Chapter 4

Sulfate transport in
*Penicillium chrysogenum*
plasma membranes


**SUMMARY**

The energetic mechanism and specificity of sulfate transport has been studied in *Penicillium chrysogenum* plasma membranes fused with liposomes containing the beef heart mitochondrial cytochrome-c oxidase. Upon energization, hybrid membranes accumulate sulfate to a high extent via a transport system with a narrow substrate specificity. Sulfate uptake is dependent on the transmembrane pH (ΔpH) gradient only. Within the external pH-range of 5.5-7.5, a linear correlation was established between the transmembrane electrochemical sulfate gradient and the -ZΔpH. Divalent cations like Ca\(^{2+}\) and Mg\(^{2+}\) are not required for sulfate transport. Studies with hybrid membranes indicate that the increased sulfate transport rate in sulfate starved mycelium is due to an elevated expression of the sulfate transport system. It is concluded that under the conditions investigated, *P. chrysogenum* accumulates sulfate via one distinct transport system that catalyzes electroneutral symport of two protons with one sulfate anion.
INTRODUCTION

Improvement of stains and cultivation procedures to obtain higher penicillin yields has been one of the main objectives in industrial penicillin research. Current industrial strains yield final penicillin titers several orders of magnitude higher than those obtained with the parental *P. chrysogenum* strain NRRL 1951 [148]. For more than 50 years, improved industrial strains have been obtained by repeated rounds of random mutation and subsequent selective screening. This has resulted in an highly increased flux through the penicillin pathway and has directed primary routes towards penicillin biosynthesis. One of the primary routes through which the flux has increased significantly is apparently the conversion of sulfate to cysteine. Sulfate is the primary sulfur source in industrial penicillin fermentations and is the precursor of cysteine which together with valine forms the backbone of the penicillin molecule [214]. A strongly increased demand for cysteine implies the need for an elevated net uptake of sulfate in industrial strains.

Studies on the mechanism of sulfate uptake by *P. chrysogenum*, related filamentous fungi and yeast have led to the conclusion that this process occurs through active transport [39, 132, 146, 192, 193, 199]. The *Penicillium* sulfate transport system possesses an high affinity for sulfate (*K_m* of approximately 30 µM) and like an analogous system in *Aspergillus*, requires a relatively high ionic strength of the medium [309]. The sulfate transport systems of *Penicillium* and *Aspergillus* species are highly specific for sulfate and analogous divalent oxyanions like thiosulfate, selenate, and molybdate [287]. In *P. notatum*, it has been suggested that sulfate is taken up in symport with one proton and one calcium ion [67]. According to such a mechanism, one positive charge is translocated and the driving forces for sulfate uptake would consequently be, the transmembrane electrical potential (ΔΨ), the transmembrane pH gradient (ΔpH) and the chemical gradient of calcium ions (ΔpCa2+). Although sulfate transport was shown to promote the uptake of Ca2+, a stoichiometric coupling could not be demonstrated. It has been suggested that the sulfate transport system exchanges SO₄²⁻/H⁺/Ca²⁺ against Ca²⁺/2OH⁻ (or HPO₄²⁻ instead of 2OH⁻) [67]. These studies have been performed with metabolically active mycelium, therefore the involvement of other plasma membrane-localized Ca²⁺-transport systems cannot be excluded. In *P. notatum*, sulfate appears to be sequestered in two distinct intracellular pools, i.e. one pool that rapidly exchanges with external sulfate and a second pool that slowly exchanges [138].

Uptake of sulfate in fungi is strictly regulated and subjected to stringent genetic and metabolic control mechanisms [146, 193]. In *Neurospora crassa*, cys-13 and cys-14 are two unlinked genes encoding for the sulfate transport systems I and II, respectively [146, 193]. The derived amino acid sequence from the nucleotide sequence
of the cys-14 gene suggest that transport system II has a molecular mass of about 90 kDa constituting 12 putative transmembrane spanning segments [146]. The cys-13 and cys-14 genes are subject to sulfur catabolic repression and controlled by the cys-3 gene. Both systems are developmentally regulated, and transport system I is found predominantly in conidiophores while transport system II is expressed mainly in mycelia. Induction of the cys-14 gene is relatively slow, presumably due to the short life-time of the mRNA [146, 193]. The half-life of both transport systems is about two hours. The limited RNA stability and dynamic turnover of the transport systems allows the cells to strictly regulate the accumulation of sulfate.

Studies on sulfate transport in filamentous fungi have so far been performed in mycelial suspensions only. The complex 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. It is concluded that sulfate uptake in P. chrysogenum proceeds via a proton coupled mechanism with a H+/SO_4^{2-} symport stoichiometry of 2:1.

**EXPERIMENTAL PROCEDURES**

**Organisms and culture conditions**

P. chrysogenum strains Wisconsin 54-1255 and Panlabs P2 (kindly supplied by Gist-brocades NV) were grown on production medium (pH 6.3) as described by Lara *et al.* (1982) supplemented with 10 mM glutamate and 10 % (mass/vol.) glucose. Cultures were incubated for approximately 70 h in a rotary shaker at 200 rpm and 25 °C. The Wisconsin 54-1255 strain was previously cultured for 24 h on production medium, with the omission of phenylacetic acid and lactose, containing 16% (mass/vol.) glucose. The P2 strain was previously cultured on YPG medium [1 % (mass/vol.) Yeast extract, 2 % (mass/vol.) Peptone and 2 % (mass/vol.) Glucose] for 72 h at pH 7.

**Plasma membrane isolation and fusion**

Bovine heart mitochondria were obtained according to the procedure described by King (1967). Cytochrome-c oxidase was isolated from these mitochondria as described by Yu *et al.* (1975), suspended in 50 mM sodium phosphate (pH 7.5) containing 1.5 % (mass/vol.) cholic acid and stored in liquid nitrogen. Cytochrome-c oxidase was reconstituted in liposomes, composed of 75 % (by mass) acetone/ether...
washed *Escherichia coli* lipids and 25% (by mass) Egg Yolk L-phosphatidylcholine, at a protein/lipid ratio of 0.16 nmol heme/µg lipid [82]. *P. chrysogenum* plasma membranes were isolated according the procedure described by Hillenga et al. (1994). Cytochrome-c-oxidase-containing liposomes (10 mg lipid) and plasma membranes (1 mg protein) were mixed, rapidly frozen in liquid nitrogen and thawed slowly at 21 °C [83]. The freeze-thaw step was repeated once, and hybrid membranes were sized with a small-volume extrusion apparatus (Avestin Inc., Ottawa, Canada) [184] using polycarbonate filters (Avestin) with pore sizes of 400 nm and 200 nm. Fused membranes had a protein/lipid ratio of approximately 0.08-0.09 (mass/mass) (relative to phospholipid).

**Electrical and pH gradients across the membrane**

The transmembrane electrical potential (Δψ, interior negative) was calculated from the distribution of the tetraphenylphosphonium ion (TPP⁺), assuming concentration dependent binding to the membranes as described [178]. The external concentration of TPP⁺ was determined with a TPP⁺-selective electrode. Cytochrome-c oxidase vesicles (corresponding to 0.23 nmol cytochrome-c oxidase) were added to 50 mM potassium phosphate of the indicated pH, containing 5 mM MgSO₄ and 2 µM TPP⁺. A proton-motive force (Δp) was generated by the addition of ascorbate (10 mM, adjusted to the desired pH), *N,N,N',N'-tetramethyl-p-phenylenediamine* (Ph(NMe₂)₂; 200 µM) and horse heart cytochrome c (20 µM). When indicated, the ionophores nigericin and valinomycin were used at concentrations of 10 nM and 100 nM, respectively. The pH gradient across the membrane (ΔpH, interior alkaline) was determined from the fluorescence of pyranine (excitation, 450 nm; emission, 508 nm), measured with a Perkin Elmer LS50B luminescence spectrophotometer. Pyranine (100 µM) was entrapped in proteoliposomes by freeze-thaw-extrusion [82]. External pyranine was removed with a Sephadex G-25 column (coarse, 1/20 cm). Valinomycin was added to a final concentration of 50 nM. A Δp was generated by addition of the electron donor system as described for Δψ-measurements. When indicated, nigericin was added to a final concentration of 1 µM.

**Transport studies**

Uptake of sulfate was studied at 25 °C and pH 6.5, unless stated otherwise. Mycelium was suspended in 50 mM potassium phosphate at a final density of 6 mg/ml (dry mass), and stored on ice until further use. [³⁵S]-sulfate (Amersham, 1 Ci/µmol) previously 50-fold diluted with non-labeled substrate, was added to the mycelial suspension to a final concentration of 10 µM, unless indicated otherwise. At given time intervals, samples of 0.5 ml were taken, added to 2 ml ice cold 0.1 M LiCl, and filtered
immediately on paper filters (296 PE, type 0860; Schleicher & Schuell). Filters were washed once with 2 ml of ice cold 0.1 M LiCl, and the amount of radioactivity was determined with a liquid scintillation counter (Packard Tri-Carb 460 CD; Packard Instruments). Cells were de-energized by preincubation with the protonophore carbamoyl-cyanide-\(m\)-chloro-phenylhydrazone (CF\(_3\)OPh\(_2\)C(CN)\(_2\), 10 \(\mu\)M) for 5 min at 25 °C. For uptake studies with hybrid membranes, the membranes were suspended to a final concentration of approximately 1.2 mg protein/ml in 50 mM potassium phosphate (pH 6.5) containing 5 mM MgCl\(_2\). After a 1-min incubation in the presence of the electron donor system ascorbate (30 mM), Ph(NMe\(_2\))\(_2\) (150 \(\mu\)M) and horse heart cytochrome \(c\) (7.5 \(\mu\)M), \([^{35}\text{S}]\)-sulfate was added to a concentration of 10 \(\mu\)M, unless indicated otherwise. Samples of 20 \(\mu\)l were taken at given time intervals and processed as described above. Samples were filtered on 0.45-µm pore-size diameter cellulose-nitrate filters (Schleicher and Scheull). Kinetic data was analyzed with the GraFit program (Erithacus Software Ltd.).

**Other methods**

Protein concentrations were determined in the presence of 0.5 % (wt/vol) SDS using a modified Lowry assay [289]. Bovine serum albumin was used as a standard.

**RESULTS**

**Driving forces in sulfate accumulation**

Studies with intact mycelium of *P. notatum* indicated that sulfate is taken up in symport with one proton and one calcium ion [67]. To determine if *P. chrysogenum* utilizes the same co-substrates, sulfate uptake was studied in hybrid membrane vesicles at pH 6.0. Initial experiments were aimed to resolve the role of the proton motive force (\(\Delta p\)) in sulfate uptake (Fig. 4.1). Upon addition of the electron donor system ascorbate, Ph(NMe\(_2\))\(_2\) and cytochrome \(c\), the hybrid membranes rapidly accumulated sulfate. Under these conditions, a high \(\Delta p\) was formed consisting of a \(\Delta \psi\) of -105 mV and a \(Z\Delta pH\) of 45 mV (data not shown, and see ref. 130). Incubation of hybrid membranes with the protonophore CF\(_3\)OPh\(_2\)C(CN)\(_2\) or omission of the electron donor system (Fig. 4.1A) prevented uptake of sulfate suggesting that this process is \(\Delta p\) dependent. Incubation with valinomycin or addition of this ionophore after 5 min of sulfate uptake did not effect the accumulation of sulfate (Fig. 4.1A and 4.1B). Since valinomycin collapses the \(\Delta \psi\), it appears that sulfate uptake occurs via an electroneutral process. The uptake of sulfate was completely abolished by preincubation of hybrid membranes with nigericin, an ionophore that collapses the \(\Delta pH\) by mediating electroneutral K\(^+\)-H\(^+\)
Fig. 4.1 Properties of Δp driven sulfate uptake in hybrid membranes. Uptake of sulfate (●, ○) by hybrid membranes (A) after preincubation with CF$_3$OPh$_2$C(CN)$_2$ (■), nigericin(▼) or valinomycin (▲). (B) Effect of the addition of nigericin (▼) and valinomycin (▲) after 5 min of sulfate uptake. (C) Effect of CF$_3$OPh$_2$C(CN)$_2$ (■) or a 30-fold excess of non-radioactive 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)$_2$, respectively.

exchange (Fig. 4.1A). Addition of nigericin or CF$_3$OPh$_2$C(CN)$_2$ after 5 min of sulfate uptake caused the release of all of the accumulated sulfate (Fig.4.1B and 4.1C). The accumulated radiolabeled sulfate was even faster released when an excess of sulfate was added to the external medium (Fig. 4.1C). These results indicate that the \textit{P.chrysogenum} sulfate transport system functions bidirectional and supports sulfate uptake through an electroneutral ΔpH-dependent mechanism.

To further define the role of the ΔpH in sulfate uptake, the pH dependency of the process was examined at external pH values ranging from pH 5.5 -7.5. The highest rates of sulfate uptake occurred at pH 5.5 and declined with increasing external pH (Fig. 4.2A). Both the steady state sulfate uptake levels and the magnitude of the ΔpH declined with increasing external pH. The transmembrane electrochemical sulfate gradient increased linearly with the ZΔpH, irrespective the external pH (Fig. 4.2B). The observed slope indicates an apparent symport stoichiometry 1.5 protons per sulfate ion. These data combined with the electroneutral nature of the process suggests a H$^+$/SO$_4^{2-}$ symport stoichiometry of 2:1.

**Effect of divalent cations on sulfate uptake**

To determine if sulfate uptake requires the presence of Ca$^{2+}$ or another divalent cation, the effect of Ca$^{2+}$ and Mg$^{2+}$ on sulfate uptake was examined at pH 6.0. For this purpose, buffers free of divalent cations were used. The presence of CaCl$_2$ or MgCl$_2$
Fig. 4.2 Relation between sulfate accumulation and the ΔpH. (A) Net sulfate uptake (energized minus non-energized) 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 electrochemical sulfate gradient and the ZΔpH generated at a specific external pH. The slope of this plot suggests an apparent H⁺/SO₄²⁻ stoichiometry of 1.5:1, the dashed line indicates a symport stoichiometry of 2:1.

Fig. 4.3 Effect of divalent cations on sulfate uptake. Uptake of sulfate (●, ○) by hybrid membranes in the absence of divalent cations. Effect of the addition of 5 mM CaCl₂ (▲), 5 mM MgCl₂ (▼) or 10 mM NaCl (■) on sulfate uptake. Closed and open symbols represent uptake of sulfate in the presence and absence of ascorbate/cytochrome-c/Ph(NMe₂)₂, respectively.

(0-10 mM) hardly affected the uptake of sulfate, only a slight stimulation occurred at a concentration of 5 mM (Fig. 4.3). Moreover, comparable results were obtained by addition of NaCl, and the presence of the chelating agent EDTA (20 mM) had no significant effect on sulfate uptake (data not shown). Similarly, neither the Δψ nor the
ΔpH levels were affected by the presence of these divalent cations or EDTA. Experiments to demonstrate sulfate uptake upon the imposition of an inwardly-directed calcium gradient were uniformly negative. It is concluded that divalent cations do not participate in the uptake of sulfate in *P. chrysogenum* and that protons are the only co-substrates in this process.

**Specificity of the sulfate uptake system**

The specificity of the sulfate uptake system was estimated from the extent of inhibition of sulfate uptake at pH 6.0 by a 30-fold excess of several inorganic sulfur and selenium compounds. The initial rate of sulfate uptake was almost completely inhibited by thiosulfate and dithionate. As shown in Table 4.1, the extent to which the inorganic sulfur compounds tested inhibited the uptake of sulfate decreased in the following order: \( \text{S}_2\text{O}_3^{2-} > \text{S}_2\text{O}_4^{2-} > \text{S}_4\text{O}_6^{2-} > \text{SO}_4^{2-} > \text{SO}_3^{2-} \). The analysis of the inhibitory effect of sulfide on sulfate transport was complexed by the partial inhibition of cytochrome-c oxidase by sulfide. When this inhibitory effect was taken into account, no significant direct inhibition of sulfate transport by sulfide was detected. Selenate showed to be only a moderate inhibitor while selenite hardly affected sulfate uptake. These results demonstrate that the sulfate transport system of *P. chrysogenum* possesses a narrow substrate specificity.

**Table 4.1** Inhibition of sulfate uptake in hybrid membrane vesicles by different sulfur compounds.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% Inhibition of sulfate uptake(^a)</th>
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<tr>
<td>( \text{SO}_4^{2-} )</td>
<td>100</td>
</tr>
<tr>
<td>( \text{S}^{2-} )</td>
<td>13(^b)</td>
</tr>
<tr>
<td>( \text{SO}_3^{2-} )</td>
<td>0</td>
</tr>
<tr>
<td>( \text{S}_2\text{O}_3^{2-} )</td>
<td>85</td>
</tr>
<tr>
<td>( \text{S}_2\text{O}_4^{2-} )</td>
<td>75</td>
</tr>
<tr>
<td>( \text{S}_2\text{O}_5^{2-} )</td>
<td>55</td>
</tr>
<tr>
<td>( \text{S}_4\text{O}_6^{2-} )</td>
<td>30</td>
</tr>
<tr>
<td>( \text{SeO}_3^{2-} )</td>
<td>30</td>
</tr>
<tr>
<td>( \text{SeO}_4^{2-} )</td>
<td>65</td>
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</table>

\(^a\) Inhibitors were added at a 30-fold higher concentration than the \([^{35}\text{S}]\text{sulfate that was used at a concentration of} 5 \mu\text{M. Inhibition by sulfate was set at} 100\%. \(^b\) Inhibition of sulfate uptake by sulfide has been adjusted for inhibition of cytochrome-c oxidase activity by this compound.
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**Induction of sulfate uptake**

As observed by Yamamoto and Segel (1966) an elevated uptake of sulfate is observed when cells are deprived from sulfate by incubating mycelium for several hours in a sulfur deficient medium (Fig. 4.4A). The elevated sulfate uptake rate could be the result from adaptations like an increased metabolic conversion of sulfate, alterations of intracellular control mechanisms at the enzyme level or a higher expression of the sulfate transport system. To discriminate between these possibilities, the sulfate transport activity was determined of hybrid membranes derived from mycelium cultured under sulfur sufficient conditions and mycelium that was subsequently starved for sulfate. As compared to sulfate uptake in sulfur sufficient hybrid membranes, higher initial rates and levels of sulfate uptake were detected in sulfur starved hybrid membranes (Fig. 4.4B). The $K_m$ of sulfate uptake of both cells types was similar, i.e., about 10 µM. The $V_{max}$ of sulfate uptake in sulfur sufficient hybrid membranes was about 0.025 nmol/min.mg protein, and increased to 0.1 nmol/min.mg protein upon sulfate starvation. The unchanged $K_m$, but 4-fold increased $V_{max}$, suggests that sulfur starvation results in an elevated expression of the sulfate transport system.

![Fig. 4.4. Induction of sulfate uptake by sulfur starvation.](image)

(A) Uptake of sulfate by mycelium grown under sulfur sufficient conditions (●) and after sulfur starvation (■). (B) Net uptake (energized minus non-energized) of sulfate by hybrid membranes obtained from sulfur sufficient (●) and sulfur starved mycelium (■). (C) Kinetics of sulfate uptake by hybrid membranes obtained from sulfur sufficient (●; $K_m$ 12 µM, $V_{max}$ 0.025 nmol/min.mg protein) and sulfur starved mycelium (■; $K_m$ 10 µM, $V_{max}$ 0.1 nmol/min.mg protein).
DISCUSSION

One of the transport processes that plays an important role in the production of penicillins is the uptake of sulfate [148, 214]. Sulfate is the primary sulfur source in industrial penicillin fermentations and thus the origin of the sulfur atom contained within the penicillin molecule. Sulfate uptake was previously extensively studied in mycelial suspensions of *Penicillium* species [67, 138, 248, 287, 309], but due to the lack of a suitable membrane isolation procedure, it has not been possible to establish the precise energetics of this system. Previously, we have reported on development of a procedure that allows the isolation of functional plasma membranes from *P. chrysogenum* [127]. These plasma membranes have been fused with cytochrome-c oxidase vesicles in order to equip them with a proton motive force generating system that can be easily activated. Recent studies on the mechanism of arginine uptake in plasma membranes of *P. chrysogenum* have demonstrated the potential of this hybrid membrane system [130]. Using this hybrid system, we now show that sulfate uptake is an electroneutral process most likely involving co-transport of the sulfate anion with two protons.

Sulfate uptake in hybrid membranes depends only on the ΔpH. The *in vitro* studies failed to demonstrate a requirement for calcium and other divalent cations. This leads us to propose that sulfate uptake in *P. chrysogenum* occurs in symport with protons. Since sulfate uptake is an electroneutral process, the $H^+/SO_4^{2-}$ symport stoichiometry must equal 2:1. The experimentally determined value is lower, but this may well be due to the heterogeneity of the hybrid membranes which could contain a subpopulation without sulfate transport systems. These membrane vesicles, however, will contribute to the measured Δp, and thus will be responsible for an underestimation of the true stoichiometry. Sulfate uptake with a $H^+/sulfate$ stoichiometry of two may be considered as an energetically expensive process. The $H^+$ translocating P-type ATPase present in the plasma membranes of *P. chrysogenum* is thought to function with a $H^+/ATP$ stoichiometry of one. The uptake of a sulfate anion would therefore, require the hydrolysis of two ATP molecules. In *P. notatum* sulfate uptake was studied in an ATP sulfurylase deficient strain to circumvent effects of metabolism on sulfate accumulation [67]. These studies suggested that both protons and calcium are involved in the uptake of sulfate in this organism. Hill plots analysis of the calcium dependency of sulfate uptake suggested that calcium acts as a co-substrate [67]. This study, however, shows that in membrane vesicles from *P. chrysogenum* such Ca$^{2+}$/sulfate co-transport does not occur. The possibility exists that the sulfate uptake system studied in *P. notatum* mycelium functions by a different mechanism, but it is also possible that the results of a combined action of Ca$^{2+}$ and sulfate transport systems have been
observed in *P. notatum* mycellium.

The *P. chrysogenum* sulfate transport system studied, possesses a narrow substrate specificity. Our results confirmed previous studies on fungal sulfate transport [287], and demonstrate that the system accepts, beside sulfate, thiosulfate, dithionate and selenate as substrates. Based on its toxicity selenate has been used in several fungi to obtain sulfate transport negative strains, consistent with its ability to compete with sulfate for transport. The kinetic analysis suggests that *P. chrysogenum* expresses the same transport system under both sulfur sufficient and deprived conditions. This transport system has a high affinity for sulfate. The sulfate concentration in the growth medium used in this study is such that the transport system is saturated, and thus functions at its maximal velocity. Upon sulfate deprivation, the $V_{\text{max}}$ is increased several fold. This most likely is the result of increased expression of the sulfate transport system to allow the cells to scavenge traces of sulfate from the medium. In addition, other regulatory mechanisms may be operationally, such as enzymatic regulation of the transport system.

In summary, these *in vitro* experiments demonstrate that under the conditions examined, sulfate uptake in *P. chrysogenum* is mediated by a specific transport system that catalyzes the symport of the sulfate anion with two protons. This process is essential for the biosynthesis of cysteine, one of the precursors of the tripeptide L-$\alpha$-aminoacidipyl-L-cysteinyl-L-valine (LLD-ACV) necessary for penicillin production.

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