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Binding of nitrate to renal brush border membranes studied with ^{14}N nuclear magnetic resonance (NMR)

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Abstract

Renal brush border membranes contain several anion exchangers that may play a role in electrolyte transport and pH regulation. To help characterize the types of exchangers present and the binding properties of these membranes, the binding of nitrate (NO_3^-) to highly purified rabbit kidney brush border membrane vesicles was studied. The method is based on the binding induced quadrupole relaxation of the ^{14}N-NMR signal of nitrate [1,2]. Brush border membrane vesicles caused a relaxation of the ^{14}N-NMR nitrate signal which could be characterized by relatively high affinity sites, $K_D = 6.7 \pm 1.5$ mM, as well as nonspecific interactions with the membranes, $K_D > 150$ mM. The anion transport inhibitor 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS) inhibited $51 \pm 6\%$ ($n = 4$) of the relaxation due to the high affinity binding sites. The DNDS inhibition could be characterized by a K_i of 10–80 μM. Both bicarbonate and formate (HCO_2^-) were found to partially inhibit the high affinity induced relaxation, with maximal inhibition of $37 \pm 8\%$ ($n = 3$) and $30 \pm 2\%$ ($n = 3$), respectively. The inhibitory effects of saturating concentrations of bicarbonate and formate were non-additive, suggesting the existence of a stilbene sensitive exchanger that can bind nitrate, as well as both bicarbonate and formate. This study indicates the usefulness of this new method for further investigation of anion exchangers on these and other membranes.

Keywords: Anion binding; Bicarbonate; Brush-border membrane; Formate; Nitrate; NMR

1. Introduction

Renal brush border membranes have been studied in detail for the presence of anion exchangers and their role in electrolyte transport. Chloride-formate, chloride-oxalate, chloride-sulfate, and chloride-bicarbonate exchangers have been described in these membranes (for a review see Brenner and Rector [3]). These anion exchangers, by functioning in parallel with the Na-H antiporter, would effect net NaCl reabsorption.

Nuclear magnetic resonance (NMR) spectroscopy has been used in the past to study the interaction of ions with a variety of proteins, both soluble [4,5] and membrane bound [6–8]. We have developed an NMR method to study the binding of bicarbonate to the human erythrocyte anion transport protein (AE1) using the ^{14}N-NMR signal of nitrate [1,2]. The NMR method is based on the transverse relaxation induced by the interaction of an anion containing a quadrupole nucleus, with a protein binding site, producing a broadening of the solution resonance of the anion. A theoretical description of this phenomenon can be found in Drakenberg and Forsen [9]. Nitrate is known to be a substrate of AE1 [1,10], and of several anion transport proteins of renal brush border membranes [11–13]. Nitrate is also an excellent structural analog of bicarbonate [14]. Using the NMR method, the affinities of bicarbonate and nitrate to human AE1 were found to be equal [1], suggesting the use of nitrate as a model compound for studying bicarbonate binding to proteins.

Abbreviations: NMR, nuclear magnetic resonance; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; AE1, anion exchanger 1; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LW, linewidth; LB, linebroadening; CA, carbonic anhydrase.

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In the present study, the ^{14}N-NMR method was employed to examine the interaction of nitrate with highly purified rabbit brush border membrane vesicles. The protein and concentration dependencies of the interaction were characterized. Competition studies were performed in which the interaction of the stilbene inhibitor (DNDS), bicarbonate and formate with the NMR visible nitrate binding sites were examined.

2. Materials and methods

2.1. Materials

4,4′-Dinitrostilbene-2,2′-disulfonate (DNDS) was obtained from Alfa Products, Danvers, MA. KNO_3 and $KHCO_3$ were reagent grade. $KHCO_2$ was prepared from 80% H_2CO_2 and KOH. Bovine erythrocyte carbonic anhydrase (CA), approx. 95% pure, and acetazolamide were obtained from Sigma, St. Louis, MO.

2.2. Renal brush border vesicle preparation

Purified brush border membranes from renal cortex were prepared from New Zealand white rabbits using differential and gradient centrifugation with ionic precipitation techniques as previously described [15]. Brush border membranes were enriched 10–15-fold in alkaline phosphatase activity with less than 10% cross contamination by basolateral membranes.

2.3. Linebroadening experiments

For the linewidth dependence on nitrate concentration, the initial sample contained 2.0 ml of vesicles added to 2.0 ml of a solution containing 6 mM KNO_3 and 5 mM Hepes (pH 8.0). The protein concentration in this sample was 11.4 mg/ml. After an NMR spectrum was obtained, the nitrate concentration was increased by the stepwise addition of small amounts of 3.0 M KNO_3. At the highest concentration of nitrate, 150 mM, the sample volume was 4.40 ml. The final pH of all the samples were measured using a Corning Orion 150 pH meter. The protein concentration in the samples was determined using the modified Lowry assay [16].

For the concentration dependence of DNDS inhibition study, the initial sample contained 2.0 ml of vesicles added to 2.0 ml of a solution containing 30 mM KNO_3 and 10 mM Hepes (pH 8.0). After each NMR spectrum was obtained, the DNDS concentration was increased by addition of small amounts of a 100 mM DNDS solution. At the highest concentration of DNDS, 1 mM, the total volume was 4.10 ml.

For the binding inhibition studies at fixed inhibitor concentration, each sample consisted of 3.0 ml of vesicles added to 1.0 ml of a solution containing 30 mM KNO_3 and 50 mM Hepes (pH 8.0). Some samples contained an additional 150 mM $KHCO_3$ or $KHCO_2$ (pH 8.0). An NMR spectrum was obtained and then concentrated solutions of DNDS, bicarbonate or formate were added to obtain 1 mM DNDS or an additional 150 mM of either bicarbonate or formate. The bicarbonate solution contained 2.0 M $KHCO_3$ and 7.5 mM KNO_3 (pH 8.0). The formate solution contained 3.0 M $KHCO_2$, 15 mM Hepes and 7.5 mM KNO_3 (pH 8.0). The DNDS solution contained 40 mM DNDS, 15 mM Hepes and 7.5 mM KNO_3 (pH 8.0).

2.4. Nuclear magnetic resonance (NMR) methods

Spectra were obtained using a Nicolet Magnetics Corporation NMC-200 WB spectrometer equipped with a 12 mm midrange probe operating at 14.45 MHz for ^{14}N. The spectral window was 750 Hz. Single 90° excitation pulses were followed by data acquisition into 512 or 1024 points. Each spectrum was signal averaged from between 512 to 42 000 transients, with an interval of 172 ms between transients. All studies were done at 12° C without any sample spinning.

The spectra were zerofilled and artificial linebroadening was added prior to Fourier transformation. The added linebroadening was between 4 and 40 Hz. After Fourier transformation the spectra were phased and the baselines were corrected. The linewidth at half maximum height (LW) for the nitrate resonances were determined using the NMCCAP program on the spectrometer to manually fit a lorentzian or gaussian lineshape.

2.5. Statistical analysis

The nitrate and DNDS concentration dependence data were fit using the non-linear least squares regression program *Inplot* (Graphpad Software, San Diego, CA). The standard errors derived from these fits are estimates provided by the program. For the binding inhibition studies, at fixed inhibitor, the data were expressed as means ± S.E.

3. Results

3.1. Effect of brush border membranes on nitrate NMR signal

The linewidth of the NMR signal is defined as the width of the signal in Hz at one-half the maximum peak height. The spectra of 10 mM nitrate in solution is shown in Fig. 1 as the solid line. The actual linewidth shown is about 19 Hz. This 19 Hz represents the true natural linewidth of the solution nitrate, 4 Hz, in addition to 15 Hz

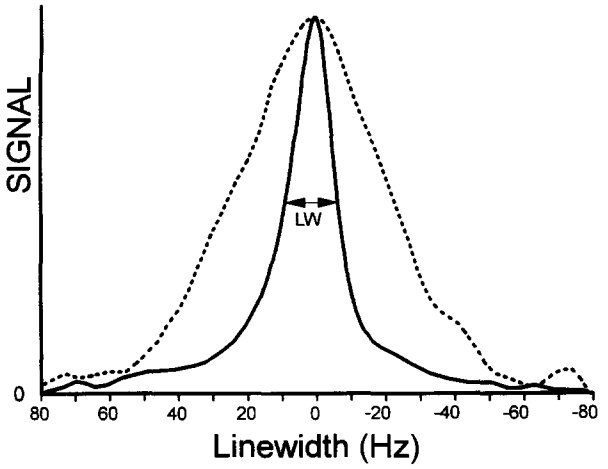

Fig. 1. Superimposed ^{14}N-NMR spectra of 10 mM KNO_3 at 12° C, pH 7.5, with and without brush border vesicles at 16.1 mg/ml protein concentration. Both spectra contain 15 Hz of computer added artificial linewidth for smoothening.

of computer added linewidth that is used to smooth the spectra for fitting. Brush border membranes alone had no NMR signal as would be expected due to the low free nitrate concentration and the wide linewidth of the nitrogen contained in the membranes (data not shown). Addition of brush border vesicles to the nitrate increased or broadened the linewidth by roughly 30 Hz, shown in Fig. 1 as the broken line. (The spectrum in the presence of the brush border vesicles also contains 15 Hz of computer added linewidth.) The spectra were obtained at 12° C and a pH of 7.5. The increase in the linewidth due to the presence of the vesicles, or 'linebroadening' (LB), is not affected by the amount of computer added linewidth used to assist in fitting.

The linebroadening of the NMR spectrum was studied as a function of brush border vesicle protein concentration at fixed nitrate concentrations of 10 mM and 150 mM, as depicted in Fig. 2. The linebroadening was linearly related to the vesicle protein concentration, with correlation coefficients, r, of 0.990 and 0.982 for 10 mM and 150 mM, respectively. The linebroadening was found to be 1.78 ± 0.07 Hz/(mg/ml) and 1.03 ± 0.06 Hz/(mg/ml) for 10 mM and 150 mM, respectively. The linear relationship between the linebroadening and protein concentration is based on theoretical grounds [9] and has been found for the ^{35}Cl [8] and ^{14}N nitrate [1] interactions with human AE1.

3.2. Effect of nitrate concentration on linebroadening

The dependence of the linebroadening on the nitrate concentration was studied at a fixed amount of vesicle protein by stepwise additions of 3.0 M KNO_3, to a single sample. Due to the volume of added nitrate, the vesicle protein concentration in the sample was not constant. Because the linebroadening is a linear function of the protein concentration, the change in sample volume results in a proportional decrease in the measured linebroadening. This decrease was adjusted for by multiplying each linebroadening by the ratio of the sample volume to the initial sample volume.

The effect of the nitrate concentration on the linebroadening is shown in Fig. 3. The experiment was conducted

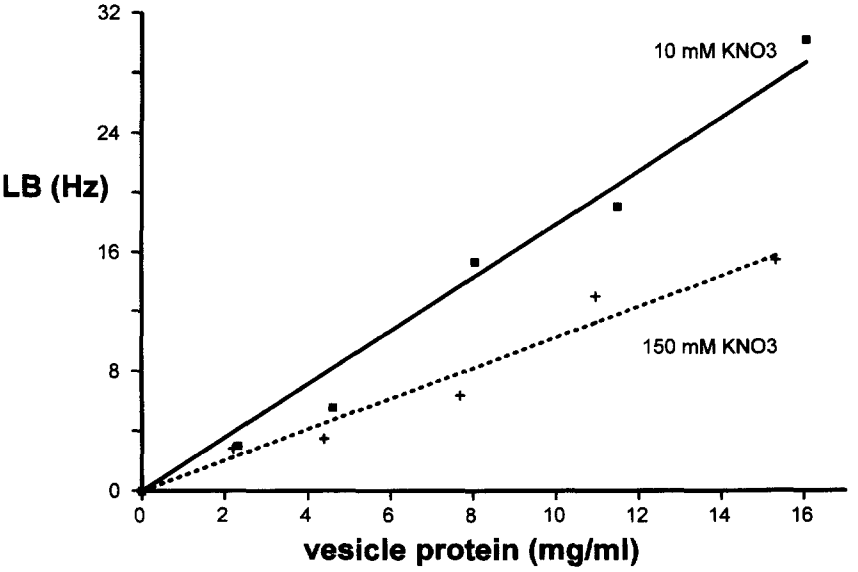

Fig. 2. The linebroadening of the ^{14}N nitrate resonance as a function of brush border vesicle protein concentration at 10 mM and 150 mM nitrate concentrations. The linebroadening is proportional to the protein concentrations with slopes of 1.78 ± 0.07 Hz/(mg/ml) and 1.03 ± 0.06 Hz/(mg/ml) for the 10 mM and 150 mM nitrate, respectively. The correlation coefficients, r, are 0.990 and 0.982 for the 10 mM and 150 mM nitrate, respectively. The pH 7.5 and the temperature 12° C.

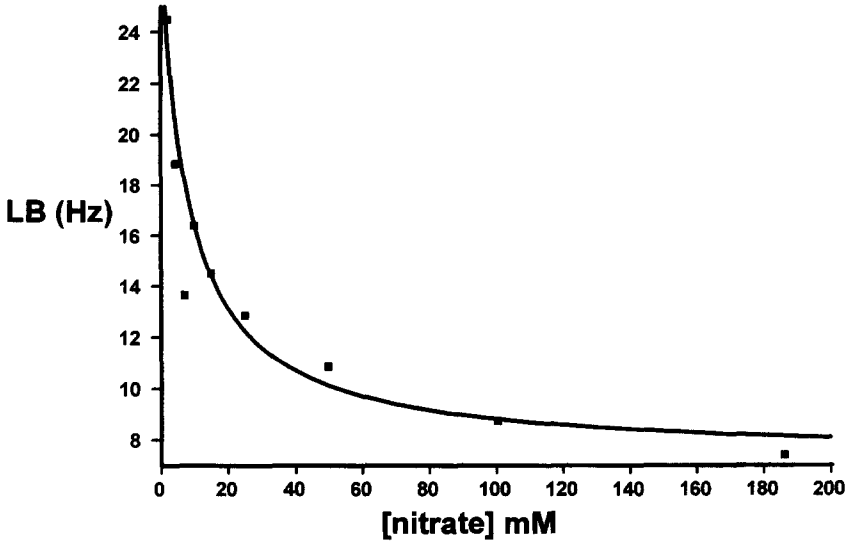

Fig. 3. The linebroadening of the ^{14}N nitrate resonance as a function of $[NO_3^-]$. The vesicle protein concentration was 11.4 mg/ml. The linebroadening is expressed as Hz. The solid line represents the nonlinear least squares best fit of the data to Eq. (1), $LB = LB_{non\text{-}spec} + LB_{spec}/(1 + [NO_3^-]/K_D)$, with $r = 0.989$. The pH was 7.5 and the temperature 12° C.

between 3 mM and 185 mM nitrate at 12° C. The linebroadening smoothly decreased as a function of nitrate concentration to an asymptotic value. At concentrations as high as 185 mM nitrate, a certain amount of linebroadening persists. This linebroadening is most likely due to low affinity non-specific interactions with the protein or lipid in the sample. The portion of the linebroadening above the asymptotic value is considered the high affinity or specific portion of the linebroadening, and is defined as that which is inhibitable by 150 mM nitrate. The value of 150 mM was chosen empirically and represents a physiologic ionic strength and osmolarity. As shown in Fig. 3, the linebroadening is near its asymptotic value at 150 mM nitrate.

Based on previous ^{35}Cl [8] and ^{14}N nitrate [1] studies,

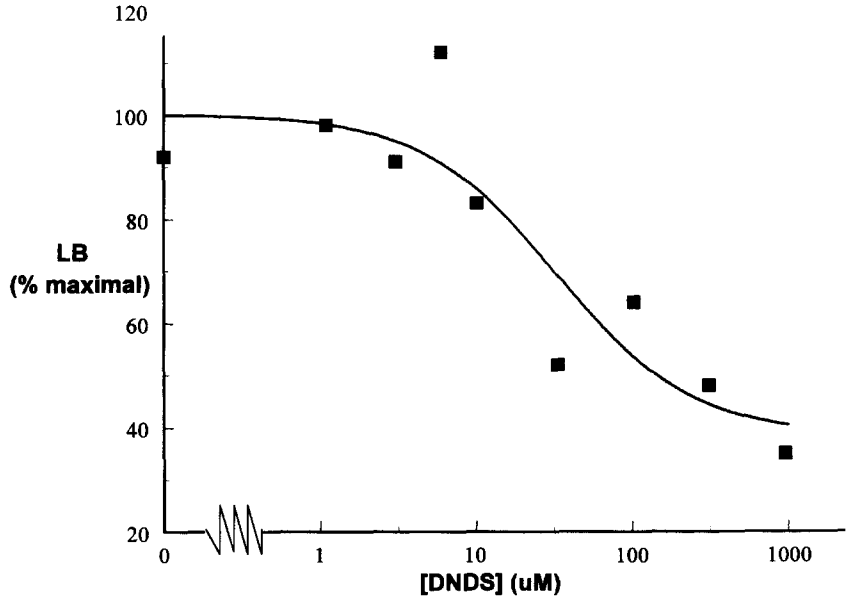

Fig. 4. The percent maximal linebroadening of the ^{14}N nitrate resonance as a function of [DNDS]. The nitrate concentration was 15.0 mM. The solid line represents the nonlinear least-squares best fit of the data to a inhibition curve with $K_i = 10–80$ μM and $r = 0.905$. The pH was 7.5 and the temperature was 12° C.

the concentration dependence of the linebroadening due to the interaction with the binding sites on the membranes is expected to follow Eq. (1):

$$LB = LB_{non-spec} + LB_{spec(max)}/(1 + [NO_3]/K_D) \quad (1)$$

Where LB is the total linebroadening, $LB_{non\text{-}spec}$ is the asymptotic value present at high concentrations of nitrate. $LB_{spec(max)}$ is the specific linebroadening interpolated to its maximum value at zero nitrate concentration and K_D is the binding constant of the interaction. A non-linear least-squares regression analysis of the data gave a K_D of 6.7 ± 1.5 mM, with an r value of 0.989. The fit is shown as the solid line in Fig. 3.

3.3. Effect of carbonic anhydrase (CA) on nitrate NMR signal

The linebroadening from purified bovine carbonic anhydrase (CA) was studied in order to investigate the possibility that the specific linebroadening from the vesicles may be in part due to the interaction of nitrate with CA. An NMR spectrum was obtained at a CA concentration of 2.7 mg/ml with 10 mM nitrate at 12° C and pH 7.5. The CA used was reported as 95% pure by weight. The linebroadening was found to be 1.06 Hz/(mg/ml) and was roughly 60% inhibited by 1 mM acetazolamide (data not shown).

3.4. Effect of DNDS, formate and bicarbonate on linebroadening

The specific linebroadening from the vesicles may be due to more than one nitrate binding site on the membrane. The K_D determined probably represents an aggregate weighted average of the affinity of all the binding sites. To help characterize these binding sites, competition and inhibition studies were conducted. The dependence of the linebroadening on the concentration of DNDS was studied at fixed nitrate, 15 mM, and vesicle protein concentration at 12° C and pH 7.5. The DNDS concentration was varied by additions of 100 mM DNDS. The change in sample volume was adjusted for as previously discussed. The results of this study are shown in Fig. 4. A non-linear least-squares regression analysis of the data (see Materials and methods) produced a K_i of 10–80 μM, with an r value of 0.905. Under these conditions, the DNDS roughly inhibited two thirds of the total linebroadening. The fit is shown as the solid line in Fig. 4.

The degree of inhibition of the specific linebroadening by fixed concentrations of formate, bicarbonate and/or DNDS was examined. Samples were prepared with 7.5 mM nitrate and fixed concentrations of vesicle protein in addition to 150 mM formate, 150 mM bicarbonate, both 150 mM bicarbonate and 150 mM formate, or 1 mM DNDS. After NMR spectra were obtained, 3.0 M KNO_3 was added to obtain a nitrate concentration of 150 mM and an additional NMR spectra was obtained. All spectra were obtained at 12° C with pH values between 7.5 and 7.8. The specific linebroadening was defined as the difference between the linebroadening at 7.5 mM nitrate and at 150 mM nitrate. As discussed previously, at 150 mM nitrate, the linebroadening is near its asymptotic value and the specific linebroadening is nearly eliminated. The lower value of 7.5 mM was chosen empirically as a compromise between the higher linebroadening present at low concentrations of nitrate versus the superior signal-to-noise ratio (S/N)

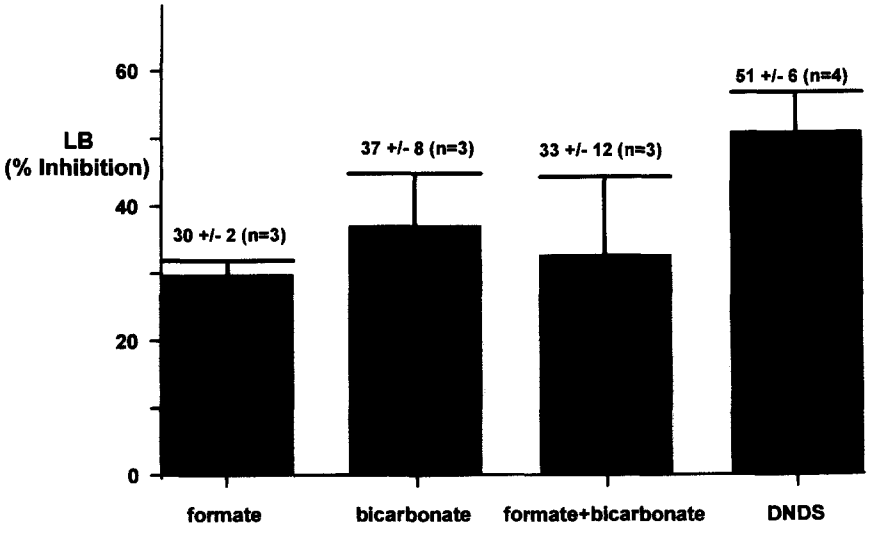

Fig. 5. The percent inhibition of the specific linebroadening of the ^{14}N nitrate resonance as a function of competitor. The specific linebroadening is defined as the difference in the linewidth between NO_3^- concentrations of 7.5 mM and 150 mM. The DNDS concentration was 1.0 mM. The formate and bicarbonate concentrations were 150 mM. The data represent an average value of n experiments denoted on the graph. The protein concentration varied, but is normalized by expressing the data as a percent of specific linebroadening inhibited. The pH varied between 7.5 and 7.8 and the temperature was 12° C.

which is obtained at higher concentrations of nitrate. The results are shown in Fig. 5.

The presence of 1 mM of the anion transport inhibitor DNDS inhibited $51 \pm 6\%$ ($n = 4$) of the specific linebroadening. Both formate and bicarbonate were incomplete inhibitors at concentrations of 150 mM. The inhibition caused by formate, $30 \pm 2\%$ ($n = 3$) was similar to that caused by bicarbonate, $37 \pm 8\%$ ($n = 3$). The inhibition due to the presence of both formate and bicarbonate at 150 mM concentration, $33 \pm 12\%$ ($n = 3$) was no greater than the inhibition produced by either anion alone, indicating that the effects were not additive.

4. Discussion

Highly purified rabbit brush border vesicles caused both specific and non-specific linebroadening of the ^{14}N-NMR signal of nitrate. The specific linebroadening indicates the existence of membrane bound protein binding sites for nitrate. This is consistent with previous reports of the ability of these membranes to transport nitrate [11–13]. The nitrate linebroadening was much less robust than previously reported for human erythrocyte membranes [1]; roughly a factor of ten smaller per mg/ml of protein. This difference is most likely due to the fact that AE1 accounts for around 25% of the erythrocyte membrane protein [17], much more abundant than the portion of vesicle membrane protein dedicated to anion transport.

Due to structural similarity between nitrate and bicarbonate, it was reasonable to suspect that nitrate might interact with carbonic anhydrase (CA), which is present in the vesicles. CA produced nitrate linebroadening which was partially inhibited by acetazolamide. Using a value found for the human kidney, the membrane bound CA present in washed microsomes represents about 0.1% of the total membrane protein by weight [18]. At the protein concentrations used in our studies, this would correspond to a few hundredths of mg/ml of CA. Based on the CA linebroadening that we determined, CA would produce on the order of a 0.01 Hz of linebroadening in our studies. Although this estimate is rough, the linebroadening from CA was most likely negligible in our studies.

The average affinity of nitrate for these NMR visible binding sites was approximately equal to that found for the human Cl^-/HCO_3^- exchanger, AE1 [1], 6.9 ± 0.8 mM. The precision of the data fit to Eq. (1) (which assumes a single binding site), cannot exclude the possibility of multiple NMR visible binding sites. Future experiments using NMR instrumentation with better signal-to-noise characteristics, as well as a greater number of data points may be useful in discriminating the actual number of NMR visible nitrate binding sites.

The results indicate the existence of a class of transport proteins that bind all three substrates; nitrate, formate and bicarbonate, as well as DNDS. The DNDS inhibition curve shows that a large portion of the nitrate binding is stilbene sensitive in the range of 10–100 μM. The Cl-oxalate [13], SO_4-CO_3 [12] and Cl-formate [11] exchangers are all known to transport nitrate, but only the Cl-oxalate and SO_4-CO_3 exchangers are inhibited strongly by DNDS [19]. The K_i of the DNDS inhibition of Cl-oxalate and SO_4-CO_3 exchangers are both in the range of 10–100 μM [19]. These data are consistent with the possibility that the binding sites examined in this study may be present on the Cl-oxalate and SO_4-CO_3 exchangers.

Since the inhibition of the linebroadening by saturating concentrations of formate or bicarbonate were not additive, the NMR visible nitrate binding sites that are inhibited by formate are likely to be the same as those inhibited by bicarbonate. Therefore, a transporter which binds both nitrate and formate, but not bicarbonate, is not detected by our study. It is important to emphasize that this study only examines binding, not transport. A protein which binds all three substrates, but only transports one or two of them can be accounted for by our data. For example, it is possible that formate and bicarbonate may compete for binding with nitrate on the Cl-oxalate exchanger, although they may not be substrates for transport. Likewise, formate may compete with nitrate for binding to the SO_4-CO_3 exchanger even though it is not reported to be a transport substrate.

A portion of the nitrate binding in not inhibited by any of the compounds studied; DNDS, formate or bicarbonate. The identification of these binding sites cannot be determined from the results of the present study. These non-inhibited sites may represent partial inhibition by the competitors studied of the known exchangers, or they may represent an interaction of nitrate with proteins not yet described.

The results presented introduce the method of ^{14}N nitrate NMR as a viable technique for studying the binding properties of anions to brush border membranes. Through competition studies, a wide range of anions can be studied as long as the anion of interest shares a binding site with nitrate. The technique has drawbacks, however, the most important being the lack of sensitivity and the large amount of protein required for good signal-to-noise. This problem can be minimized in the future by using NMR spectrometers with greater field strengths, producing spectra with better signal-to-noise, while requiring smaller samples and less time required for measurement.

This linebroadening method is also limited because no direct observations can be made regarding the transport of the substrates being studied. On the other hand, transport studies only measure transport and direct information regarding binding is usually not obtained. Thus, these binding studies can provide information complementary to that provided by transport studies.

This study has shown that the ^{14}N-NMR linebroadening method can be used to detect nitrate binding to renal brush border membranes. The concentration dependence of the

interaction of nitrate with these membranes was determined to be 6.7 ± 1.5 mM. DNDS, formate and bicarbonate were all found to be partial inhibitors of the nitrate binding. The pattern of the inhibitory effects of these compounds suggests the presence of stilbene sensitive membrane exchange proteins which bind nitrate, bicarbonate and formate. These binding sites may represent one or both of the Cl-oxalate and SO_4-CO_3 exchangers. In the future, the use of more powerful NMR instrumentation will allow for more thorough evaluation of the concentration dependence of both the nitrate interaction with the membranes as well as the competition produced by other anions and inhibitors. Evaluation of the pH dependence of the linebroadening may also be of interest. These types of studies will help to characterize the types of binding sites present and their substrate and inhibitor specificity. This technique should also be of use in the evaluation and characterization of the anion binding proteins on a variety of other physiologically significant membranes.

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