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The *Escherichia coli* CysZ is a pH dependent sulfate transporter that can be inhibited by sulfite

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ABSTRACT

The *Escherichia coli* inner membrane protein CysZ mediates the sulfate uptake subsequently utilized for the synthesis of sulfur-containing compounds in cells. Here we report the purification and functional characterization of CysZ. Using Isothermal Titration Calorimetry, we have observed interactions between CysZ and its putative substrate sulfate. Additional sulfur-containing compounds from the cysteine synthesis pathway have also been analyzed for their abilities to interact with CysZ. Our results suggest that CysZ is dedicated to a specific pathway that assimilates sulfate for the synthesis of cysteine. Sulfate uptake via CysZ into *E. coli* whole cells and proteoliposome offers direct evidence of CysZ being able to mediate sulfate uptake. In addition, the cysteine synthesis pathway intermediate sulfite can interact directly with CysZ with higher affinity than sulfate. The sulfate transport activity is inhibited in the presence of sulfite, suggesting the existence of a feedback inhibition mechanism in which sulfite regulates sulfate uptake by CysZ. Sulfate uptake assays performed at different extracellular pH and in the presence of a proton uncoupler indicate that this uptake is driven by the proton gradient.

1. Introduction

The proper functioning of a wide range of cellular processes relies on the continuous supply of sulfur-containing compounds. In the aerobic biosphere, inorganic sulfate is the most abundant source of sulfur that can be utilized by cells. The synthesis of biologically important sulfur containing molecules such as cysteine, methionine and *S*-adenosylmethionine (SAM) all depends on the transport of sulfate into the cell. Indeed, sulfate assimilation is an important physiological process in the cells and sulfate transporters mediate the first step of sulfate uptake [1]. Both sulfate and the closely related thiosulfate can gain entrance into the bacterial cells via four different types of known sulfate transporters: the ABC type CysTWA transporter, the proton:sulfate symporter or putative sulfate: bicarbonate antiporter SulP, the sulfate transporter CysP (PiT) that is related to inorganic phosphate transporter and finally the high affinity, high specificity transporter CysZ [2–7]. After being taken up into the cytoplasm, sulfate is further converted to a series of sulfur-containing intermediates in the cysteine synthesis pathway, including sulfite and sulfide, before being incorporated into cysteine by cysteine synthase CysK (Fig. 1). An alternative pathway to synthesize cysteine involves producing *S*-sulfocysteine from thiosulfate and *O*-acetylserine. *S*-sulfocysteine can be further converted to cysteine [8]. Besides being an important amino acid, cysteine also serves as a precursor for the synthesis of methionine and *S*-adenosylmethionine.

Homologs of CysZ are found primarily in proteobacteria [9,10], including many pathogenic bacteria, but can also be found in yeast and fungi. Sulfate uptake and assimilation are also regarded as important processes in food industry. For example, the CysZ-containing *Brevibacterium aurantiacum* has been used for a long time by the cheese industry for cheese ripening and has been shown to represent more than 5% of total bacteria on cheese surfaces [11], giving distinct sulfur aromas to cheeses and preventing the growth of pathogenic bacteria.

Early studies have shown that *Escherichia coli* carrying mutated Ec*cysZ* gene is deficient of sulfate transport [4,12]. CysZ in other organisms is also annotated as a transporter dedicated to the uptake of sulfate. It has been further proposed to be a high-affinity sulfate transporter based on the observation that when CgCysZ in *Corynebacterium glutamicum* was knocked out, the bacteria failed to survive in media containing <10 mM sulfate, while the growth could be restored with the addition of >20 mM sulfate [10]. This indicates that other types of sulfate transporters in this organism were not able to carry out sulfate uptake at relatively low sulfate concentrations. In Nature, sulfate concentrations vary substantially in different environments where bacteria are exposed. For example, the sulfate concentration in the seawater is approximately 28 mM [13] whereas in fresh water, as well as in the gastrointestinal tract of animals, the concentration is mostly in the range of micromolar to a few millimolar [14]. The bacterial sulfate uptake therefore relies specifically on CysZ in many environments with relatively low sulfate concentrations.

The process of transporting a divalent anion such as sulfate into the cell is energetically unfavorable. Under physiological conditions, the *E. coli* cells maintain a pH gradient across the plasma membrane,

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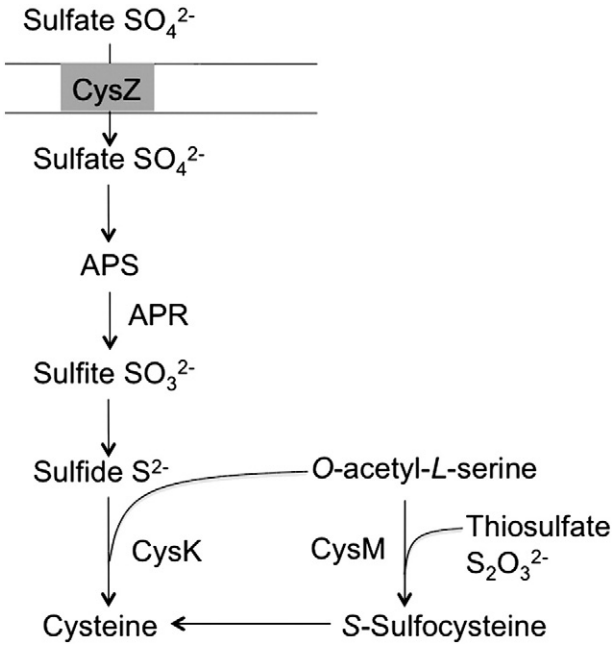

Fig. 1. The cysteine synthesis pathway in bacteria.

where the periplasm is slightly more acidic than the cytosol, along with an electric potential difference of approximately 80 mV. In order to overcome the repelling effect of this electrical potential during the uptake of anions, secondary transporters such as CysZ require existing chemical gradients to facilitate the transport. One hypothesis is that the sulfate ions cross the membrane together with cations, thereby temporarily neutralizing negative charge during transport. The existing pH gradient offers an excess of protons at the periplasmic side of the membrane, and the proton gradient is therefore an interesting candidate to be tested as the driving force for sulfate uptake by CysZ.

To date, little is known regarding the structure and function of CysZ. In pursuit of understanding the transport mechanisms of this important sulfate transporter, we have over-expressed and purified CysZ from *E. coli*. The purified protein is functionally active and stable in detergent solutions. To further characterize the transporter, we quantified interactions between the transporter and its substrates as well as substrate analogs. The sulfate transport activity of CysZ was investigated and the proton gradient was tested as a driving force for transport.

2. Material and methods

2.1. Protein over-expression and purification

The PCR product of *cysZ* from *E. coli* K12 (Swiss-Prot accession number P0A6J3) was cloned into pET-21a with a hexa-histidine tag at the C-terminus to create the construct *C43 (pET21a-cysZ)*. Typically, cell culture was grown by inoculating 0.5 L 2YT media with 4 mL overnight culture and incubated at 37 °C and 200 rpm until the absorbance at 600 nm reached 0.7. Protein expression was then induced with 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cells were grown for another 5 h at 25 °C for protein production. After harvesting, the cell pellet was resuspended in 50 mM Tris pH 7.8, 100 mM NaCl with protease inhibitor (Roche) and DNase I (Sigma). Cell disruption was carried out at 4 °C with Cell Disruptor (Constant Systems, UK) by two passes at 30 kpsi. Unbroken cells were removed by 40 min of centrifugation at 20,000 ×*g*. The membrane was then collected by ultracentrifugation at 100,000 ×*g* for 3 h. Membrane pellets were stored at −20 °C for further use. The isolated membrane was homogenized in 50 mM Tris pH 7.8, 100 mM NaCl and 10% Glycerol (v/v) in the presence of protease inhibitor (Roche, EDTA free) to a volume 10 times of its weight with a manual homogenizer. n-Dodecyl-beta-D-maltopranoside (DDM) (Anatrace) was added to 1% (w/v) to the homogenized membrane and incubated for 1 h under constant agitation. The unsolubilized membrane was then removed by 1 h of ultracentrifugation at 100,000 ×*g*. Before loading the supernatant onto a 1 mL Hi-Trap Ni-affinity column (GE Healthcare), 5 mM imidazole was added to the supernatant. The column was washed with 15 mL of Buffer O (50 mM Tris pH 7.8, 100 mM NaCl, 10% Glycerol, 0.03% DDM) containing 50 mM imidazole. A further wash of 15 mL of Buffer O containing 100 mM imidazole was followed by the elution of the his-tagged CysZ with Buffer O containing 300 mM imidazole. The Ni-affinity purified CysZ was then concentrated to 10–15 mg/mL before loading to a Superdex 10/300 200 gel-filtration chromatography column (GE Healthcare) and eluted with Buffer O containing 0.5 mM EDTA. The protein concentration was initially determined by both bicinchoninic acid (BCA) assay (Thermo Scientific) and absorbance at 280 nm to obtain the extinction coefficient at 280 nm. Protein concentrations were subsequently measured by absorbance at 280 nm.

2.2. Western Blot

CysZ protein samples were run on SDS-PAGE gel (Any kD™, BioRad) and transferred onto a polyvinylidene fluoride (PVDF) membrane (BioRad) with a wet transfer cassette (BioRad). The membrane was blocked by 3% Bovine Serum Albumin (BSA) at room temperature for 1 h and then at 4 °C overnight with gentle rotating. The blocked membrane was incubated with primary antibody of Anti-His antibody (GE healthcare) at room temperature for 1 h. A cross-absorbed fluorescent secondary antibody IRDye 680RD Goat Anti-Mouse IgG (LI-COR Biosciences) was then incubated with the membrane for 1 h at room temperature and visualized in its compatible Odyssey Clx infrared imaging system (LI-COR Biosciences).

2.3. Isothermal Titration Calorimetry (ITC) measurements

ITC measurements were carried out at 25 °C using a VP-ITC MicroCalorimeter (MicroCal), which is equipped with a 1.437 mL cell and a 300 μL syringe. CysZ protein samples were prepared by dialysing for 2 h in 20 mM Tris pH 7.8, 100 mM NaCl, 10% v/v Glycerol, 0.03% w/v β-DDM and 0.5 mM EDTA. All the substrates were freshly prepared, dissolved in the dialysate to minimize artifacts from buffer mismatch. A titration experiment was initiated with a single 2 μL injection followed by a series of 8 μL injections of substrates into the protein solution (0.02–0.06 mM) in the cell. Control experiments titrating dialysate into protein as well as ligand into dialysate were carried out. The results were corrected for the heat changes arising from injection of substrates into dialysate before data analysis with ORIGIN software (MicroCal).

2.4. Whole cell uptake assay with ^{35}S labeled sulfate

The uptake assays were carried out as described before [15], with modifications. Generally, *C43 (pET21a-cysZ)* and control cells *C43 (pET21a)* were grown in M9 minimal medium supplemented with 0.25 μM glutathione as the sole sulfur source, 2 mM magnesium chloride (in place of magnesium sulfate) and 0.1 mg/mL ampicillin. When cell growth reached the exponential phase, 1 mM IPTG was added to induce the over-expression of CysZ. Five hours after induction, cells were harvested, washed and resuspended in M9 medium at 10 mg (wet weight)/mL and incubated for 10 min at 37 °C. The transport was initiated by adding 0.5 μCi of sodium [^{35}S] sulfate (250–1000 mCi/mmol; Perkin Elmer) into the cell suspension, followed by vortex. Fractions of 0.1 mL cell suspension were taken at different time points, mixed with 1 mL of stop solution (M9 minimal medium containing 2 mM magnesium sulfate and 2 mM sodium thiosulfate) and collected by centrifugation. After washing with 1 mL of stop solution, cells were resuspended in 0.5 mL stop buffer and then transferred to polyethylene

vials containing 2 mL Optiphase 'HiSafe 3' scintillation fluid (Perkin Elmer). The radioactivity was counted in a Perkin Elmer liquid scintillation analyzer.

2.5. Sulfate uptake in the presence of sulfite

Uptake experiments were carried out with and without sulfite to examine the effect of sulfite binding on sulfate transport. Suspensions of *C43 (pET21a-cysZ)* cells were divided into two equal portions. Uptake of sodium [^{35}S] sulfate at various concentrations was recorded from 0.1 mL cells with and without pre-incubation of 5 μM sodium sulfite. All uptakes were stopped after 5 min and the reactions were washed and counted as described above.

2.6. Whole cell sulfate uptake at different pH and in the presence of a proton ionophore

The uptake efficiency was compared in pH 7.0, 7.5 and 7.9. The CysZ over-expressing *C43 (pET21a-cysZ)* and control cells *C43 (pET21a)* were washed and re-suspended in buffers with desired pH. After incubation at 37 °C for 20 min with or without 50 μM proton ionophore carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone (FCCP) (Sigma), 0.05 μCi of sodium [^{35}S] sulfate was added to 0.1 mL of cell suspension and the uptake reactions were allowed for 5 min before being quenched, washed and counted. The V_{max} and K_m were calculated by using GraphPad Prism 6.

2.7. Uptake assays with purified CysZ reconstituted into proteoliposomes

LMVs (large multilamellar vesicles) were prepared following the protocol of Geertsma et al. [16] in an inside vesicle buffer (15 mM Hepes pH 7.2, 150 mM KCl, 5% Glycerol, 1 mM DTT). Before use, 1 mL of 20 mg/mL LMVs were extruded 11 times through a 400 nm polycarbonate filter (Whatman) fitted to a Mini-Extruder (Avanti Polar Lipids). Triton X-100 (Fluka) 10% solution was added to a final concentration of 0.5% (w/v). Purified CysZ was added to a protein/lipid ratio of 1:100 (wt/wt). For a negative control, protein purification buffer was added instead of protein. Bio-Beads (Bio-Rad) (0.8 g) were then added in 4 batches to remove detergent. Finally, the proteoliposomes were harvested by centrifugation at 180,000 ×*g* at 20 °C for 1 h and then resuspended in 0.4 mL loading buffer. Reconstituted vesicles were used for uptake assay within 1 h. For uptake assays, 20 μL of vesicles was mixed with 180 μL desired reaction buffers, followed by adding 0.05 μCi of sodium [^{35}S] sulfate. To monitor the [^{35}S] sulfate uptake assay over time, pH 6.9 buffer with Hepes 15 mM, 150 mM NaCl and 5% Glycerol was used and 50 μM of valinomycin was added. The same buffer with pH 6.9 and pH 7.5 was used to investigate the influence of proton gradient on the uptake. In the inhibitory study, 5 μM of freshly made sodium sulfite was included in the inside vesicle buffer and mixed with the reaction buffer prior to the assay, and then the uptake was performed by using [^{35}S] sulfate at different concentrations, with 50 μM valinomycin. In these two assays, the sulfate uptake was terminated after 5 min by mixing with 2 mL of ice-cold buffer and quickly passing the reactions through membrane filters (Millipore). The membranes were immediately washed by 2 mL of ice-cold stop solution and counted in a Perkin Elmer liquid scintillation analyzer after being dried up.

3. Results

3.1. The purified CysZ

The gel-filtration elution profile of CysZ is shown in Fig. 2A, and SDS-PAGE (10%, home-made) and Western Blot (Any kD^{TM}, BioRad) of the purified protein sample are shown in Fig. 2B and C. Depending on the type of gel used, the apparent molecular weight of CysZ is around 25 kDa as appose to the calculated 30 kDa. This is because additional SDS molecules incorporate into the DDM detergent micelles, charging the protein more than average.

3.2. ITC measurements confirm strong interactions between CysZ and sulfur-containing compounds

CysZ in different organisms has been previously assigned as a sulfate transporter based on mutagenesis studies at the whole-cell level [4,10]. To investigate the interactions between sulfate and the putative sulfate transporter CysZ, we measured the binding of sulfate to purified EcCysZ in detergent solutions using ITC and analyzed thermodynamic changes during this process (Fig. 3A). The fitted ITC data suggests the binding affinity of sulfate to CysZ to be 0.4 μM (Table 1). Interactions between CysZ and the cellular cysteine synthesis intermediates sulfite, sulfide, thiosulfate as well as the product cysteine were also measured by ITC titrations (Fig. 3).

Our ITC results also showed that with a K_d of 0.04 μM, sulfite interacts with CysZ with a higher affinity than that of sulfate (Fig. 3B). Furthermore, the binding of sulfite demonstrated an inhibitory effect on the transit of sulfate in CysZ; in the presence of 20 mM sulfite, the thermal changes upon titrating sulfate into CysZ were no longer observed (Fig. 3F). In contrast, another sulfur-containing intermediate in the cysteine synthesis pathway, thiosulfate, which can be incorporated into cysteine via cysteine synthase CysM, did not show any interactions with CysZ within the detection range of our ITC experiment (Fig. 3C). Sulfide, an intermediate only present in the CysK branch of the pathway, as well as the final product cysteine, can also interact with CysZ (Fig. 3D, E). The binding of sulfate, sulfite as well as sulfide to CysZ all

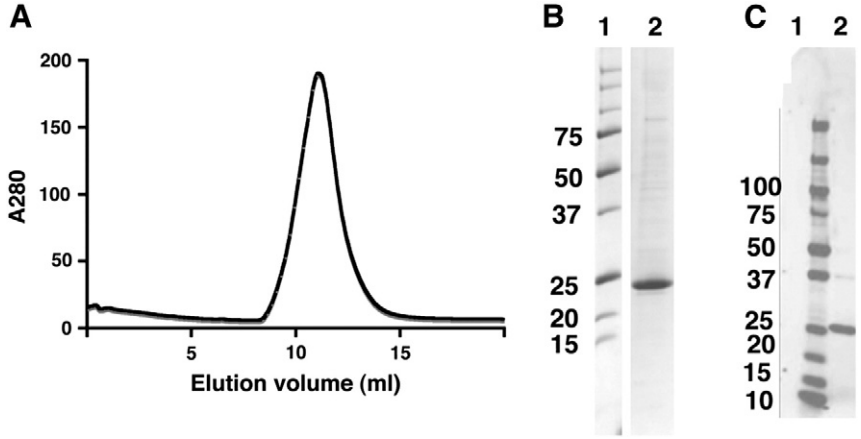

Fig. 2. Expression and purification of CysZ. Gel-filtration elution profile (A) SDS-PAGE (B) and Western Blot (C) of CysZ.

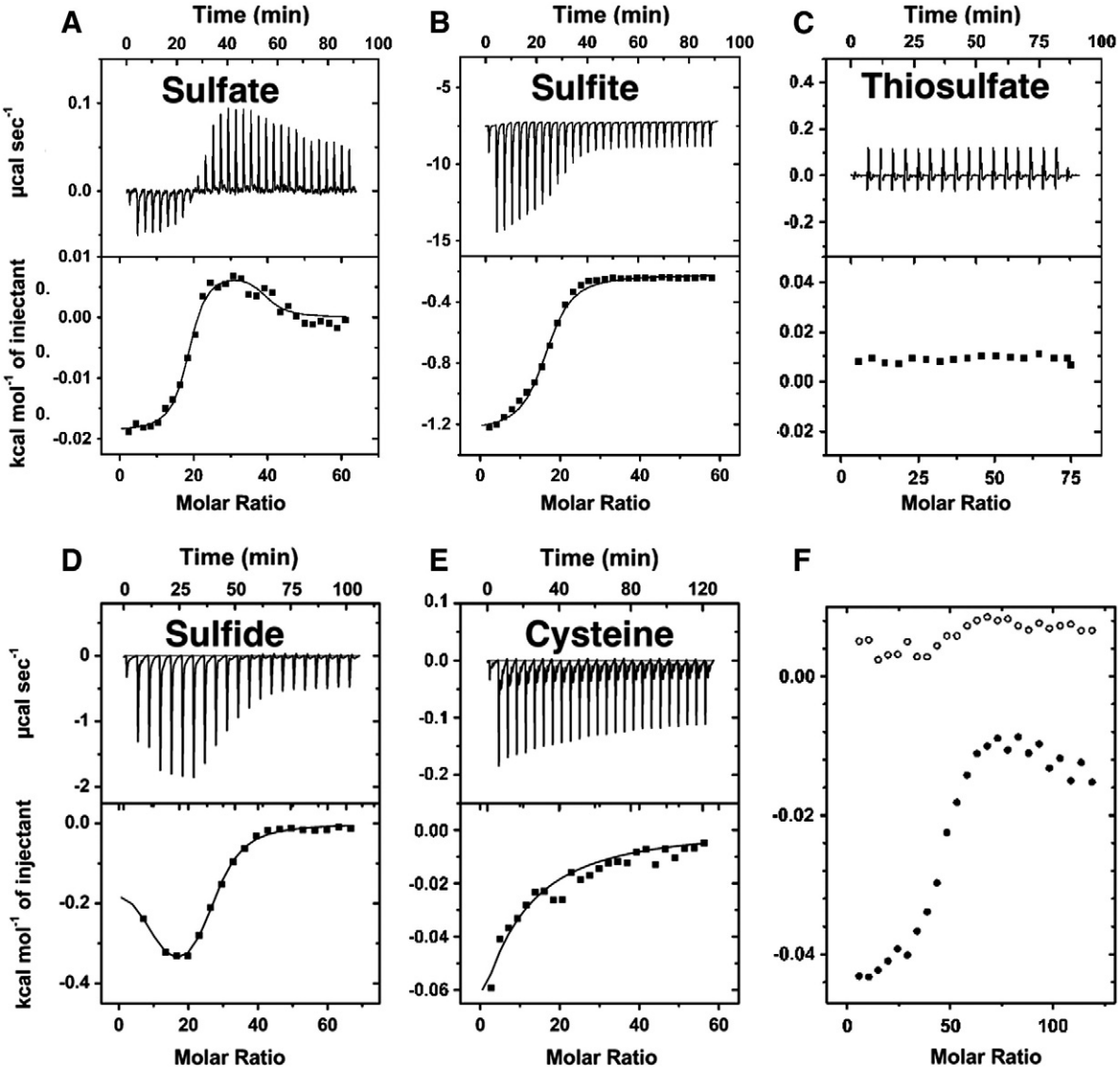

Fig. 3. ITC analysis of the interaction of CysZ with sulfate and other sulfur-containing compounds, as well as the comparison of the thermodynamic changes between CysZ and sulfate with/without sulfite. Sodium sulfate (A), sodium sulfite (B), sodium thiosulfate (C), sodium sulfide (D) and cysteine (E) were titrated into 0.02–0.06 mM CysZ, in a VP-ITC MicroCalorimeter and the heat exchanges were determined at 25 °C. In each dataset, the upper panel shows the raw energy changes during the titration, while the lower panel represents the derived integrated total energy change as a function of the molar ratio of the interactions. Non-linear regression fitting of the data (shown as a solid line through the data points in the lower panel) to a biphasic two-site model yielded the thermodynamic parameters for the interactions (Table 1). In (F) gel filtration purified CysZ (0.03 mM) from the same batch was used for two ITC experiments: one part was pre-mixed with 20 mM fresh made sodium sulfite for 20 min and then titrated with 20 mM sodium sulfate (open circle). For the other protein sample, 20 mM sodium sulfate was titrated into CysZ directly (filled circle).

demonstrated biphasic isotherms, with the second affinity being significantly lower than the first (Table 1), whereas cysteine titration fits to a monophasic binding profile (Fig. 3E).

Table 1
Thermodynamic parameters for sulfate and other sulfur-containing compounds binding to CysZ.

Substrates	K_a (M^{-1})[a]	K_d (μM)[b]	ΔH (cal/mol)[a]	ΔS (cal/mol K)[a]	ΔG (kcal/mol)[c]
Sulfate	2.6×10^6	0.4	−18.9 ± 0.7	29	−8.7
	4.2×10^5	2.4	8 ± 1	21	−6.3
Sulfite	2.5×10^7	0.04	−1250 ± 38	30	−10
	9.9×10^2	1010	−225 ± 37	13	−4.1
Sulfide	2.4×10^5	4.2	−170 ± 45	24	−7.3
	2.0×10^4	50	−478 ± 68	18	−5.8
Cysteine	1.8×10^3	537	−636	13	−4.5

[a] Determined experimentally as described in the text.
[b] Disassociated constant, K_d (= 1 / K_a).
[c] Calculated using Eq. (1) (T = 298 K).

To further analyze the properties of the protein–ligand interactions, the free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) terms of the interactions were calculated and presented in Table 1.

3.3. CysZ can mediate sulfate uptake into E. coli cells and proteoliposomes

In the sulfate uptake assay carried out with whole cells, *C43 (pET21a-cysZ)* cells demonstrated a significant increase in sulfate uptake compared to *C43 (pET21a)* (Fig. 4A). Since the same *E. coli* expression host was used in the control experiment and the amount of cells in each measurement was kept the same, we can therefore attribute the increased uptake of sulfate to the over-expressed CysZ.

To isolate the sulfate uptake rate of CysZ from other sulfate transporters and sulfate metabolism of the whole cell, the uptake assay was also performed on CysZ reconstituted in proteoliposomes. In these experiments the uptake rate of sulfate was significantly higher in the CysZ vesicles compared to the empty vesicles, especially within the first 2 min (Fig. 4B).

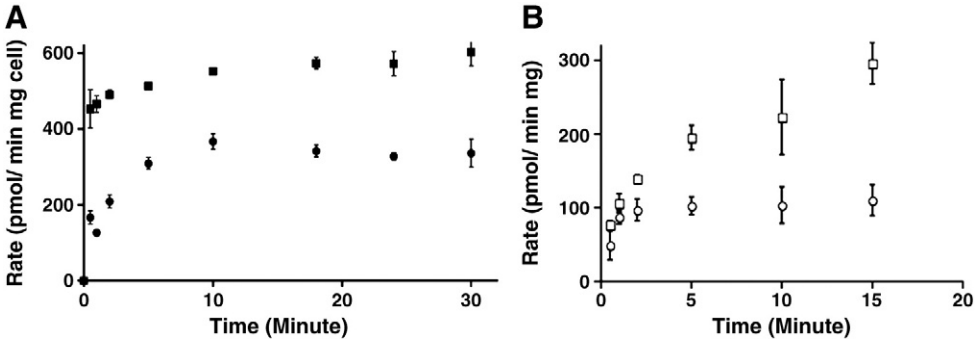

Fig. 4. Uptake assays with [^{35}S] Sulfate. (A) Isotopic sulfate uptake of *E. coli* with (■) and without (●) over-expressed CysZ at different time points. (B) CysZ proteoliposome uptake assays. Uptake rates of CysZ vesicles (□) and control vesicles (○) at different time points. Each point represents the mean ± standard deviation for three determinations.

3.4. Sulfate uptake by CysZ is inhibited by sulfite

The sulfate analog sulfite has been previously reported as an inhibitor for sulfate uptake in other organisms [17,18]. Our ITC experiments indicated that sulfite could diminish the heat changes in the reaction between sulfate and CysZ. To further characterize sulfite as an inhibitor on sulfate uptake by CysZ, we also included sulfite in uptake assays using both whole cell and proteoliposomes.

As shown in Fig. 5A, when 5 μM sulfite was mixed with ^{35}S labeled sulfate in the uptake assay, a dramatic reduction of sulfate uptake rate was observed in comparison with the control group in the absence of sulfite. For this dataset, the V_{max} of the uptake rate in no and 5 μM sulfite groups are 160 ± 8 and 88 ± 4 pmol/mg cell/min with the K_m of 0.72 ± 0.2 μM and 1.3 ± 0.3 μM. Due to the fact that there are other sulfate transporters in *E. coli* cells, the inhibition of sulfite was also studied using proteoliposomes. Similar to the results of whole cell uptakes, the presence of sulfite could inhibit the uptake of sulfate with a V_{max} of 200 ± 12 pmol/mg/min and 178 ± 11 pmol/mg/min, K_m of 8.9 ± 1.1 (no sulfite) and 6.8 ± 0.9 (5 μM sulfite) μM respectively (Fig. 5B).

3.5. CysZ mediated sulfate transport is pH dependent

To test the hypothesis of protons being a driving force for the sulfate transport in CysZ, we carried out sulfate uptake assay firstly in whole cells at three different extracellular pH levels. It has been previously reported that mildly acidifying the extracellular solution could cause an immediate pH decrease in both the cytoplasm and the periplasm of *E. coli*, but the former would recover after approximately 3 min [19]. Prior to the uptake assay, cells were pre-incubated in pH 7.0, pH 7.5 and pH 7.9 medium for 10 min respectively. The results showed that at pH 7.0, the amount of sulfate transported into the *C43 (pET21a-cysZ)* cells is higher than those at pH 7.5 and pH 7.9 (Table 2). Considering that the physiological *E. coli* intracellular pH is in a range of 7.2–7.8 [20,21], this data suggests that inward proton gradient across the inner membrane could elevate the uptake of sulfate, which supports the hypothesis that proton gradient may facilitate the CysZ mediated sulfate transport.

To further examine the effects of the proton gradient and the membrane potential on sulfate uptake, the proton ionophore FCCP was introduced to the assay system by adding 50 μM FCCP to the uptake reactions. At both pH 7.0 and 7.5, sulfate uptake reduced in the presence of FCCP, whereas at pH 7.9, sulfate uptake increased. To verify that the above-observed effect was indeed related to CysZ, the same experiments were also carried out in proteoliposomes with both artificial pH gradient and the presence of FCCP. Valinomycin was also included to eliminate the effect of proton buildup during multiple transport cycles. Using reconstituted CysZ proteoliposome at pH 7.2, the in vitro transport assays showed similar results as the whole cells uptake assays. In both pH 6.9 and pH 7.5 reaction buffers, CysZ vesicles could uptake significantly more [^{35}S] sulfate than the control empty vesicles (Fig. 6). However, with 50 μM of FCCP and/or valinomycin, the sulfate uptake decreased at both pH.

4. Discussion

4.1. Interactions between CysZ and its putative substrate sulfate have been confirmed

In comparison to other known sulfate transporters, CysZ demonstrates two interesting properties. Firstly, CysZ binds sulfate with a relatively high affinity of 0.4 μM, which is approximately 10 times higher than the recently reported high-affinity sulfate transporter SulP from *Mycobacterium tuberculosis* [22]. Secondly, unlike CysTWA and CysP, which are capable of binding as well as transporting both sulfate and

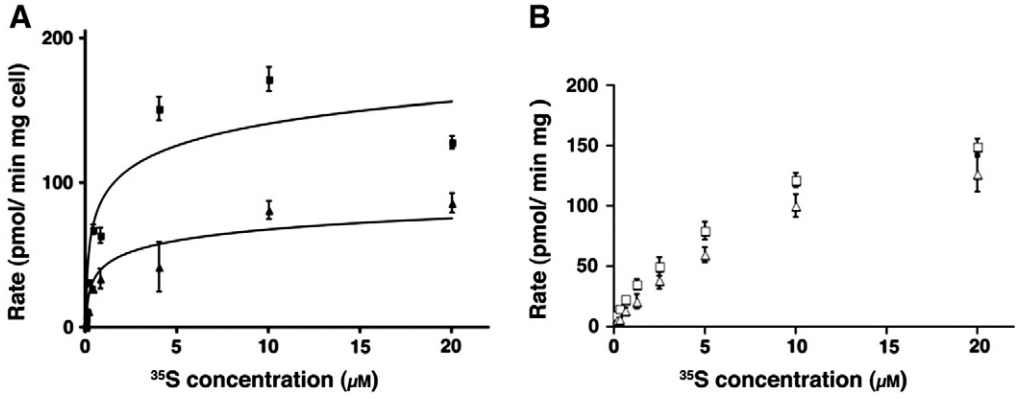

Fig. 5. The inhibition of sulfate uptake by sodium sulfite. (A) The Michaelis Menten plots of the uptake rates in *E. coli* whole cells without (■) and with 5 μM (▲) sodium sulfite. (B) Sulfate uptake rate in CysZ proteoliposome vesicles without (□) and with 5 μM (△) sodium sulfite.

Table 2
Whole cell sulfate uptake rates (pmol/min/mg cell) at pH 7.0, pH 7.5 and pH 7.9 with and without FCCP.

	pH 7.0		pH 7.5		pH 7.9	
	No FCCP	FCCP	No FCCP	FCCP	No FCCP	FCCP
C43 (pET21a-CysZ)	612 ± 66	570 ± 31	482 ± 18	439 ± 16	484 ± 19	542 ± 35
C43 (pET21a)	348 ± 18	358 ± 19	290 ± 22	304 ± 41	326 ± 17	312 ± 57

thiosulfate [2,5,23], CysZ binds sulfate but not thiosulfate, in agreement with observations from earlier studies [10]. The high sulfate affinity indicates that CysZ plays important physiological roles when the cell is exposed to environments with low sulfate concentrations [10], while the binding selectivity suggests that CysZ is not involved in the synthesis of cysteine from the *O*-acetylserine and thiosulfate pathway.

Energetically, the free energy change (ΔG) of sulfate binding to CysZ can be separated into contributions from enthalpy (ΔH) and entropy (ΔS):

$$\Delta G = \Delta H - T\Delta S = -nRT\ln K. \quad (1)$$

If we consider non-specific interactions to be negligible, the enthalpy ΔH can be directly measured and the binding constant K can be calculated from ITC experiments, thus the net ΔG and ΔS can be deduced. For interactions between sulfate and CysZ, we observed a ΔG of −8.7 kcal/mol. Since the interaction process is an exothermic process, where the ΔH is only −18.9 cal/mol, the majority of the free energy change comes from the −TΔS term. In the protein–ligand interaction process, the enthalpic component mainly stems from hydrogen bond formation (a few kcal/mol) and protonation events, whereas the entropic component arises from hydrophobic interactions, water/ion release and conformational changes in the protein [24]. Electrostatically, sulfate and its analogs are divalent anions at neutral pH. The ligand binding data with a relatively small enthalpic component and a large entropic component therefore suggest that upon the binding of sulfate, CysZ undergoes significant conformational changes [25].

The ITC data for the binding of sulfate, sulfite as well as sulfide to CysZ are all fitted to biphasic models, suggesting the existence of a second substrate binding site with cooperative binding. However, this would need to be analyzed in future studies; right now we refrain from drawing conclusions from the ITC data alone.

4.2. CysZ is a sulfate transporter that mediates sulfate uptake into E. coli cells

CysZ has previously been assigned as a sulfate transporter based on the fact that certain Ec*cysZ* mutants of *E. coli* are deficient in sulfate transport [4,10,22]. In both the whole cell and proteoliposome transport assays, although only about 0.5 μM of [^{35}S] sulfate was used in each measurement, CysZ could still effectively mediate sulfate uptake, which also confirms previous reports of CysZ being able to transport sulfate into cells at very low sulfate concentrations [10]. Noteworthily, the control measurement carried out on cells transformed with empty pET-21a plasmids also demonstrated certain amount of sulfate uptake, which is attributed to endogenous CysZ and other sulfate transporters. In the whole cells, the amount of sulfate being taken up in the first 1.5 min is very similar to that at 20 min. Interestingly, this phenomenon was not observed from the proteoliposome uptake results. This can be due to the fact that the *E. coli* cells used in this experiment were cultured in minimal medium and the cells were starving for sulfate. In this case, CysZ was recruited to acutely correct this impoverishment. However, we cannot rule out the possibility of the influence from other sulfate channels and transporters despite the over-expression of CysZ. In the vesicle uptake, the uptake rate increased linearly and was slower than in the whole cell assay, which could be caused by the sidedness of the proteoliposome. As reported in previous studies, membrane proteins are normally randomly oriented during reconstitution [26–28]. People habitually choose to neglect the sidedness because membrane transporters are suppose to transfer the substrate under certain driving forces. Molecules orientated against the driving force or necessary conditions, like the presence of ATP, can be considered as non-effective. Together with the previously mentioned findings from ITC, we conclude that CysZ can bind as well as actively import sulfate into *E. coli*, and this transport can be conducted at very low extracellular sulfate concentrations.

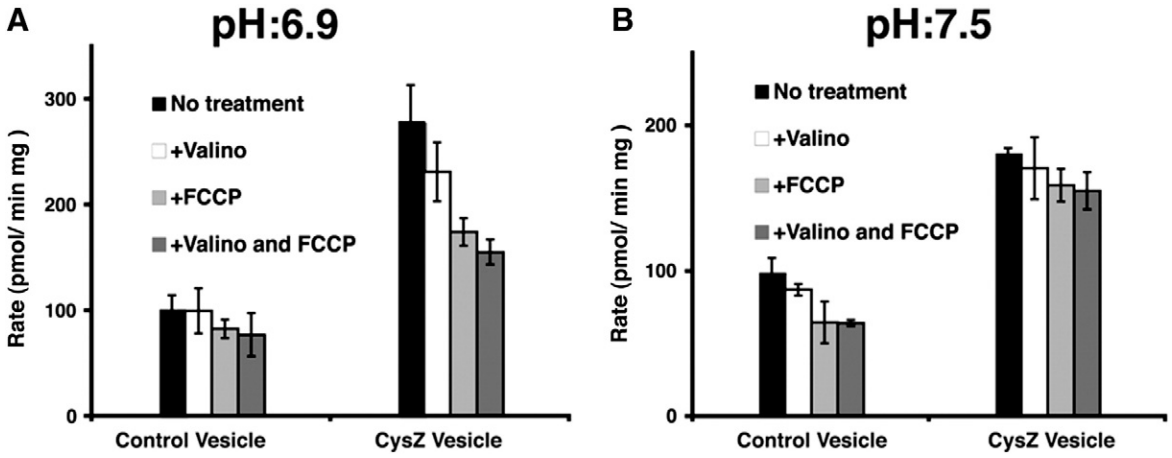

Fig. 6. CysZ proteoliposome sulfate uptake rates (pmol/min/mg) at outside vesicle pH 6.9 (A) and pH 7.5 (B) with and without valinomycin and FCCP. Aliquot of 20 μL vesicles was mixed with reaction buffers containing 15 mM pH 6.9 or pH 7.5 Hepes, 150 mM NaCl and 5% Glycerol, with/without FCCP (50 μM) and valinomycin (50 μM). All the reactions were stopped after 5 min.

4.3. Sulfite is an inhibitor of CysZ and contributes to the regulation of sulfate uptake

Generally, the cysteine synthesis pathway is regulated by demand [29], which means that it is repressed when reduced sulfur compounds start to accumulate. In the sulfate assimilation pathway, after being transported into the cell, sulfate is reduced to sulfite through multiple enzymatic reactions [30] (Fig. 1). Besides its transcriptional activator CysB, which positively regulates genes of the *cys* regulon [31,32], reducing APS (adenosine 5′-phosphosulfate) by APR (APS reductase) [33,34] to sulfite has been regarded as one regulating step, while other enzymes in the pathway are not regulated. Comprehensive studies have been done in plants to elucidate the molecular mechanisms of APR in regulating the sulfate assimilation pathway, and it has been shown that APR can be transcriptionally regulated by its catalytic product sulfite [35]. However, we have observed that the inhibition of sulfate uptake by sulfite occurred immediately after mixing the cells with sulfite, indicating that the uptake of sulfate can be inhibited by the direct binding of sulfite to the sulfate transporter CysZ, thus regulating the very first step in the cysteine synthesis pathway.

Verified in both whole cell and proteoliposome uptake assays, here we present direct evidence for the first time that sulfite, which is an intermediate product in the sulfate assimilation pathway, can repress the *E. coli* sulfate uptake via direct binding with CysZ, which could not have been due to transcriptional regulation since the inhibition occurred promptly after the cells were exposed to sulfite. As an intermediate, sulfite is produced in the cytosol whereas in our whole-cell uptake experiment it was introduced from the extracellular side. However, for CysZ to successfully bind and release sulfate, the translocation pathway must be accessible from both sides of the membrane. We therefore reason that sulfite can gain access to the sulfate-binding site from both the cytosolic and periplasmic sides. The interactions between sulfite and CysZ thus suggest the existence of a feedback mechanism, in which sulfite inhibition of CysZ down-regulates the influx of sulfate and prevents further accumulation of the toxic sulfite inside the cells.

Nevertheless, the ITC signal for sulfate binding to the sulfite-bound CysZ is diminished (Fig. 3F), which again suggests that the heat exchange occurred upon the binding of ligands as observed in the ITC data comes from the subsequent conformational changes triggered by ligand binding rather than the actual protein–substrate interactions.

4.4. The driving force of sulfate uptake

As mentioned in the Introduction, a positively charged ion gradient could effectively serve as a driving force for sulfate uptake into the cell against the existing membrane potential. The proton gradient across bacterial cell membranes is a likely candidate. Previous studies have also shown that in both plants and bacteria, sulfate uptake is pH dependent and can be abolished by adding either an inhibitor of the proton-translocating F_1F_0-ATPase – DCC (N,N-dicyclohexylcarbodiimide) [17] or a proton ionophore – CCCP (carbonyl cyanide m-chlorophenyl hydrazone) [36]. Although all of these uptake assays were carried out on whole cells that may contain multiple sulfate transporters, these observations still point to the proton gradient being the sulfate transport driving force and is therefore a top candidate to be investigated.

As shown in Table 2, within the physiological pH range of *E. coli* cells, the lower the extracellular pH is, the higher the sulfate uptake rate becomes in the CysZ over-expressing cells. The same conclusion is also reached with data obtained with CysZ containing proteoliposomes (Fig. 6). Since these pH values are within the range of cell growth conditions for typical *E. coli* strains, the difference in uptake can therefore reflect the effect of different proton concentrations rather than detrimental effect of extreme pH on the cell or protein's overall structural integrity. This result suggests that protons are involved in the sulfate transport mediated by CysZ.

Furthermore, when the proton ionophore FCCP was present, the uptake rates at both pH 7.0 and 7.5 decreased in comparison to rates without FCCP, demonstrating the negative effect on sulfate uptake by CysZ when the proton gradient across the cell membrane is destroyed. However, in the whole cell uptake, the presence of FCCP increased the uptake rate at pH 7.9. This is due to the fact that the intercellular pH of *E. coli* cells is typically around 7.5, and when the extracellular pH is raised to 7.9, the natural proton gradient across the cell membrane is reverted. When FCCP is present, the unfavorable proton gradient is removed, thus proton-coupled sulfate uptake increased.

To further explore the mechanism of pH-dependant transport, the effect of the proton ionophore, FCCP, on sulfate uptake was examined using proteoliposome. FCCP alone, or in the presence of valinomycin, drastically reduced CysZ-mediated sulfate uptake at outside vesicle buffer pH 6.9, while this phenomenon was not as obvious when the external pH is 7.5. These observations are consistent with the conclusion from the whole cell uptake assays that sulfate uptake is coupled to proton influx.

In general, the symport of proton and sulfate would help temporarily neutralize the negatively charged sulfate theoretically and help the substrate to overcome the electrostatic potential across the cell membrane. The stoichiometry of proton and sulfate during transport, the possible involvement of additional ions in the transport process, as well as the detailed transport mechanisms, require further investigation.

5. Conclusions

In summary, based on our results from both the ITC and sulfate uptake assays, the sulfate transporter CysZ is a high-affinity, high-specificity sulfate transporter that provides the sulfur source for the cysteine synthesis pathway. It is essential for the survival of bacteria under low sulfate conditions. The transporter makes direct interactions with the toxic intermediate sulfite from the sulfate assimilation pathway. This interaction serves as a feedback regulation mechanism in the sulfate transport. The effect of pH on sulfate uptake suggests that CysZ transport might be facilitated by the proton gradient across the plasma membrane.

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