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Lysosomal sulfate transport: inhibitor studies

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Abstract

Sulfate derived from the degradation of macromolecules is released from lysosomes via a carrier mediated process. In order to further characterize this process, recognized inhibitors of the erythrocyte band 3 anion transporter were examined for their effects on the lysosomal system. Studies with band 3 transport site inhibitors such as DIDS, SITS and phenylglyoxal indicated that, similar to the case for the band 3 protein, the lysosomal transporter has critical lysine and arginine residues. Band 3 translocation pathway or channel blocking inhibitors had mixed effects on the lysosomal system. 1,2-Cyclohexanedione, which covalently modifies a band 3 arginine residue distinct from that modified by phenylglyoxal, inhibited lysosomal sulfate transport. In contrast, the potent band 3 inhibitor dipyridamole had no effect on lysosomal sulfate transport indicating that there are some structural differences between the erythrocyte and lysosomal anion transporters. The band 3 translocation inhibitors niflumic acid and dinitrofluorobenzene were both effective inhibitors of the lysosomal system. Cupric ion inhibited sulfate transport while Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} had no inhibitory effects. Exposure of intact lysosomes to trypsin largely ablated transport of sulfate. This information should be useful in efforts to further elucidate the structure and function of the lysosomal sulfate transporter.

Keywords: Lysosome; Transport; Sulfate; Membrane vesicle

1. Introduction

The transport of sulfate across the lysosomal membrane is a process that is sensitive to pH and is most active when sulfate is transported down a proton gradient. At pH 5.0, uptake of sulfate by resealed lysosomal membrane vesicles (pH 7.0 inside) has a K_m of 160 μM and is insensitive to changes in membrane potential. In contrast, at pH 7.0 uptake becomes sensitive to membrane potential. Counter-transport of sulfate can be demonstrated in the absence of a proton gradient with a $t_{1/2}$ of 30 s and a K_m identical to that of uptake at pH 5.0. Competition and *trans*-stimulation of sulfate by other anions have been demonstrated, although sulfate produced by the activity of various lysosomal sulfatases seems to be the primary substrate for this system [1].

A great deal of information regarding the structure and function of the band 3 anion protein and gene has been amassed [2–20], which may be useful for study of the lysosomal transport system. The band 3 protein has a molecular mass of 90 kDa and is the product of one (AE1) of a family of genes (AE1, AE2 and AE3) that code for tissue specific plasma membrane anion transporters [21]. A truncated transcript of the AE1 gene codes for a transport protein with a shortened amino-terminus in rat kidney [22,23]. In general, a region containing the carboxyl-terminus of the anion transporters appears to play an essential role in transport and is highly conserved [24]. Proteins which cross react with selected anti band 3 antibodies have also been found in Golgi apparati and mitochondria [25,26] while similar anion transport systems have been described in plasma membranes from lung fibroblasts and Chinese hamster ovary cells [27–29].

The band 3 transporter shows pH dependence of anion transport, including increased proton-sulfate co-transport at low pH. At higher pH, monovalent anions such as bicarbonate are recognized preferentially. Similar to the lysosomal system, band 3 is regulated by pH, exhibits counter-transport of sulfate and is sensitive to membrane potential at neutral pH. However, band 3 accepts monovalent anions

Abbreviations: DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; NAP-taurine, *N*-(4-azido-2-nitrophenyl)-2-aminoethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid.

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such as bicarbonate as substrates, while this does not appear to be the case for the lysosomal transporter. Band 3 mediated anion transport is affected by three major classes of inhibitors that are characterized as: (1) transport site inhibitors, (2) translocation pathway or channel blockers, and (3) translocation inhibitors [30–32]. Information regarding the sites and mechanisms of action of these and other inhibitors has been useful for the development of models of the structure and function of the transporter. We have examined the effects of band 3 inhibitors on lysosomal sulfate transport in order to gather information regarding the structure and function of the lysosomal transport protein. These studies show that the lysosomal sulfate and band 3 anion transporters are sensitive to many of the same inhibitors, although differences in their responses can be demonstrated.

2. Experimental procedures

2.1. Materials

With the exception of NAP-taurine (Pierce), all inhibitors were obtained from Sigma. $Na_2^{35}SO_4$ was purchased from ICN Biochemicals, Scintiverse II (Fisher), Dulbecco's phosphate-buffered saline without calcium (Irvine Scientific) and GFF glass fiber filters (Whatman).

2.2. Lysosomal purification

Female 150–200 g Sprague-Dawley rats (Harlan) were injected with 1000 units of heparin by tail vein just prior to sacrifice with CO_2 narcosis. The livers were removed and flushed of blood using ice-cold normal saline containing 1 mM EDTA. Lysosomes were then purified from liver homogenates by differential centrifugation followed by Percoll density gradient centrifugation in the presence of 1 mM PMSF [33]. Protein was determined spectrophotometrically [34].

2.3. Membrane vesicle formation

The dense, approx. 1 ml fraction containing lysosomes was removed from the Percoll gradient and mixed with an equal volume of a buffered solution to achieve the following concentrations: 0.25 M sucrose, 20 mM Hepes, pH 7.0, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 500 μM PMSF, 2 mM $MgCl_2$, and 2.0 mg/ml bovine serum albumin. Addition of 5 mM methionine methyl ester and incubation at 25° C for 15 min resulted in the specific osmotic rupture of lysosomes. In order to prepare sulfate loaded vesicles for countertransport (*trans*-stimulation) experiments, 10 mM Na_2SO_4 (final concentration) was added to the buffer mixture used during osmotic rupture of lysosomes by methionine methyl ester. The much less dense membrane vesicles were collected from the top of a second Percoll density gradient that had been prepared similarly to the first gradient. They were washed with 20 volumes of 0.25 M sucrose and collected by centrifugation at $18\,000 \times g$ for 30 min prior to use. Vesicles prepared in this manner are largely free of contaminants such as plasma membrane, Golgi, endoplasmic reticulum, mitochondria, and internal hydrolases and exhibit a number of specific transport processes for substrates such as vitamin B_{12}, *N*-acetylglucosamine and sulfate [29,35–37]. The membrane vesicles have a random membrane orientation and have an intravesicular environment consisting of 0.25 M sucrose, 20 mM Hepes, pH 7.0, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 500 μM PMSF, 2 mM $MgCl_2$, and 2.0 mg/ml bovine serum albumin.

2.4. Sulfate transport

Sulfate transport assays were conducted as previously described [1]. Standard assays for determination of sulfate uptake were performed in microcentrifuge tubes. Incubations were initiated by the addition of 20 μl of membrane vesicles or lysosomes suspended in 0.25 M sucrose to 30 μl of assay buffer consisting of 0.25 M sucrose, 167 μM Na_2SO_4, 33 mM Mes, pH 5.0 with 2 μCi of $Na_2^{35}SO_4$. For determination of K_I (inhibitor), the concentration of sulfate in the assay buffer was varied. The tube contents were mixed by trituration and allowed to incubate for 1 min at 25° C. Membrane vesicles were then collected by filtration through a GF/F filter and washed with 10 ml of Dulbecco's phosphate-buffered saline (without calcium) at 4° C. Filters were dried under a heat lamp and subjected to analysis by scintillation counting. Uptake was defined as μmol of sulfate/mg protein of membrane vesicles per min at 25° C based on the specific activity of sulfate in the assay buffer.

Countertransport (*trans*-stimulation) assays were performed using membrane vesicles that had been previously loaded with sulfate as described above. Incubations were initiated by the addition of 20 μl of membrane vesicles suspended in 0.25 M sucrose to 30 μl of assay buffer consisting of 0.25 M sucrose, 167 μM Na_2SO_4, 33 mM Hepes, pH 7.0 with 2 μCi of $Na_2^{35}SO_4$. Membrane vesicles were incubated, collected by filtration, washed and subjected to scintillation counting as described for sulfate uptake. Uptake was defined as μmol of sulfate/mg protein of membrane vesicles per min at 25° C based on the specific activity of sulfate in the assay buffer.

2.5. Kinetics

K_I was calculated by Dixon plot analysis using a minimum of three sulfate concentrations and a minimum of six points per curve at a fixed sulfate concentration. Sulfate concentrations ranged from 0–200 μM.

2.6. Binding of irreversible inhibitors

Inhibition studies with phenylglyoxal and 1,2-cyclohexanedione were performed by incubating whole lysosomes ± the appropriate inhibitor for 1 h at 37° C in 80 mM boric acid and 0.20 M sucrose, pH 8.0, prior to forming vesicles. Similar studies were performed with lysosomes incubated for 30 min at 37° C in 0.18 M sucrose, 50 mM NaCl, and 20 mM Hepes, pH 7.4 ± dinitrofluorobenzene. In all cases, lysosomes were washed free of inhibitor prior to forming vesicles.

2.7. Trypsin treatment of lysosomes

Intact lysosomes were suspended in 0.25 M sucrose, 2 mM Hepes, pH 7.0 and incubated for 10 min at 30° C with 50 μg/ml of trypsin ± 50 μg/ml soybean trypsin inhibitor. Controls were incubated in the same manner ± 50 μg/ml trypsin inhibitor but without trypsin. Following incubation, the lysosomes were assayed for sulfate uptake. Latency studies were performed using the impermeant substrate *O*-β-D-galactopyranoside ± 0.1% Triton X-100 to ensure that there was no lysosomal breakage caused by trypsin exposure. Samples were incubated in 0.25 M sucrose, 50 mM sodium citrate, pH 5.0 for 15 min at 37° C. Latency was defined as 100 − (substrate hydrolysis without Triton X-100/substrate hydrolysis with Triton X-100).

3. Results

3.1. Band 3 transport site inhibitors

Consistent with our preliminary studies [1], transport was readily inhibited by DIDS, an impermeant disulfonic acid stilbene known to inhibit band 3 function by binding to a specific lysine residue [8,38]. The K_I for inhibition of lysosomal transport by DIDS was approx. 3.5 μM (Fig. 1). Irreversible inhibition of transport was observed in membrane vesicles which were prepared from intact lysosomes that were pre-incubated with DIDS suggesting that there is an accessible site for DIDS binding on the lysosomal exterior (Fig. 2). SITS, a compound with a similar mechanism of action, also inhibited sulfate countertransport in membrane vesicles although the K_I was somewhat higher at 70 μM. Phenylglyoxal, a compound known to inhibit band 3 transport by covalent modification of a specific arginine residue [9,39], also inhibited lysosomal anion transport (Table 1). These findings suggest that the lysosomal transporter has accessible lysine and arginine residues that are critical for function. In the case of the erythrocyte band 3 anion transporter, a lysine residue is required for irreversible binding of DIDS although this is not required for reversible inhibition by DIDS [38].

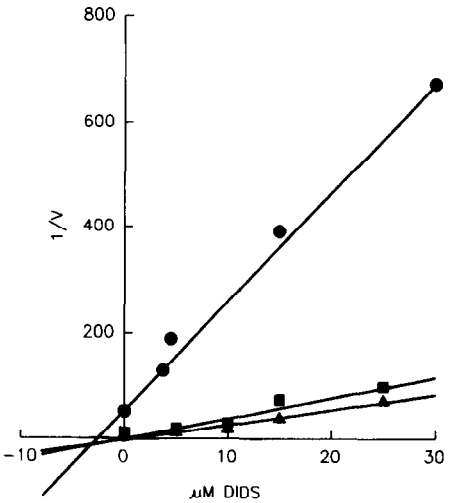

Fig. 1. Dixon plot of inhibitory effects of DIDS. Samples were prepared for countertransport as described in Experimental procedures, containing varying concentrations of inhibitor and varying concentrations of substrate (sulfate) in the incubation buffer. Membrane vesicles were exposed to DIDS only for the one minute assay interval. ●, 10 μM Na_2SO_4; ■, 50 μM Na_2SO_4; and ▲, 100 μM Na_2SO_4. Each point is the mean of three determinations. V = μmol SO_4^{2-}/mg protein per min. K_i is calculated based on the assumption of reversible inhibition.

3.2. Band 3 translocation pathway channel blockers

Lysosomal sulfate transport was inhibited by 1,2-cyclohexanedione (Table 2) which irreversibly inhibits band 3 mediated anion transport by covalent modification of an arginine required for translocation of bound substrate [31,39]. This arginine residue is thought to be discrete

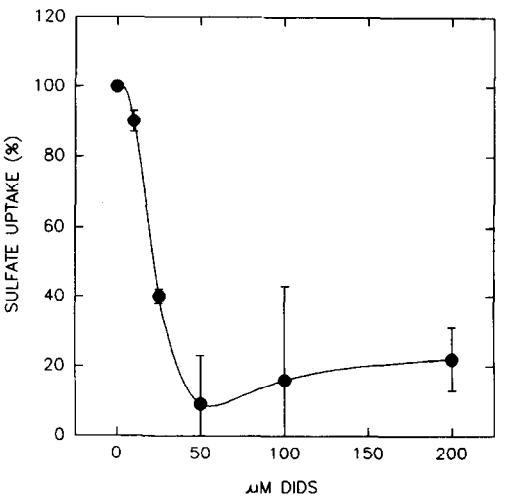

Fig. 2. Irreversible inhibition by DIDS. Whole lysosomes were incubated at pH 8.0 for 30 min at 37° C with 0.25 M sucrose, 20 mM Hepes, and the indicated concentrations of DIDS without sulfate. Subsequently, the lysosomes were washed free of any excess DIDS, and vesicles loaded with 10 mM Na_2SO_4 were prepared for countertransport as described in Experimental procedures. DIDS was not added again at the time of countertransport. Transport was calculated as μmol SO_4^{2-}/mg protein per min. Values represent % of control uptake from lysosomes incubated without DIDS. Points are means ± S.D., $n = 3$.

Table 1
Lysosomal sulfate countertransport and band 3 transport site inhibitors

Inhibitor	Countertransport (%)	K_I (μM)
Control	100	–
25 μM DIDS	12±5	3.5
120 μM SITS	4±2	70
15 mM phenylglyoxal	11±8	–

Vesicles were prepared for assay of countertransport as described in Experimental procedures and incubated at 25° C for 1 min in 0.25 M sucrose, 20 mM Hepes, pH 7.0 with 100 μM Na_2SO_4, 2 μCi of $Na_2^{35}SO_4$ and the indicated inhibitors. For phenylglyoxal, membrane vesicles were prepared from intact lysosomes which had been incubated for 1 h at 37° C in 0.16 M sucrose, 80 mM boric acid, pH 8.0 with phenylglyoxal and then washed to remove unbound inhibitor. Results were compared to transport in comparably treated controls. Each value represents the mean ± S.D., $n = 3$.

from the arginine modified by the transport site inhibitor phenylglyoxal. In contrast, dipyridamole, a potent reversible inhibitor of erythrocyte band 3 transport with a K_I of approx. 0.7 μM [40] had no discernable effect on lysosomal sulfate transport. This was observed for both sulfate countertransport and sulfate uptake by lysosomal membrane vesicles in the presence of up to 100 μM dipyridamole. Lysosomal membrane vesicles that were pre-loaded with 100 μM dipyridamole exhibited normal uptake of sulfate ruling out differential effects due to membrane orientation. In erythrocytes, inhibition of anion transport by dipyridamole may be dependent upon the presence of chloride. However, dipyridamole had no effect on lysosomal sulfate transport in the presence of chloride. Thus, unlike the case for the erythrocyte transporter, the lysosomal sulfate transporter is not sensitive to inhibition by dipyridamole.

3.3. Band 3 translocation inhibitors

Translocation inhibitors appeared to have similar effects on the band 3 and lysosomal sulfate transporters. Niflumic acid, a reversible inhibitor of band 3 action that increases the free energy of formation of the rate-limiting translocation intermediate [32], inhibited the lysosomal system with a K_I of 36 μM. NAP-taurine, a photoreactive anion with K_I values of 20 μM (external side) and 740 μM (internal side) for the band 3 protein [41] also inhibited lysosomal sulfate transport. Similar results were obtained with 2,4-dinitrofluorobenzene, an irreversible band 3 inhibitor which covalently modifies a lysine residue on band 3 that is discrete from the lysine modified by DIDS. For the band 3 transporter, inhibition is directly related to substrate concentration (chloride) presumably because dinitrophenylation occurs during carrier translocation thus locking the transporter into a stable intermediate conformation [32]. This is a markedly different mechanism of action from compounds such as DIDS where available substrate can protect against inhibition by competing for the transport site. Membrane vesicles prepared from lysosomes that had been incubated with 2,4-dinitrofluorobenzene without chloride had 74 ± 9% (mean ± S.D., $n = 3$) of control uptake while those incubated with 2,4-dinitrofluorobenzene and 50 mM chloride had 40 ± 12% (mean ± S.D., $n = 3$) of control uptake. This is consistent with the mechanism of action proposed for the band 3 transporter (Table 3).

Table 2
Lysosomal sulfate countertransport and band 3 translocation pathway (channel) blockers

Inhibitor	Countertransport (%)
Control	100
100 μM dipyridamole	98±9
40 mM 1,2-cyclohexanedione	10±4

Sulfate transport was assayed as described for Table 1. Membrane vesicles prepared from 1,2-cyclohexanedione treated lysosomes were handled as described in Table 1 for phenylglyoxal. Each value represents the mean ± S.D., $n = 3$.

Table 3
Lysosomal sulfate countertransport and band 3 translocation inhibitors

Inhibitor	Countertransort (%)	K_I (μM)
Control	100	–
60 μM niflumic acid	13± 2	36
500 μM NAP-taurine	57±12	400
1 mM dinitrofluorobenzene	3± 2	–

Sulfate transport was assayed as described for Table 1. Membrane vesicles prepared from dinitrofluorobenzene treated lysosomes were handled as described in Table 1 for phenylglyoxal. Each value represents the mean ± S.D., $n = 3$.

3.4. Other inhibitors

The organic anion pyridoxal 5-phosphate is a poorly transported substrate for the erythrocyte band 3 protein that functions as an inhibitor of transport with a K_I of 860 μM. Additional band 3 inhibitors include 2,4-dinitrophenol, phloretin and phloridzin [39,42]. The mechanisms of action of phloretin and phloridzin are not fully defined although they are recognized to inhibit a variety of plasma membrane transport systems. All of these substances inhibited sulfate countertransport in lysosomal membrane vesicles (Table 4).

Table 4
Lysosomal sulfate countertransport and miscellaneous band 3 inhibitors

Inhibitor	Countertransport (%)
Control	100
120 μM phloretin	15±5
3 mM phloridzin	24±8
600 μM pyridoxal 5-phosphate	65±3
3 mM 2,4-dinitrophenol	45±3

Lysosomal sulfate transport was assayed as described for Table 1. Each value represents the mean ± S.D., $n = 3$.

3.5. Cations

A variety of divalent cations were screened for their effects on sulfate transport. Sulfate uptake was slightly increased in the presence of 100 μM Ca^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+}, and Zn^{2+}. Transport increased by 35% with 100 μM Mn^{2+}. Of the divalent cations that were screened, only cupric ion had a significant inhibitory effect on transport. This effect was eliminated when the reducing agent, dithiothreitol was present during incubation at a concentration of 1 mM. The effect of cupric ion on sulfate uptake by membrane vesicles was similar for sulfate countertransport by membrane vesicles and uptake by intact lysosomes (data not shown).

3.6. Trypsin digestion

Brief exposure of intact lysosomes to trypsin resulted in an 82% decrease in sulfate uptake for the assay interval. This marked inhibition of uptake was completely prevented when trypsin inhibitor was present during exposure of lysosomes to trypsin. Incubation with trypsin inhibitor alone did not result in inhibition of transport. Lysosomal latency was not significantly affected by the exposure to trypsin, remaining approx. 80%.

3.7. Inhibition and membrane orientation

Similar to what had previously been observed in lysosomal membrane vesicles, pH-dependent sulfate uptake was demonstrated using intact Percoll density gradient purified lysosomes (data not shown). While lysosomal membrane vesicles have a random membrane orientation, intact lysosomes have a uniform membrane orientation with the bulk of glycosylated protein on the inner surface. Results of inhibition studies were similar in both preparations in that those substances that inhibited sulfate countertransport by membrane vesicles also inhibited sulfate uptake by intact lysosomes (Table 5). As observed for membrane vesicles, dipyridamole had no effect on sulfate transport by intact lysosomes. Lysosomal latency was unaffected by the inhibitors in the concentrations used in these experiments.

Table 5
Inhibition of sulfate uptake in intact lysosomes

Condition	Uptake (% control)
Control	100 ± 6
120 μM SITS	9 ± 5
50 μM DIDS	1 ± 1
300 μM dipyridamole	103 ± 17
900 μM NAP-taurine	14 ± 8
96 μM phloretin	39 ± 11
3 mM phloridzin	21 ± 2
600 μM pyridoxal 5-phosphate	35 ± 2
3 mM dinitrophenol	38 ± 12
60 μM niflumic acid	21 ± 4

Intact lysosomes were incubated for 1 min at 25° C in 50 μl of 0.25 M sucrose, 20 mM Mes, pH 5.0, 100 μM Na_2SO_4 with 1 μCi of $Na_2^{35}SO_4$. Lysosomes were collected by filtration on GF/C glass fiber filters and washed as described for membrane vesicles. Each value represents the mean ± S.D., $n = 3$.

4. Discussion

Our studies indicate that, with one notable exception, lysosomal sulfate transport is sensitive to a variety of known inhibitors of band 3 mediated anion transport. This is particularly interesting because studies of band 3 have indicated that these inhibitors have markedly different sites and mechanisms of action. The inhibitory effects of SITS, DIDS, dinitrofluorobenzene, and pyridoxal 5-phosphate all indicate that at least one unoccupied external lysine residue is critical for lysosomal sulfate transport. The effects of 1,2-cyclohexanedione and phenylglyoxal indicate that at least one and possibly two arginine residues are important for function of the lysosomal system. Phenylglyoxal binds preferentially to arginine residues with relatively low pK_a values such as are found in anion binding site of the band 3 protein [43]. Although there is general similarity between inhibition of the erythrocyte band 3 anion transporter and the lysosomal sulfate transporter, more specific differences in the patterns of inhibition are apparent. For instance, while both proteins are inhibited by DIDS, the band 3 protein is much more sensitive to the effects of this inhibitor than is the lysosomal sulfate transporter. In addition, tryptic digestion of the cytoplasmic side of the band 3 anion protein results in two protein fragments although the transporting ability of the protein is retained [44]. While the fragmentation pattern achieved with tryptic digestion of the lysosomal transporter is as yet uncertain, it is clear that such transport activity is greatly diminished by such treatment.

Membrane orientation is a factor that must be considered when assessing the effects of impermeant inhibitors on transport. Inhibition constants for impermeant compounds may vary from one side of the membrane to the other. For example, differential inhibition of erythrocyte band 3 chloride transport by DIDS and NAP-taurine is related to the membrane orientation of the protein [41,45]. Such effects are also possible for lysosomal sulfate transport since the lysosomal membrane is highly asymmetric as indicated by glycosylation patterns of the inner and outer surfaces. It is interesting to note that inhibition of sulfate transport by intact lysosomes is similar if not identical to inhibition of sulfate transport by vesicles with random membrane orientation. However, whether the lysosomal sulfate transporter is symmetrical in its patterns of inhibition cannot be determined from our studies. Investigation of this issue will require the development of techniques to control the membrane orientation of vesicles prepared from lysosomes.

Most of the divalent cations that were tested had either no effect or were slightly stimulatory with the exception cupric ion which had pronounced inhibitory effects on transport. One possible explanation for the effect of cupric ion relates to its ability to interact with sulfhydryl groups. Although *N*-ethylmaleimide had no notable effect on sulfate countertransport in membrane vesicles [1], it is possible that other sulfhydryl groups are accessible to cupric ion. Cupric ion is also recognized for its ability to interact with histidine residues but the fact that dithiothreitol partially reverses the effect of cupric ion suggests that the effect is due to sulfhydryl interaction. Interestingly, it has been suggested that copper is a substrate for transport by the erythrocyte anion transporter [46]. Additional studies will be required to further explore the relationship between cupric ion and lysosomal sulfate transport.

The information derived from these inhibitor studies indicate that the band 3 and lysosomal sulfate transporters have considerable functional similarities raising the possibility that these proteins may share sequence homology as well. Should this hypothesis prove to be correct, the lysosomal sulfate transporter would join the growing family of related proteins responsible for anion transport. However, the lysosomal and erythrocyte systems are clearly not identical as best demonstrated by their discrepant sensitivity to dipyridamole. Since study of inhibition is insufficient to resolve the nature of the relationship between these proteins, other techniques will be required. Molecular characterization will be critical for more complete understanding of the structure, processing and function of the lysosomal sulfate transporter and any possible relationship to the band 3 protein.

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