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Direct Measurement of Nitrite Transport Across Erythrocyte Membrane Vesicles Using the Fluorescent Probe, 6-Methoxy-*N*-(3-sulfopropyl) quinolinium

Richard Shingles,[1] Michael H. Roh,[1] and Richard E. McCarty[1]

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Nitrite was shown to quench the fluorescence of 6-methoxy-*N*-(3-sulfopropyl) quinolinium (SPQ) almost twofold more than chloride. SPQ loaded inside vesicles prepared from asolectin and isolated erythrocyte ghosts allowed for the direct measurement of nitrite movement across these membranes. Movement of nitrite across asolectin occurred by diffusion as HNO_2 in a pH-dependent manner. By contrast, erythrocyte ghosts had very low diffusion rates for nitrous acid. Erythrocyte ghosts preloaded with 50 mM nitrite to quench SPQ fluorescence were utilized to study heteroexchange with externally added anions. SPQ fluorescence increases (becomes unquenched) with added bicarbonate and nitrate, indicating that nitrite is moving out of the preloaded vesicles. The pH optimum for this exchange was approximately 7.6 and exchange was inhibited by *N*-ethylmaleimide (NEM) and dihydro-4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS). These data indicate that nitrite moves across erythrocyte plasma membranes as NO_2^- by a heteroexchange mechanism with other monovalent anions.

KEY WORDS: Nitrite transport; erythrocyte membranes; anion exchange.

INTRODUCTION

Acquired methemoglobinemia is the most common form of methemoglobinemia and follows exposure to drugs or toxins (Mansouri and Lurie, 1993). Nitrite has been reported to cause methemoglobinemia by the oxidation of hemoglobin to methemoglobin (Martin and Huisman, 1963). While the effects of nitrite on hemoglobin have been the subject of a number of investigations (Tomada *et al.*, 1981; Wallace *et al.*, 1982; Unnikrishnan and Rao, 1992), little is known about nitrite transport across the erythrocyte membrane.

Nitrite could move across biological membranes by one or a combination of mechanisms. The first is by simple diffusion of HNO_2 across membranes. In a recent study, utilizing chloroplast inner envelope membrane vesicles, movement of nitrite across these membranes was shown to occur by diffusion as nitrous acid (Shingles *et al.*, 1997). This movement was dependent upon a pH gradient (alkaline inside) across the membranes. A second mechanism for nitrite transport may involve a carrier or exchanger in which nitrite transport could occur by either a symport or antiport mechanism with other ions. Erythrocyte membranes have an anion exchange protein (AE1)[2] also known as band 3 (Fairbanks *et al.*, 1971) or capnophorin (Wieth and Bjerrum, 1983). The protein typically catalyzes the exchange of bicarbonate with chloride although it can exchange with other anions such as sulfate and phosphate at greatly diminished rates (Illsley and Verkman, 1987; Calafut and Dix, 1995). A third possible mechanism for nitrite transport could be through a membrane channel.

One of the methods used to measure bicarbonate/chloride exchange in erythrocyte ghosts is to follow the fluorescence of the halide-sensitive fluorophore, SPQ,

[1] Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218-2685.

[2] Abbreviations: AE1, anion exchange protein; DIDS, dihydro-4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; NEM, *N*-ethylmaleimide; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate; SPQ, 6-methoxy-*N*-(3-sulfopropyl) quinolinium.

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loaded into isolated membrane vesicles (Illsley and Verkman, 1987; Calafut and Dix, 1995). This method allows for the direct measurement of anion exchange with time resolution of less than 2 ms and it was shown that SPQ has minimal effect on the exchange activity itself (Illsley and Verkman, 1987). In this study the movement of nitrite across asolectin vesicles and erythrocyte ghosts, loaded with SPQ, were compared using stopped-flow spectrofluorometry. Movement of nitrous acid and heteroexchange of nitrite with other monovalent anions were measured in erythrocyte ghosts to determine the mechanism by which nitrite moves into erythrocytes.

MATERIALS AND METHODS

Materials

SPQ was purchased from Molecular Probes (Eugene, Oregon). Asolectin was obtained from Associated Concentrates (Woodside, New York). Erythrocyte ghosts were isolated from horse blood (Carolina Biological Supply, Burlington, North Carolina) according to the method described by Steck and Kant (1974) for preparation of right-side out vesicles.

SPQ Loaded Vesicles

For measurement of proton-linked nitrite movement SPQ-loaded vesicles were prepared by freeze/thawing 10–20 mg protein of erythrocyte ghosts or suspending 20 mg of asolectin in resuspension buffer consisting of 5 mM SPQ, 5 mM K-HEPES (pH 8.0), and 50 mM K_2SO_4. For anion exchange measurements vesicles were prepared in resuspension buffer consisting of 5 mM SPQ, 5 mM K-HEPES (pH 8.0) and 50 mM KCl, or 50 mM KNO_2. The SPQ-loaded vesicles were passed through a 1.5 × 10 cm Sephadex G-50 column equilibrated with resuspension buffer (minus SPQ). The eluted vesicles were then dialyzed against 250 ml of resuspension buffer using Spectra/Por membrane tubing with pore size of 6–8 kDa (Spectrum Medical Industries, Houston, Texas) at 4°C for 2 h prior to use to remove external fluorophore.

Fluorescence Measurements

An Olis modified SLM-SPF-500C spectrofluorometer and an Olis USA-SF stopped-flow apparatus (Bogart, Georgia) were used to measure SPQ fluorescence emission at 443 nm with excitation at 350 nm. All slits were set at 10 nm with an LP34 cutoff filter (Oriel, Stamford, Connecticut) placed over the entrance to the emission monochromator. Chamber A of the stopped-flow apparatus contained 2.0 ml of the vesicle suspension at pH 8.0. Chamber B contained 2.0 ml of buffer of a predetermined composition and pH in a manner such that the intravesicular osmotic and ionic strengths were closely balanced with those of the external medium. Mixing of samples was achieved by a nitrogen-driven piston at 80 p.s.i. All measurements were taken at 25°C.

Data Reduction and Handling

The rates of nitrite flux were calculated from the initial rate of change of SPQ fluorescence and the Stern–Volmer quench constant K. The quenching of SPQ fluorescence by nitrite is described by the relationship

$$F = F_0/(1 + K\,[NO_2^-]) \qquad (1)$$

where F_0 is the fluorescence intensity of intravesicular SPQ in the absence of nitrite and F is the fluorescence intensity of SPQ at equilibrium after mixing in the stopped flow apparatus. To obtain $d[NO_2^-]/dt$, Eq. (1) is differentiated with respect to time:

$$(dNO_2^-/dt)_{t=0} = -F_0[dF/dt\}_{t=0}/K(F^2) \qquad (2)$$

where $[dF/dt\}_{t=0}$ is estimated from the initial slope of a single exponential fitted to the first 1.5 s of the collected data. Curve fitting was carried out using the graphing program Kaleidagraph (Synergy Software, Reading, Pennsylvania). The correlation of the data to the fits was greater than 0.95 as determined by the least squares method. The initial rates of anion exchange are expressed as the absolute values in the tables and figures.

RESULTS

Direct Measurement of Nitrite

SPQ has been reported to be a chloride-sensitive fluorophore although its fluorescence is quenched by halides in general (Krapf *et al.*, 1988). The effect of nitrite on SPQ fluorescence emission was measured as

a function of nitrite concentration (Fig. 1). The linearity of the Stern–Volmer plot indicates that SPQ quenching by nitrite occurs by a collisional mechanism (Illsley and Verkman, 1987) with a quenching constant of 0.180 mM^{-1}. As expected, chloride also showed a linear quenching effect; however, the quenching constant was only 0.076 mM^{-1}. Bicarbonate and nitrate are ineffective quenchers of SPQ fluorescence.

Proton-Linked Nitrite Movement Measured with SPQ

To directly measure nitrite transport, experiments were conducted using SPQ-loaded asolectin vesicles and erythrocyte ghosts. When SPQ-loaded asolectin vesicles at pH 8.0 were mixed with pH 7.0 buffer in the presence of 3 mM KNO_2, a rapid decrease in SPQ fluorescence was observed during the first 1.5 s (Fig. 2). When the pH inside and outside of the vesicles was equivalent, there was no change in SPQ fluorescence (data not shown). In assays utilizing SPQ-loaded erythrocyte ghosts with an inward-directed proton gradient, little change in fluorescence was observed when KNO_2 was added (Fig. 2). The offset in initial fluorescence between asolectin and erythrocyte ghosts is likely due to small differences in SPQ loading and/or the presence of residual external SPQ. In our stopped-flow system any external SPQ would be quenched within the mixing time of the apparatus (~ 4 ms).

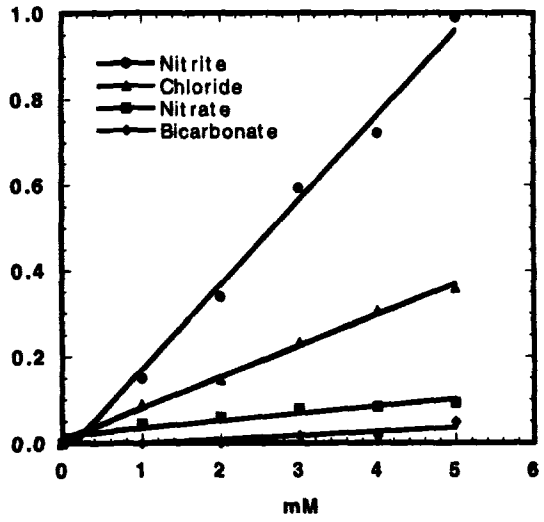

Fig. 1. Stern–Volmer plot of SPQ fluorescence quenching by various anions. The fluorescence of a solution of 1.5 μM SPQ and varying concentrations of anions was measured at 25°C. Data were fit to a linear function.

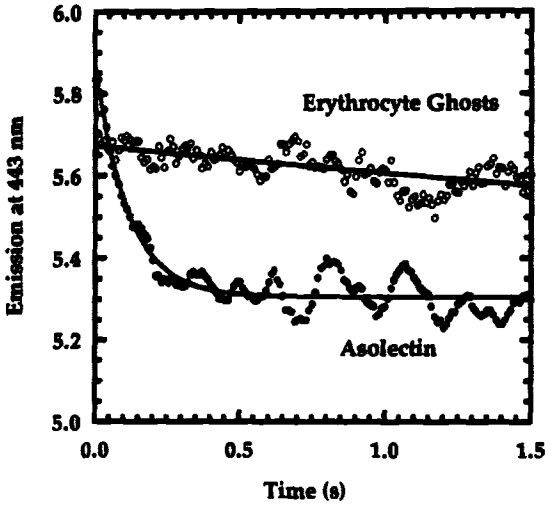

Fig. 2. Direct measurement of nitrous acid diffusion across asolectin and erythrocyte membranes. Asolectin and erythrocyte ghosts were loaded with 50 mM K_2SO_4, 5 mM K-HEPES (pH8.0), and 5 mM SPQ as described in Materials and Methods. These membrane vesicles were rapidly mixed with buffer at pH 7.0 in the presence of 3 mM KNO_2. The change in SPQ fluorescence was monitored for 1.5 s.

Measurement of Nitrite Exchange in Erythrocyte Ghosts

SPQ-loaded erythrocyte ghosts have been used to monitor chloride-bicarbonate exchange via the anion exchange protein (Illsley and Verkman, 1987; Calafut and Dix, 1995). Erythrocyte ghosts were loaded with 5 mM SPQ and 50 mM nitrite or 50 mM chloride (pH 8.0) and then mixed with 50 mM bicarbonate. Upon mixing the vesicles with bicarbonate, a rapid increase in SPQ fluorescence was observed over the first 0.5 s (Fig. 3). As intravesicular nitrite or chloride was exchanged for external bicarbonate, SPQ fluorescence was "unquenched." The extent of the fluorescence change was much greater for bicarbonate/nitrite exchange than for bicarbonate/chloride exchange, reflecting the differential sensitivities of SPQ quenching to nitrite and chloride. The initial rate of nitrate/nitrite exchange was similar to that of bicarbonate/nitrite exchange, indicating that nitrate can also exchange for nitrite (Table I).

pH Optimum for Anion Exchange

SPQ can be utilized to measure anion exchange at different pH values since this fluorophore is pH insensitive (Krapf *et al.*, 1988). The initial rates for bicarbonate/nitrite exchange were determined under

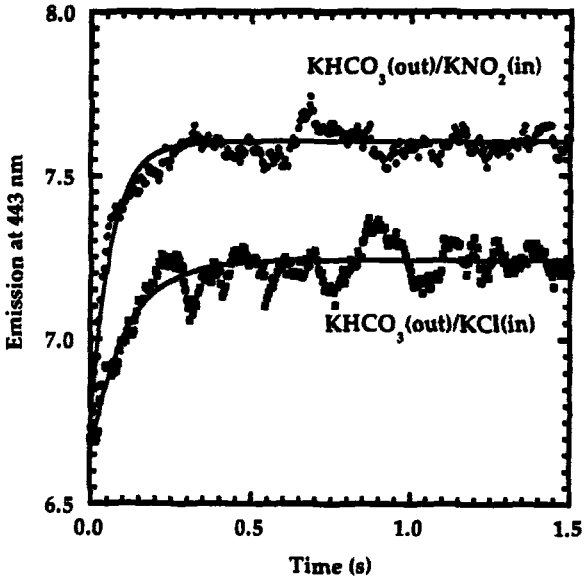

Fig. 3. Unquenching of SPQ fluorescence in erythrocyte ghosts. Erythrocyte ghosts (60 μg membrane protein) were loaded with 5 mM SPQ and either 50 mM KNO_2 or 50 mM KCl. Rapid mixing with 50 mM $KHCO_3$ using stopped-flow spectrofluorometry initiated the exchange of anions and resulted in the unquenching of SPQ fluorescence. Data were fit to the equation describing a single exponential rise (solid line).

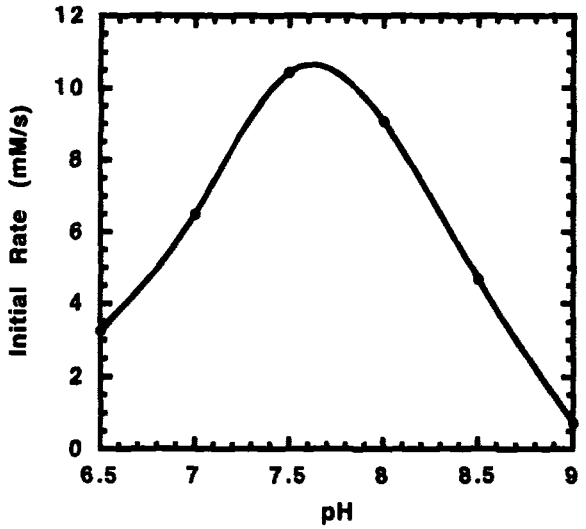

Fig. 4. pH dependence of anion exchange in erythrocyte ghosts. Experimental conditions were the same as for Fig. 3. Erythrocyte ghosts (60 μg membrane protein) were mixed with buffer at the desired pH for 10 min before being rapidly mixed with 50 mM $KHCO_3$ at the same pH. Initial rates of anion exchange were determined over the first 1.5 s after mixing as described in Materials and Methods.

different pH conditions. The results shown in Fig. 4 illustrate that the optimum for bicarbonate/nitrite exchange was approximately pH 7.6. At one pH unit above or below the pH optimum the initial exchange rate falls to less than one half the optimum initial rate.

Table I. Initial Rates of Anion Exchange in Erythrocyte Ghosts Preloaded with Nitrite[a]

	Initial rate (mM/s)
Bicarbonate	9.07
+ NEM	0.19
+ DIDS	0.25
Nitrate	9.50
+ NEM	0.16

[a]Erythrocyte ghosts (60 μg membrane protein) were loaded with 5 mM SPQ and 50 mM KNO_2 as described in Materials and Methods. Membrane vesicles were rapidly mixed with 50 mM potassium salts of bicarbonate or nitrate. Erythrocyte ghosts were preincubated for 30 min with either 2 mM NEM or 20 μM DIDS. Initial rates of anion exchange were determined from the spectrofluorometric traces as described in Materials and Methods.

Inhibition of Anion Exchange

The AE1 protein of erythrocytes is known to be sensitive to disulfonic stilbene inhibitors such as DIDS and SITS (Calafut and Dix, 1995). In addition there is some sensitivity of anion exchange to maleimides such as eosin maleimide (Liu and Knauf, 1993). In this study inhibitors from these two classes of compounds were utilized to determine if bicarbonate/nitrite and nitrate/nitrite exchange were also sensitive to inhibition. The bicarbonate/nitrite exchange rate was inhibited over 97% by the addition of 20 μM DIDS (Table I). In addition 2 mM NEM inhibited bicarbonate/nitrite exchange and nitrate/nitrite exchange by 98%.

DISCUSSION

SPQ is one of the few commercially available fluorophores that is sensitive to particular anions such as chloride (Illsley and Verkman, 1987; Verkman *et al.*, 1989). In this study we have demonstrated that SPQ fluorescence is also quenched by nitrite. The quenching constant determined from a Stern–Volmer plot (Fig. 1) is 0.180 mM^{-1}, more than twice that for chloride: 0.076 mM^{-1} in this study and 0.071 mM^{-1} measured by Calafut and Dix (1995). As

a result SPQ can be used as a very sensitive indicator for the nitrite ion.

SPQ entrapped within membrane vesicles has been used to directly measure chloride movement across phospholipid membranes (Verkman *et al.*, 1989). SPQ was loaded inside asolectin vesicles, a membrane containing no proteinaceous components, to demonstrate that this fluorophore can be used to measure nitrite formed from the dissociation of HNO_2 after diffusion across these membranes. The diffusion of HNO_2 occurs only when an inwardly directed proton gradient is applied across the membranes (Fig. 2), similar to the results obtained for chloroplast inner envelope vesicles (Shingles *et al.*, 1997) where the proton formed from the dissociation of nitrous acid was measured. In contrast erythrocyte ghosts show little evidence of nitrous acid diffusion across their membranes (Fig. 2). The diffusion of a compound across a lipid membrane will vary with the composition and structure of a membrane. Asolectin is composed largely of phospholipids and has low levels of other integral membrane components such as sterols. The chloroplast inner envelope is a membrane which also has a very low sterol content (Douce and Joyard, 1990; Uemura and Steponkus, 1997). Finkelstein and Cass (1967) showed that the addition of cholesterol to lecithin–decane bilayers decreased the permeability of small nonelectrolytes by reducing membrane fluidity. The plasma membrane of erythrocytes is known to have high levels of cholesterol and this may account for the fact that this membrane is relatively impermeable to HNO_2 diffusion.

By loading SPQ inside erythrocyte ghosts movement of nitrite across these membranes as a result of a carrier or exchange activity can be measured. Since nitrite transport across erythrocyte membranes was low in the presence of an inward-directed proton gradient (Fig. 2), it is unlikely that nitrite movement across these membranes is linked to proton-symport or hydroxyl-antiport activity. Even in the absence of an inwardly directed proton gradient there was no measurable movement of nitrite into the erythrocyte ghosts, which indicates that there was no "open" nitrite channel in these membranes (data not shown).

The erythrocyte plasma membrane has an anion exchange protein (AE1) which typically exchanges bicarbonate for chloride (for review see Jennings, 1989). Erythrocyte membranes were shown to have low chloride/sulfate exchange rates (Illsley and Verkman, 1987; Calafut and Dix, 1995), an observation which validates the use of sulfate-loaded vesicles in the diffusion studies. Over the time course of the diffusion experiments (1.5 s) very little nitrite/sulfate exchanged (Fig. 2).

Erythrocyte ghosts loaded with 50 mM nitrite to quench internal SPQ fluorescence show rapid exchange when 50 mM bicarbonate was added (Fig. 3), an indication that nitrite may be transported by a hetero-exchange mechanism. The initial rate of bicarbonate/ nitrite exchange was apparently higher than that for bicarbonate/chloride exchange, an observation which reflects the differential sensitivity of SPQ to nitrite and chloride. From the Stern–Volmer plot (Fig. 1) the initial rate of bicarbonate/nitrite exchange was determined to be 151 mM/s/mg protein and for bicarbonate/chloride exchange the initial rate was 152 mM/s/mg protein, which indicates that there is little overall difference in the exchange rates of nitrite versus chloride. For human erythrocyte membranes the initial rate of bicarbonate/ chloride exchange was calculated to be 400 mM/s/mg protein (Illsley and Verkman, 1987), a rate more than twice that measured in horse erythrocyte ghosts. The difference in the initial exchange rates might be related to variations in affinities or quantities of the anion exchange protein itself or might be due to different temperature and pH conditions under which the two studies were performed. For human erythrocytes monovalent halides show a broad maximum at pH 7.8 for anion exchange (Dalmark, 1975). In horse erythrocyte ghosts the initial bicarbonate/nitrite exchange rate also has a broad pH optimum with a similar maximum at pH 7.6 (Fig. 4). The presence of a pH maximum for bicarbonate/nitrite exchange would be indicative of this exchange occurring on a proteinaceous transporter such as AE1. Since nitrite also exchanged for nitrate at rates equivalent to bicarbonate/nitrite exchange (Table I) this indicates that nitrate may also be exchanged on the same transporter.

Nitrite exchange across the erythrocyte plasma membrane was further characterized using two classes of known inhibitors of the AE1 protein. Bicarbonate/ chloride exchange in erythrocyte plasma membranes is sensitive to disulfonic stilbenes such as SITS and DIDS (Illsley and Verkman, 1987; Calafut and Dix, 1995; Verkman *et al.*, 1989). In this study DIDS also inhibited bicarbonate/nitrite exchange (Table I), further suggesting that nitrite may also be carried on the AE1 protein. Sulfhydryl modification of cysteine residues of AE1 has been shown to have little role in anion exchange capabilities (Knauf and Rauthstein, 1971). In fact, changing all of the cysteine residues to serine residues had little effect on anion exchange of the reconstituted protein (Casey *et al.*, 1995). However, eosin maleimide has been shown to bind covalently to a lysine residue at

position 430 of AE1, a position not located at the substrate binding site but located close enough to other monomers of AE1 to prevent transport (Liu and Knauf, 1974). A similar effect could possibly account for the strong inhibition of bicarbonate/nitrite and nitrate/nitrite exchange observed when NEM was added to our erythrocyte ghost preparations (Table I). Taken together these results suggest that nitrite transport across erythrocyte membranes does occur by exchange with other monovalent anions such as bicarbonate, chloride, and nitrate probably via the AE1 protein.

The erythrocyte membrane requires the rapid entry and exit of bicarbonate and chloride to facilitate the removal of CO_2 from the tissues and its delivery to the alveoli. A membrane freely permeable to ions would require an energetically costly ion pumping system to balance the accumulation of ions (Purczeld *et al.*, 1978; Shingles *et al.*, 1997). Erythrocyte membranes have a selective membrane with low permeability to cations and a specialized system for rapid exchange of anions. Unfortunately the low specificity of the anion exchange system can result in the uptake of less desirable compounds which can have debilitating effects, such as the development of acquired methemoglobinemia in response to nitrite.

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