Production and characterization of monoclonal antibodies directed against native epitopes of NhaA, the Na^+/H^+ antiporter of *Escherichia coli*

Etana Padan[a], Miro Venturi[b], Hartmut Michel[b], Carola Hunte[b,*]

[a] *Division of Microbial and Molecular Ecology, The Hebrew University of Jerusalem, Jerusalem, Israel*
[b] *Max-Planck Institut für Biophysik, Abteilung Molekulare Membranbiologie, Heinrich-Hoffmann-Straße 7, D-60528 Frankfurt/M, Germany*

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Abstract Monoclonal antibodies (mAbs) recognizing native epitopes are an important tool for functional and structural studies of proteins, yet they have rarely been used with transport proteins. In an attempt to raise monoclonal antibodies against the NhaA Na^+/H^+ antiporter of *Escherichia coli* we encountered difficulties in the screening procedure, which is based on the standard enzyme-linked immunosorbent assay (ELISA). Here we report a rapid and efficient method of screening for anti-NhaA mAbs which recognize native epitopes of the antiporter. The method is based on the use of His-tagged protein, Ni^{2+}-nitrilotriacetic acid coated plates and non-denaturing conditions in the assay. With this procedure four mAbs were obtained, three of which recognize the NhaA in its native conformation and one preferentially recognizes the denatured form. The latter mAb is Western blot positive, the others are Western blot negative and bind the detergent solubilized NhaA as assayed by gel filtration chromatography. Competition experiments show that the native epitopes are common to both the His-tagged and the wild-type protein. We suggest that in the standard ELISA the NhaA protein is not presented to the antibody in the native conformation whereas the His tag based protocol favors this presentation. Indeed, we could remarkably improve the low reactivity of the standard ELISA by coating the plates with anti-NhaA mAb and use it to present NhaA ('sandwich' ELISA or two antibodies assay). Remarkably, two of the mAbs (5H4, 2C5) which bind native NhaA inhibit drastically the ΔpH driven ^{22}Na uptake mediated by His-tagged NhaA reconstituted in proteoliposomes. Hence, these mAbs afford a new tool to study the structure/function relationship of the antiporter.

Key words: Active transport; Na^+/H^+ antiporter; NhaA; Monoclonal antibody; *Escherichia coli*

1. Introduction

Sodium/proton antiporters are ubiquitous membrane proteins found in the cytoplasmic and organelle membranes of cells of many different origins, including plants, animals and microorganisms. They are involved in cell energetics and play primary roles in the regulation of intracellular pH, cellular Na^+ content and cell volume (review in [1]).

Escherichia coli has two antiporters, NhaA [2,3] and NhaB [4], which specifically exchange Na^+ or Li^+ for H^+ [1]. NhaA is indispensable for adaptation to high salinity, for challenging Li^+ toxicity and for growth at alkaline pH (in the presence of Na^+ [3]). Both NhaA and NhaB are electrogenic antiporters which have been purified to homogeneity and reconstituted in a functional form in proteoliposomes [5–7]. The H^+/Na^+ stoichiometry of NhaA is $2H^+/Na^+$ [7] and that of NhaB is $3H^+/2Na^+$ [6]. The activity of NhaA is drastically dependent on pH. Between pH 6 and 8 the V_{max} changes by over three orders of magnitude and the protein undergoes conformational changes [5,8,9]. Accordingly, the expression of *nhaA* which is dependent on NhaR is induced by Na^+ in a pH dependent manner [10].

Similar to other transport proteins which use the energy of electrochemical ion gradients for active transport, the three-dimensional structure of NhaA is completely unknown. Although many of these integral membrane proteins have now been overexpressed and purified, neither 3D nor 2D crystals of these proteins were obtained to allow structural analysis with either X-ray or electron diffraction, respectively.

There are inherent problems in generation of crystals from membrane proteins because they are soluble only in detergent solutions. The detergent covers hydrophobic parts of the membrane protein surface. Polar regions needed for protein-protein contacts to establish a three-dimensional crystal lattice are comparatively small and are partially covered by polar headgroups of the detergents. In addition, it has been suggested that transport proteins exist in multiple conformations needed for translocation of substrates, a detrimental property for crystal formation.

A novel approach [11,12] recently led to the successful crystallization of cytochrome *c* oxidase from *Paracoccus denitrificans* and to the determination of its high resolution structure. In this approach monoclonal antibodies (mAbs) were produced against a native conformation of this enzyme. Subsequently, the DNA encoding the corresponding F_v fragment was cloned, overexpressed and the F_v fragment purified and used for co-crystallization. This fragment is critically involved in the crystal packing. Additionally, F_v fragments may have the capacity of locking the membrane protein in one conformation. This approach raises hopes regarding crystallization of other integral membrane proteins such as transporters including the NhaA antiporter.

We have previously shown that NhaA can be very efficiently overexpressed from the *tac* promoter. When fused at its C-terminus to six histidines (His-tagged NhaA) the trans-

*Corresponding author. Fax: (49) (69) 96769-423.
E-mail: hunte@biophys.mpg.de

Abbreviations: mAb, monoclonal antibody; LM, dodecyl-β-D-maltoside; OG, *N*-octyl-β-D-glucopyranoside; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Ni^{2+}-NTA, Ni^{2+}-nitrilotriacetic acid; PL, phospholipids; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPES, *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid]; PBS, phosphate buffered saline; RT, room temperature

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porter is fully active and is readily purified by affinity chromatography using a Ni^{2+} column [13]. We therefore used this His-tagged NhaA to produce monoclonal antibodies recognizing the antiporter in its native conformation.

Monoclonal antibodies have been raised against numerous membrane proteins and they are a versatile tool for their biochemical and structural characterization. If the membrane protein of interest can be solubilized and purified in an active form in milligram amounts, these protein preparations can be used for immunization as well as for screening purposes based on the enzyme linked immunosorbent assay (ELISA) to raise monoclonal antibodies [14,15]. However, antibodies selected via conventional ELISA screening often recognize nearly exclusively the denatured protein as e.g. formed under the conditions used in Western blot analysis [16,15]. In a typical experiment 35 Western blot positive mAbs recognizing the denatured form of the yeast cytochrome bc_1 complex and only one recognizing the native protein were obtained (C. Hunte and H. Michel, unpublished results). This bias towards mAbs recognizing the denatured protein might be due to the ELISA. Although less harsh in comparison to Western blot analysis which involves SDS denaturation, in the ELISA protocol the antigen is directly bound to the polystyrene matrix under alkaline conditions. This binding to the solid phase is known to cause partial denaturation of proteins [17–19]. To obtain conformation specific monoclonal antibodies against the NhaA antiporter we developed an efficient modified ELISA technique. This approach utilizes plates coated with Ni^{2+}-NTA resin. The plates are decorated with the His-tagged NhaA under physiological conditions allowing the presentation of the antigen in a native conformation. Thereby, three out of the four mAbs obtained recognized only the native protein. Two of the mAbs binding to the native protein inhibit drastically Na^+/H^+ antiporter activity.

2. Materials and methods

2.1. Purification of NhaA

His-tagged NhaA and wild-type NhaA were purified [5,13] and stored in aliquots at −80°C in 20% glycerol.

2.2. Production of monoclonal antibodies

Female BALB/c mice (Harlan/Winkelmann) were immunized by intraperitoneal injection of 100 μg purified His-tagged NhaA antiporter protein, mixed with the same volume (100 μl) of adjuvant (ABM-2, PAN-SYSTEMS). Blood sera, taken 10 days later, showed positive signals specific against NhaA, in an ELISA (Section 2.5). Boosting was performed three times at 4 week intervals. After the final boosting, the spleen was removed aseptically and the spleen cells were fused with P3/NSI/1-Ag4-1 (DSM ACC145) myeloma cells by using 50% polyethylene glycol 1500 (Boehringer-Mannheim). Hybridoma cell selection was performed with hypoxanthine and azaserine. The cells were cultured and cloned according to standard protocols [20,21].

2.3. Immunological sub-typing

The immunological sub-type of each monoclonal antibody was determined with the help of an immunological sub-typing kit (Boehringer Mannheim).

2.4. Purification of mAbs

60 ml of hybridoma supernatants were concentrated using 100 kDa cut-off Centriprep filters (Amicon, Beverly, MA, USA) to approximately 10 ml at 4°C and then titrated to pH 5. The mAbs were purified on a protein G affinity column (Pharmacia) pre-equilibrated with 5 column volumes of 0.1 M Na^+-acetate buffer (pH 5) and washed with 5 column volumes of the same buffer. Elution was carried out in 0.1 M glycine-HCl (pH 2.8). The mAbs were immediately buffered to neutral pH, concentrated by ultrafiltration (Centricon, 50 kDa cut-off, Amicon) at $4000 \times g$, diluted 1/100 in PBS and stored at 4°C.

2.5. Standard ELISA assay

For the standard ELISA assay [21], 96 well polystyrene plates (Greiner) were coated with NhaA (12 μg/ml in 0.2 M Na_2CO_3 coating buffer, pH 9.8) for 1 h at RT. Washing steps were performed using a saline solution containing 0.9% NaCl, 0.1% Tween-20. Blocking was done with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% BSA. Blood serum, hybridoma supernatants or purified mAbs diluted in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% BSA were added (100 μl/each well) and incubated for 45 min. For probing the bound antibodies, a secondary antibody anti-mouse IgG conjugated to alkaline phosphatase (whole molecule, Sigma) was used with a dilution of 1:1000 in the blocking buffer. The color reaction was developed with *p*-nitrophenylphosphate (1 mg/ml) in buffer containing 10% (w/v) diethanolamine, pH 9.8 and 0.5 mM $MgCl_2$. After 30 min of incubation at RT, the signals were measured with an ELISA reader at 405 nm, using 450 nm as reference wavelength.

2.6. His tag based ELISA

His-tagged NhaA was bound to Ni^{2+}-NTA coated 96 well microtiter plates (Qiagen) using a final concentration of 12 μg/ml in coating buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% (w/v) LM (Calbiochem)) and gently shaking the plates at RT for 45 min. Washing steps were performed with 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.03% (w/v) LM and 0.1% (w/v) BSA. Ni^{2+}-NTA coated plates do not need any blocking since they are pre-blocked by the manufacturer. The washing buffer was also used for dilution and subsequent incubation of purified mAbs on the plates. All other steps were performed as described in the standard ELISA protocol.

2.7. Sandwich assay (two antibodies assay)

For this assay, the IgG1κ-type mAb 2C5 (isolated in this study) was bound to polystyrene 96 well microtiter plates (Greiner) according to the standard ELISA protocol. His-tagged NhaA was then diluted in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% (w/v) LM buffer and incubated for 45 min on these plates to allow binding of the proteins. Washing steps were performed with the same buffer. A second mAb isolated in this study (5H4 or 6F9; of IgG2b or IgG2a sub-types respectively) was then added and incubated for 45 min at RT to allow binding to NhaA. Anti-mouse IgG2a or IgG2b specific antibodies conjugated to horseradish peroxidase were finally added to probe binding of the secondary antibody. The peroxidase activity was measured using 2,2′-azino-di-(3-ethylbenzothiazoline) sulfonic acid substrate (Boehringer Mannheim) and quantified in an ELISA reader at 405 nm wavelength after 30 min incubation.

2.8. Competitive binding assay

This assay was performed by incubating mAb solutions at a final concentration of 12 μg/ml with different concentrations of wild-type NhaA in a volume of 200 μl overnight at 4°C. The probes were then loaded onto Ni^{2+}-NTA plates, which were previously coated with His-tagged NhaA (12 μg/ml). The subsequent steps were as described for the His tag based assay.

2.9. SDS-PAGE and Western blot

SDS-PAGE was done according to [22]. Proteins were transferred onto a nitrocellulose membrane using a semi-dry blotting apparatus by applying 50 mA/gel for 1 h at 4°C. After transfer the membrane was blocked overnight in PBS buffer containing 3% (w/v) BSA, 0.1% (w/v) Triton X-100. All washing steps were carried out in PBS buffer containing 0.05% (w/v) Triton X-100. For incubation with primary antibodies, blood sera and secondary antibody (anti-mouse IgG alkaline phosphatase conjugated) the probes were diluted 1:1000 in blocking buffer. The nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate color reaction was performed in 20 mM Tris, pH 9.8, 500 mM NaCl, 0.5 mM $MgCl_2$.

2.10. Gel filtration chromatography

Gel filtration chromatography was carried out using a Superose 6 column (Pharmacia) at a flow rate of 50 μl/min at 4°C on a SMART system (Pharmacia). To assess molecular weights a calibration curve was calculated based on separation of purified proteins (aldolase,

catalase, ferritin and thyroglobulin, Sigma). The running buffer contained 25 mM potassium acetate pH 4.5, 100 mM KCl, 5 mM $MgCl_2$, 7.5% (w/v) glycerol, 0.03% (w/v) LM. For each run 30 μg of proteins in the same buffer were loaded onto the column. For forming the complex between mAbs and His-tagged NhaA the proteins were mixed in running buffer, incubated for 90 min at 4°C and then applied onto the column.

2.11. Reconstitution of proteoliposomes

Reconstitution was performed essentially as described [5]. A mixture (300 μl) containing 26.7 mg/ml of *E. coli* phospholipids (PL), 1.2% octylglucoside (OG), 100 mM K^+-MOPS (pH 7) was presonicated in a bath type sonicator (G1128PIT, Laboratory Supplier Co., New York) until clear and mixed with 400 μl of a solution containing 80 μg His-tagged NhaA, 32.5% glycerol, 1.2% OG, 0.5 mg/ml PL, 1 mM DTT, and 50 mM MOPS (pH 7). The mixture was briefly sonicated again and rapidly diluted (36-fold) into the appropriate dilution buffer. After 20 min at RT proteoliposomes were sedimented by centrifugation at 250 000 × *g* for 1 h, resuspended in 100 μl of the dilution buffer, frozen in liquid nitrogen, and stored at −70°C. Frozen proteoliposomes were thawed at RT, and where indicated, purified mAbs added at a molar ratio of 4/1 (mAb/NhaA). The solution was sonicated briefly until clear, incubated for 1 h at 37°C and briefly sonicated again before use.

2.12. ΔpH driven sodium uptake in proteoliposomes

Proteoliposomes were made by dilution into a buffer containing 150 mM NH_4Cl, 1 mM DTT, and 15 mM Tris-HCl, pH 7.5. The assay [6] was conducted at room temperature by dilution of 10 μl proteoliposomes (1.3 μg His-tagged NhaA) into 500 μl containing either 150 mM choline chloride or NH_4Cl (control without ΔpH) and in addition 2 mM $MgSO_4$, 10 mM Tris HEPES (pH as indicated) and 50 μM $^{22}NaCl$ (1 μCi/ml). The reaction was stopped by adding 2 ml of the above ice-cold solution without sodium, filtered on 0.2 μm Schleicher and Schuell filters and washed with an additional 2 ml. Radioactivity on the filter was measured in a γ counter. Non-specific binding was determined by dilution into NH_4Cl buffer (no ΔpH) and used for correction. Experiments were done in triplicate.

2.13. Protein determination

Protein was determined according to Zor and Selinger [23].

3. Results and discussion

3.1. Production of anti-NhaA mAbs

To obtain anti-NhaA mAbs mice were immunized with purified His-tagged NhaA. Blood sera tested in the standard ELISA protocol showed antigen specific positive signals up to a dilution of $1:10^5$. A control performed with His-tagged calsequestrin was negative (data not shown). Accordingly, Western blot analysis performed with the antiserum (diluted 1:1000) showed a positive reaction with His-tagged NhaA, whereas no signal was found using the pre-immune serum (Fig. 1, lanes 9 and 10, respectively). The spleen cells isolated from the immunized mouse were fused to myeloma cells and 1800 hybridoma colonies were obtained from half of the fused cells.

In the first screen for antibody producing cells we used the standard ELISA with no detergent in the coating buffer. Very few positive clones were obtained and the majority of these later turned out to be false positive. We considered two possibilities to explain the difficulty in applying the standard ELISA: either the His-tagged NhaA does not bind to the plates or it is not properly presented to the antibodies. The former hypothesis seems unlikely since the binding of detergent solubilized membrane proteins has been shown in several cases [14,15] and allowed successful raising of monoclonal antibodies. A supplementation of the coating buffer with non-ionic detergents to improve the antigen presentation in the standard ELISA protocol was not pursued, as it might interfere with decorating the protein to the polystyrene plates. Furthermore, the alkaline pH required for binding is known to cause aggregation of the NhaA (data not shown).

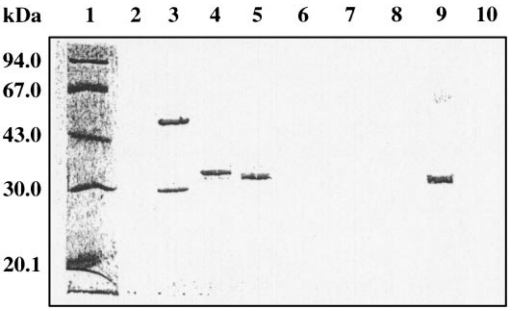

Fig. 1. Western blot analysis of the anti-NhaA mAbs after SDS-PAGE. Lane 1: low molecular weight markers; lane 2: negative control using BSA; lane 3: positive control using 1F6 IgG1 κ-chain mAb; lane 4: His-tagged NhaA probed with 1F6 mAb; lane 5: wild-type NhaA probed with 1F6 mAb; His-tagged NhaA probed with 2C5 mAb (lane 6), with 6F9 mAb (lane 7), with 5H4 mAb (lane 8); lane 9: His-tagged NhaA probed by the immunized mouse serum in a dilution 1:1000; lane 10: His-tagged NhaA probed with pre-immune serum (dilution 1:1000). For each lane 3 μg of protein were applied.

To achieve the native presentation of the antigen we used Ni^{2+}-NTA coated plates to bind NhaA via its His tag. The antigen coating, primary and secondary antibody binding as well as the washing steps are milder in the new protocol, i. e. pH 7.5 and presence of detergent (0.1% (w/v) LM). The conditions are based on the procedure we have developed to purify the His-tagged NhaA in an active form via Ni^{2+}-NTA affinity chromatography [13]. In addition, as the protein is bound at the C-terminus via the His tag, the antigen should be better accessible for the antibodies. Indeed with this assay (henceforth referred to as His tag based ELISA) supernatants obtained from 15 hybridoma clones gave strong signals at least 10-fold above the background. Single cell cloning resulted in four hybridoma cell lines with reproducibly positive signals in the His tag based ELISA. They were designated

Table 1
Comparison of the standard, His tag based and sandwich ELISA protocols in the detection of His-tagged NhaA with four mAbs

mAb	Immunological sub-type	Standard ELISA assay	His tag based assay	'Sandwich' assay
2C5	IgG1κ	0.71 ± 0.02	2.27 ± 0.03	–
5H4	IgG2bκ	0.25 ± 0.04	1.21 ± 0.05	1.06 ± 0.16
6F9	IgG2aκ	0.34 ± 0.08	1.01 ± 0.09	1.80 ± 0.36
1F6	IgG1κ	0.85 ± 0.03	1.53 ± 0.02	–

The different anti-NhaA mAbs were tested in the indicated assays as described in Section 2. Results are reported as mean absorption values ± S.D. ($n = 4$). Saturating conditions with respect to the antibody (12 μg/ml) and antigen (12 μg/ml) were used. Mean background levels were determined to be 0.07 absorption units for the standard and 'sandwich' ELISAs and 0.1 absorption units for the His tag based assay.

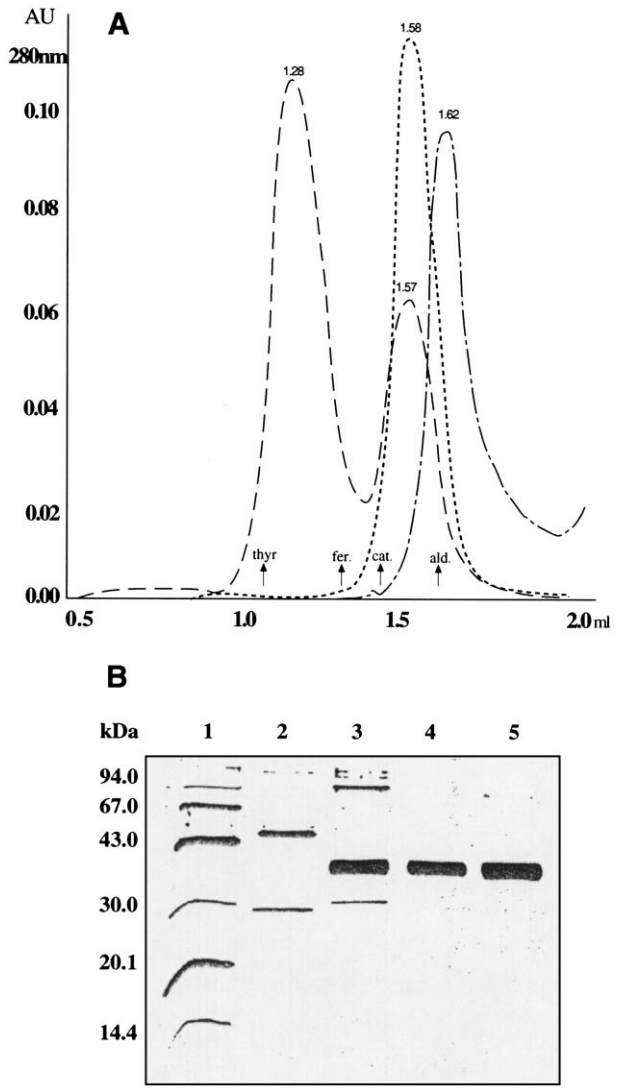

Fig. 2. Binding of 2C5 mAb to His-tagged NhaA. A: Gel filtration. His-tagged NhaA and 2C5 mAb protein samples were run separately (dotted and dash-dotted lines, respectively) on a Superose 6 gel filtration column as described in Section 2. Their elution profiles are shown together with the respective retention volumes (ml). To obtain the 2C5 mAb His-tagged NhaA complex the proteins (20 μg each) were mixed in 25 mM potassium acetate (pH 4.5), 100 mM KCl, 0.1% (w/v) LM, 5 mM $MgCl_2$ and incubated for 90 min at 4°C prior to applying to the column. The elution profile (dashed line) is shown. B: Silver stained gel after SDS-PAGE analysis of gel filtration chromatography runs (see A). Fractions were taken and pooled from the main peaks indicated with the retention volumes in ml. Lane 1: low molecular weight markers; lane 2: fractions of the 1.62 ml retention peak (dash-dotted line) containing 2C5 IgG1 κ-chain mAb; lane 3 and lane 4: fractions of the NhaA-antibody mixture (dashed line) containing the mAb-His-tagged NhaA complex (1.28 ml) and unbound His-tagged NhaA (1.57 ml); lane 5: fractions of the run containing His-tagged NhaA (dotted line, 1.58 ml). SDS-PAGE samples of lanes 1 and 2 were boiled prior to electrophoresis, while samples of lanes 3–5 were heated for 20 min at 37°C to prevent NhaA aggregation.

1F6, 2C5, 5H4, 6F9 and their respective monoclonal antibodies were purified.

A comparison between the results obtained for the purified monoclonal antibodies using the standard ELISA and the His tag based protocol is shown in Table 1. It is evident that, while the background signals were similar, the His tag based assay gave signals 16–23 times higher than the background. Those obtained with the standard ELISA were much lower in most cases. In addition, the results obtained with the new assay were very reproducible in contrast to the results with the standard ELISA. The four mAbs were immunologically sub-typed: 1F6 (IgG1), 2C5 (IgG1), 5H4 (IgG2b), 6F9 (IgG2a) as shown in Table 1.

3.2. Three out of the four mAbs bind only to the native NhaA

A Western blot analysis of the mAbs is shown in Fig. 1. The 1F6 mAb (lanes 4 and 5) was found to be Western blot positive to both His-tagged NhaA as well as to the wild-type protein which is 20 amino acids shorter [13]. In marked contrast the other three mAbs, although being positive in the His tag based protocol, were negative in a Western blot with either the wild-type (not shown) or the His-tagged NhaA (Fig. 1, lanes 6–8). These results suggest that these mAbs bind the native conformation of the antiporter but not the denatured protein.

To confirm this binding capacity we measured direct binding of the antibody to the antigen. Gel filtration was used to separate the complex of mAb and His-tagged NhaA and each of the single components. Fig. 2A shows that His-tagged NhaA and the anti-NhaA mAb (2C5) run on the gel filtration column with retention volumes of 1.58 ml and 1.62 ml, respectively. Calibration of the column with the indicated MW standards shows that these retention volumes correspond to 180 kDa and 150 kDa molecular weights, respectively. The molecular weight of His-tagged NhaA is 43.5 kDa as calculated from the primary sequence [13]; assuming that a LM micelle of about 100 molecules contributes to the apparent molecular mass of the protein in the gel filtration assay, it is suggested that NhaA runs as a dimer. When the 2C5 mAb and His-tagged NhaA were mixed and incubated for 90 min prior to the application to the column, two peaks were ob-

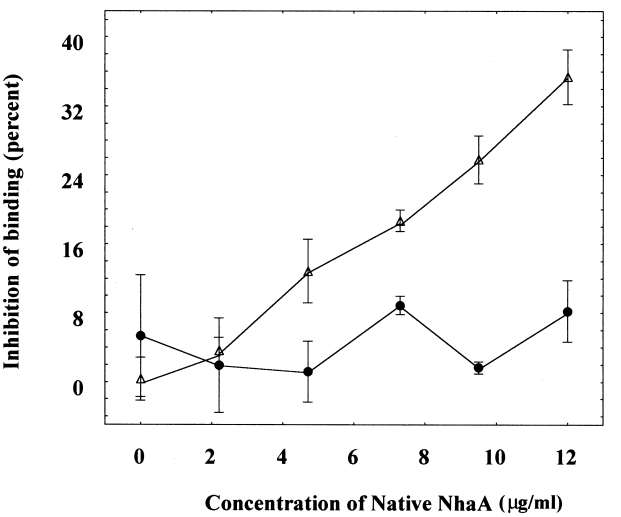

Fig. 3. Competition assay showing the recognition of native epitopes for both wild-type and His-tagged Nha by mAbs 2C5 and 1F6. 2C5 (△) or 1F6 (●) (10 μg/ml each) mAbs were mixed with the indicated concentrations of wild-type NhaA in 25 mM potassium acetate pH 5, 300 mM KCl, 0.1% (w/v) LM and incubated overnight at 4°C. These mixtures were then loaded onto His-tagged NhaA precoated Ni^{2+}-NTA plates for the His tag based ELISA. Individual points represent mean values of triplicate trials with standard deviations (error bars).

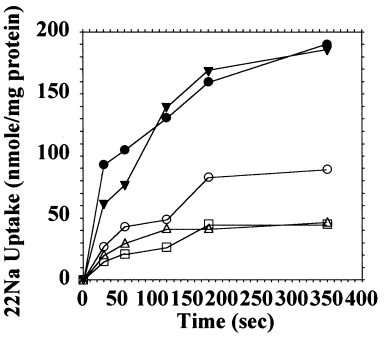

Fig. 4. Effect of mAbs on ΔpH driven ^{22}Na uptake into proteoliposomes. Proteoliposomes loaded with ammonia were prepared to measure ΔpH dependent $^{22}Na^+$ uptake and stored at −70°C. Aliquots were thawed, preincubated with mAbs and assayed for ΔpH dependent ^{22}Na uptake as described in Section 2 (●, control; ▼, 6F9; ○,1F6; △, 5H4; □, 2C5).

tained: one at higher MW (retention volume of 1.28 ml which corresponds to about 500 kDa) with respect to either His-tagged NhaA or 2C5 mAb, the other of a molecular weight corresponding to that of His-tagged NhaA. SDS-PAGE analysis of the two peak fractions (Fig. 2B) shows that the high molecular weight peak contains the complex of the 2C5 mAb and His-tagged NhaA (lane 3). The lower peak of this run contains the residual non-bound His-tagged NhaA (lane 4), which migrates identically to fractions of pure His-tagged NhaA (lane 5). The identity of the bands corresponding to the heavy and light chains of the antibody molecule in the complex were confirmed by Western blot (data not shown). These bands show a different mobility behavior in the gel as compared to the control sample of the pure 2C5 mAb (lane 2), as all samples containing NhaA were not boiled prior to electrophoresis whereas the antibody probe was. NhaA aggregates upon boiling in SDS-PAGE sample buffer as is known for other membrane proteins.

Similar results were obtained for the other two antibodies (5H4 and 6F9) binding to the native NhaA, while remarkably 1F6 did not show any complex formation with His-tagged NhaA in the gel filtration assay (data not shown).

To test whether the native epitopes in the His-tagged NhaA exist in the wild-type protein, a competition experiment was conducted using both proteins (Fig. 3). In this experiment a constant saturating amount of each mAb was mixed with increasing amounts of wild-type NhaA and incubated overnight to reach binding equilibrium. The uncomplexed mAb, left free in this binding mixture, was then assayed by subjecting the mixture to the His tag based assay. Fig. 3 shows the results obtained with the 2C5 mAb: as the concentration of the wild-type NhaA in the binding mixture increased, a decrease in the signal of the untitrated 2C5 mAb (reflecting inhibition of binding) was observed. Similar results were obtained with 5H4 and 6F9 mAbs (data not shown). It is concluded that both the wild-type and the His-tagged NhaA contain the native epitopes recognized by all three native conformation specific mAbs. The same experiment performed with the 1F6 mAb (Fig. 3) did not show any significant decrease in the signal.

3.3. The His tag based protocol is a promising alternative to the 'sandwich' ELISA

The results show that the Ni^{2+}-NTA plates are very efficient for the detection of mAbs recognizing the native conformation of the antiporter. We suggest that in the standard ELISA the NhaA is very poorly presented in its native conformation to the mAbs. To verify this point we used the 'sandwich' or 'two antibodies' ELISA in which an antibody bound to the polystyrene plates is used to present the antigen in a native conformation. In this experiment the 2C5 mAb was used to present the NhaA. The binding of another mAb (5H4 or 6F9) to the antiporter was quantitated following the His tag ELISA protocol (Section 2.7). The results clearly show that similar positive signals were obtained, whether NhaA was presented by the Ni^{2+}-NTA or the 2C5 mAb (Table 1). Hence, the His tag based protocol is a readily available and easy alternative to the 'sandwich' ELISA protocol and it could be extremely useful for other membrane proteins which can be produced as recombinant His-tagged proteins.

3.4. The effect of the mAbs on the Na^+/H^+ antiporter activity of NhaA

The effect of the anti-NhaA mAbs on the Na^+/H^+ antiporter activity was tested in proteoliposomes containing purified His-tagged NhaA. The proteoliposomes were preincubated with the mAbs before measuring ΔpH driven ^{22}Na uptake. The results summarized in Fig. 4 show that the 6F9 mAb has no effect on the antiporter activity: neither the initial rate of uptake nor the steady state level of accumulation is affected. Hence, the native epitope recognized by this mAb is not essential for antiporter activity. In this respect it is similar to many of the known mAbs which recognize native conformations of membrane proteins but do not affect their activity [15,24].

1F6 mAb inhibits both the initial rate as well as the steady state level of the antiporter activity about 3-fold, i.e. mAb 1F6 binds to His-tagged NhaA under the reconstitution conditions. As shown above this mAb also binds to the His-tagged NhaA either presented on Ni^{2+}-NTA coated plates (Table 1) or after SDS denaturation in Western blot analysis (Fig. 1). In marked contrast, 1F6 mAb does not bind to the detergent solubilized antiporter as measured either by gel filtration (Fig. 2A) or the competition assay (Fig. 3). Hence, these results imply that the epitope recognized by the 1F6 mAb is important for antiporter activity and is exposed only upon binding of the His-tagged Nha to the Ni^{2+}-NTA matrix or when the protein is reconstituted into proteoliposomes. With respect to the latter finding, it is very interesting to mention the marked effect of lipids on the exposure of an epitope of the Lac permease to mAb 4B1 [25,26].

The most dramatic effect on the antiporter activity was found with mAbs 5H4 and 2C5. They inhibit both the initial rate and the steady state level of activity by about 5–6-fold. Both 5H4 and 2C5 mAbs bind to native antiporter epitopes which are critical for activity and are exposed on the detergent solubilized antiporter (Fig. 2 and Table 1).

The mAbs isolated in this work are the first directed against an antiporter. 4B1 mAb, which uncouples the *lac* permease, is the only mAb known to affect a symporter [24,27]. While 6F9 mAb will help in the study of the topology of NhaA, the mAbs recognizing essential epitopes (5H4, 2C5, and 1F6) provide a new important tool to study the mechanism of the Na^+/H^+ antiporter and the relationship between its structure and function.

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