 6). The uptake of sulfate was blocked by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (FCCP) (Fig. 2A). Preincubation with valinomycin to collapse the $\Delta\Psi$ or addition of this ionophore after 5 min of sulfate uptake had no effect on sulfate accumulation (Fig. 2A and B). Uptake of sulfate was completely abolished by nigericin, an ionophore that dissipates the ΔpH (Fig. 2A). Addition of nigericin (Fig. 2B), FCCP, or an excess of sulfate (Fig. 2C) after 5 min of sulfate uptake caused the release of the accumulated radiolabeled sulfate. These results indicate that sulfate is taken up via an electroneutral ΔpH-driven process.

Sulfate uptake was measured as a function of the external pH, i.e., pH 5.5 to 7.5. The highest rates of sulfate uptake occurred at pH 5.5, and the rate declined with increasing pH (Fig. 3A). A plot of the transmembrane chemical gradient of sulfate ions ($\Delta\bar{\mu}_{SO_4^{2-}}/F$) against the $Z\Delta pH$ revealed a linear relationship (Fig. 3B), with a slope indicative of an apparent stoichiometry of 1.5 protons per sulfate ion. Since sulfate uptake is an electroneutral process, it is concluded that the mechanistic H^+/sulfate symport stoichiometry must be equal to 2:1. Underestimation of the experimental determined value is likely due to the presence of nonfused cytochrome *c* oxidase vesicles in the hybrid membranes (3). These vesicles contribute to the measurement of the ΔpH but do not participate in sulfate uptake.

Effect of divalent cations on sulfate uptake. To determine if Ca^{2+} or another divalent cation participates in the uptake of sulfate, the effect of Ca^{2+} and Mg^{2+} on sulfate uptake was examined at pH 6.0. For this purpose, buffers that were free of divalent cations were used. $CaCl_2$ or $MgCl_2$ (1 to 10 mM) hardly affected the uptake of sulfate, and at the most, a marginal stimulation was observed at a concentration of 5 mM (Fig. 4). EDTA (20 mM) had no significant effect on sulfate uptake (data not shown), nor did the divalent cations or EDTA affect the $\Delta\Psi$ and ΔpH levels (data not shown). Experiments to demonstrate sulfate uptake upon the imposition of an inwardly directed calcium gradient were uniformly negative. These data demonstrate that divalent cations do not participate in the uptake of sulfate in *P. chrysogenum*. This contrasts with reports of studies with *P. notatum* (2, 7) in which it was suggested that both protons and calcium ions are involved in the uptake of sulfate. The possibility exists that the sulfate uptake system of *P. notatum* functions by a fundamentally different mechanism, but it is also possible that the result is from the combined action of Ca^{2+} and sulfate transport systems. Such interference is not expected in membrane vesicles, as the plasma membrane Ca^{2+} ATPase is not active since there is not ATP present.

Specificity of the sulfate uptake system. The specificity of the sulfate uptake system was examined by analyzing the inhibitory effect of a 30-fold excess of several inorganic sulfur and selenium compounds on the initial rate of sulfate uptake. The compounds inhibited the sulfate uptake in the following order (percent inhibition is in parentheses): $S_2O_3^{2-}$ (85%) > $S_2O_4^{2-}$ (75%) > SeO_4^{2-} (65%) > $S_2O_5^{2-}$ (55%) > $S_4O_6^{2-}$

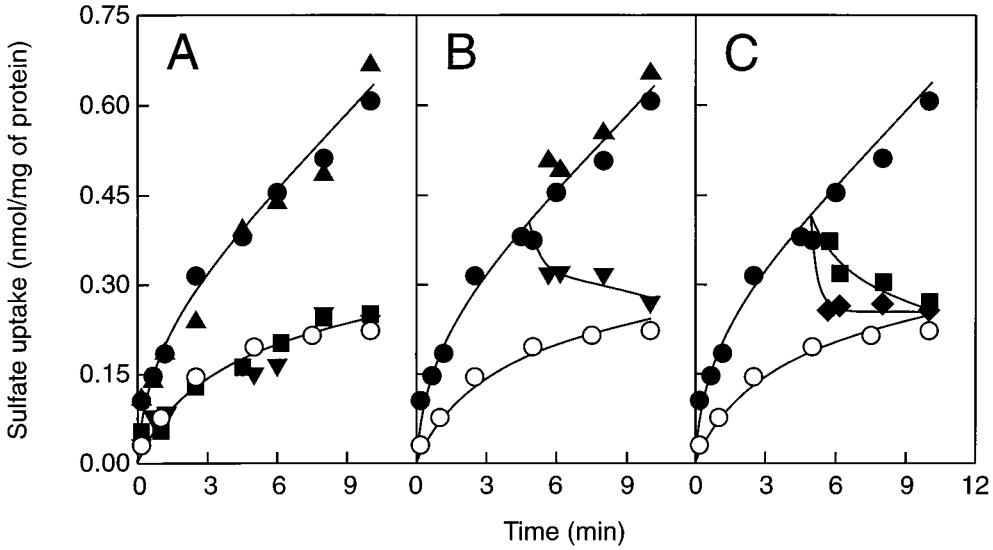

FIG. 2. Properties of Δp-driven sulfate uptake (●, ○) in hybrid membranes. (A) After preincubation with $CF_3OPh_2C(CN)_2$ (■), nigericin (▼), or valinomycin (▲). (B) Effect of the addition of nigericin (▼) and valinomycin (▲) after 5 min of sulfate uptake. (C) Effect of $CF_3OPh_2C(CN)_2$ (■) or a 30-fold excess of nonradioactive sulfate (◆) after 5 min of sulfate uptake. Closed and open symbols represent uptake of sulfate in the presence and absence of ascorbate-cytochrome *c*-$Ph(NMe_2)_2$, respectively.

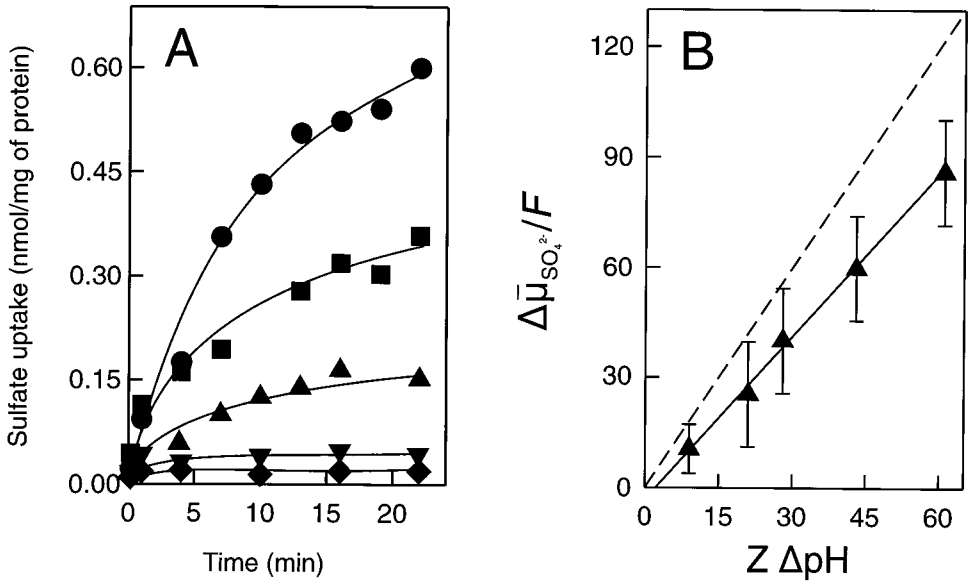

FIG. 3. Relation between sulfate accumulation and the ΔpH. (A) Net sulfate uptake (energized minus nonenergized) by hybrid membranes at pH 5.5 (●), pH 6 (■), pH 6.5 (▲), pH 7 (▼), and pH 7.5 (◆). (B) Correlation between the steady-state transmembrane chemical sulfate gradient and the $Z\Delta pH$ generated at a specific external pH. The slope of this plot suggests an apparent H^+/SO_4^{2-} stochiometry of 1.5:1; the dashed line indicates the theoretically predicted symport stoichiometry of 2:1.

and SeO_3^{2-} (30%) ⩾ SO_3^{2-} (0%). Sulfide had a strong inhibitory effect, but control experiments showed that this was due to the partial inhibition of cytochrome *c* oxidase. These results demonstrate that the sulfate transport system of *P. chrysogenum* possesses a narrow substrate specificity, in agreement with results of previous studies using mycelia (18, 19).

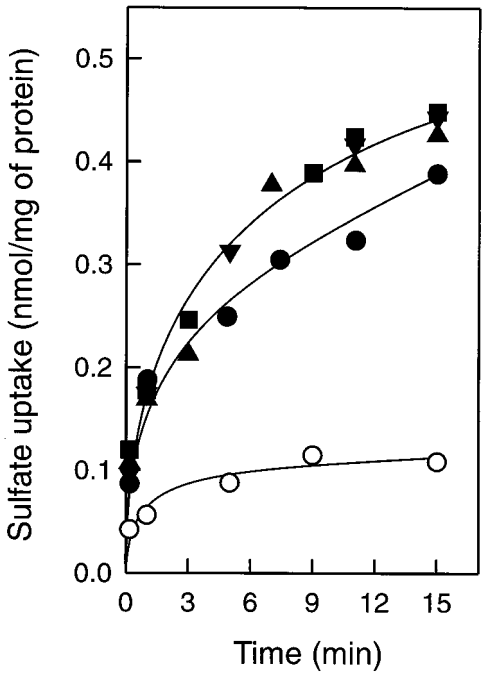

FIG. 4. Effect of divalent cations on sulfate uptake. Shown is uptake of sulfate (●, ○) by hybrid membranes in the absence of divalent cations and the effect of the addition of 5 mM $CaCl_2$ (▲), 5 mM $MgCl_2$ (▼), or 10 mM NaCl (■) on sulfate uptake. Closed and open symbols represent uptake of sulfate in the presence and absence of ascorbate-cytochrome *c*-TMPD, respectively.

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