# Roles of Histidine 752 and Glutamate 699 in the pH Dependence of Mouse Band 3 Protein-Mediated Anion Transport†

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ABSTRACT: In the accompanying paper we have shown that four different histidine residues are involved in the maintenance of mouse band 3 in a state in which it is able to execute its anion transport function. Here we focus on the functional significance of His 752 and demonstrate that this residue, together with Glu 699, plays a key role in the control of pH dependence of $Cl^-$ transport. Mouse band 3-encoding cRNA was expressed in Xenopus oocytes, and band 3-mediated $Cl^-$ transport was measured at zero membrane potential over the pH range 6.0−9.2. Transport decreased with increasing $H^+$ concentration and was governed by a single p$K$ of 5.8. After correction for temperature differences, this result agrees well with measurements in erythrocyte ghosts of $Cl^-$ flux by Funder and Wieth [Funder, J., & Wieth, J. O. (1976) *J. Physiol. 262,* 679−698] and our own determinations by $^{35}Cl$ NMR spectroscopy of $Cl^-$ exchange between the substrate binding site and the medium. After mutation of either Glu 699 to Asp or of His 752 to Ser, the maximal rate of transport is reduced and the rate of anion exchange is now governed by a single p$K$ of about 6.8−6.9. This suggests that the formation of a hydrogen bond between His 752 and Glu 699 is essential for the decrease of band 3-mediated $Cl^-$ transport at low pH. We suggest that in the wild type band 3 both the decrease of the chloride exchange between the medium and the substrate binding site and the inhibition of chloride translocation across the membrane are dominated by a common rate-limiting step and that this step involves hydrogen bond formation between Glu 699 and His 752.

The pH dependence of band 3 protein-mediated anion transport across the red blood cell membrane follows a characteristic pattern [Gunn et al., 1973; Funder & Wieth, 1976; Wieth & Bjerrum, 1982; Bjerrum et al., 1989; Bjerrum, 1992; summarized by Salhany (1990) Figure 34, p 125]. If measured at 0 °C, the transport of monovalent anions, as exemplified by the physiologically important substrate $Cl^-$, increases with increasing pH until at pH values above 7 a plateau is reached. This plateau extends up to about pH 10.5, where another increase of transport rate occurs. The transport rate passes through a maximum around pH 11.5, which is followed by a sharp decrease to a similarly low level as that seen at the lower end of the pH range covered in the published work. The curve relating transport to pH can be approximated by the assumption that it is the consequence of the action of at least three dissociating groups with p$K$ values of 6, 10.5, and 12.

Attempts have been made to identify by chemical modification with group specific reagents the amino acid residues in the transport protein that are responsible for the observed pH dependence. Determinations of the pH dependence of the rate constants for the rate of irreversible inhibition of chloride transport by phenyl glyoxal strongly suggest that the inhibition at high pH is caused by the modification of an arginine residue, as originally inferred from the existence of the p$K$ of about 11−12 (Wieth & Bjerrum, 1982; Bjerrum, 1992). Susceptibility to inhibition with carbodiimides was taken to support the notion that carboxyl groups are involved in the control of anion transport at low pH values and that the modifiable group is identical to the group that is responsible for the titration of $Cl^-$ transport with a p$K$ of about 6 [discussed by Bjerrum et al. (1989)]. The participation of a carboxyl group was quite convincingly demonstrated by experiments with Woodward's Reagent K and borohydride, which converts COOH groups into alcoholic OH groups. Microsequencing revealed glutamate 699 as the site of action (Jennings, 1992). Finally it was shown that a histidine specific reagent, diethyl pyrocarbonate (DEPC),[1] produces inhibition of anion transport. This gave rise to the view that one or several histidine residues are also required for the maintenance of the transport function (Hamasaki et al., 1992). A requirement of four out of five histidines (His 721, 752, 837, 852) in the hydrophobic, anion-transporting domain of mouse band 3 was subsequently demonstrated by site-directed mutagenesis in the accompanying paper (Müller-Berger et al., 1995).

In the present work we used site-directed mutagenesis of both His 752 and Glu 699 to elucidate further the molecular basis of the pH dependence at the lower end of the pH scale studied in the past. We confirm that Glu 699 is important

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[1] Abbreviations: $H_2$DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; DEPC, diethyl pyrocarbonate; WRK, Woodward's reagent K.

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Table 1: Nucleotide Sequences of the Oligonucleotides Used for PCR Experiments[a]

| | | |
|---|---|---|
| | +2086 +2106 | strand |
| E699D1 | 5'-ATTTTCCTCGACTCTCAGATC-3' | plus |
| | +2106 +2086 | |
| E699D2 | 5'-GATCTGAGAGTCGAGGAAAAT-3' | minus |
| E699A1 | 5'-ACCTACACACAGAAACTCTCC-3' | plus |
| E699A2 | 5'-CTGTTCCTTGACCTGTTGGAT-3' | minus |
| T7 vector primer | 5'-TAATACGACTCACTATAGGGAGA-3' | |

[a] Mutated sites are underlined.

for the maintenance of the capacity of band 3 to accomplish chloride transport. We show in addition that His 752 is also involved and suggest that the breaking of a hydrogen bond between the two residues is involved in the control of anion transport in the pH range below pH 8.3.

## MATERIALS AND METHODS

Expression of wild type and mutated mouse band 3-encoding cRNA and the subsequent measurements of chloride efflux were performed in Xenopus oocytes essentially as described in the accompanying paper (Müller-Berger et al., 1995).

*Preparation of Mutant E699D.* The cRNA encoding for the mutant H752S was identical to that used in the previous work. Like the mutant H752S, the mutant E699D was prepared by the PCR method (Higuchi, 1990). Amplification of the PCR products was carried out with the primer combinations E699A1-E699D1 and E699A1-E699D2 (see Table 1). The fragments obtained were purified by gel electrophoresis and used as templates for a further PCR reaction with the primer combination E699A1-E699A2. This PCR fragment was cut with *Xma*I and ligated to pSPT19-Bd3-5xmut [see the accompanying paper of Müller-Berger et al. (1995)] treated with the restriction enzymes *Xma*I and *Mlu*I. The mutation was verified by sequencing, and it was shown that appropriate translation products were obtained in cell free reticulocyte lysates. The expression of the mutant in the oocyte was verified 2 days after microinjection of the cRNA by immunoprecipitation, using the method described previously (Grygorczyk et al., 1989). Flux measurements were performed after microinjection of strictly standardized quantities of cRNA (accuracy about 5%) under the conditions described below.

*NMR Measurements.* Chloride binding to isolated, purified red cell membranes was measured by means of $^{35}Cl$ NMR. Preparation of "white" ghosts [according to Dodge et al. (1963)] and determination of the DNDS sensitive line broadening as a measure of chloride binding to band 3 was performed as previously described (Glibowicka et al., 1988). All measurements were carried out in a Brucker AM 270 spectrometer. The $^{35}Cl$ resonance frequency was 26.47 MHz. The measurements were performed in NMR tubes with a diameter of 10 mm containing 2.5 mL samples of 50% ghost suspensions. The suspension medium consisted of 120 mM NaCl and 20 mM EDTA in which 20% of the $H_2O$ was replaced by $D_2O$. The spectral width was 1200 Hz and the size of the spectra 2K points. Depending on conditions, 500−15000 scans were sampled for each spectrum. The receiver gain was first determined automatically with a Brucker subroutine and then adjusted to values between 200 and 800. The repetition rate was automatically controlled for any given time domain. The 90° pulse was 25 $\mu$s. The probe head was matched and tuned before each new set of measurements. The temperature control was set to 23 °C. The line broadening of the chloride spectrum was calculated by subtracting the half-height widths of the lines determined in the presence and absence of DNDS. The final DNDS concentration was 1 mM. This concentration sufficed to produce a maximal effect on inhibition of anion transport over the pH range covered in these experiments. The $K_i$ value for inhibition of sulfate transport increased from 5 $\mu$M at pH 7.4 to 25 $\mu$M at pH 5.5 (S. Lepke and H. Passow, unpublished results) and hence never exceeded $^1/_{40}$ of the DNDS concentration used. The line width at half-height of the spectra was calculated with a Bruker fitting program after phase and basis line correction for a window of 300 data points. The pH titration was carried out by addition of 1 N HCl or 1 N NaOH to the concentrated ghost suspensions. Hence the changes of chloride concentration in the course of the titration procedure remained negligibly small.

*The specific protocols for the flux measurements* at different internal and external pH values are based on the results of pilot experiments concerning (1) the adjustment of intracellular pH by ammonium chloride (Lee & Steinhardt, 1981) and (2) the influence of membrane potential on band 3-mediated chloride efflux (Grygorczyk et al., 1987; Schwarz et al., 1992). The techniques used and the results obtained in these experiments can be summarized as follows:

(1) Determinations of membrane potential and intracellular pH in the oocytes were performed in perfusion chambers made from Lucite, which permitted a continuous exchange of the external medium during the whole time of experimental observation. The chamber was grounded via an AgCl/Ag electrode. The potential electrodes had a resistance of 10 MΩ. They were made from borosilicate glass (Hilgenberg, Germany) and filled with 1 M KCl. For intracellular pH measurements essentially similar electrodes were used. However, their internal surfaces were made hydrophobic by exposure to dimethylchlorosilane vapors at 150 °C for 1 h. They were subsequently filled with a pH-sensitive liquid ion exchanger (hydrogen ionophor I, Fluka) and beveled until a resistance of about 1 GΩ was achieved. For further details see Amman et al. (1981). For the simultaneous measurements of intracellular pH and membrane potential, a differential electrometer (Duo 773, WPI) was used.

(2) Membrane potential and intracellular pH, respectively, were varied by partial or complete, nearly isosmotic substitution of the NaCl in the external medium (Barth's solution) by KCl and/or ammonium chloride.

(*i*) *Adjustment of Membrane Potential and Intracellular pH.* After expression of the band 3 protein, oocytes were incubated for 90 min in Barth's media of different pH. Subsequently intracellular pH and membrane potential were determined. Table 2 shows that exposure to external pHs between 8.3 and 6.1 had virtually no effect on intracellular pH, which remained constant at 7.3. However, the membrane potential decreases considerably with decreasing pH.

The membrane potential is susceptible to variations of the external potassium concentration. When the NaCl in the Barth's solution (90 mM) is replaced by KCl (110 mM),

Table 2: Intracellular pH ($pH_i$) and Membrane Potential ($V_m$) in *Xenopus* Oocytes[a]

| $pH_e$ | $pH_i \pm SD$ | $V_m \pm SD$ | $n$ |
|---|---|---|---|
| 8.3 | 7.33 ± 0.1 | −69 ± 12 mV | 8 |
| 7.6 | 7.33 ± 0.1 | −51 ± 14 mV | 10 |
| 6.1 | 7.36 ± 0.1 | −27 ± 9 mV | 8 |

[a] Two days after microinjection of band 3 wild type-encoding cRNA, the oocytes were incubated in Barth's medium (90 mM NaCl) at 23 °C for 90 min. Subsequently, membrane potential and intracellular pH were measured with intracellular potential and pH electrode at three different pH values in the external medium ($pH_e$). $n$ represents the number of different oocytes used for the measurements.

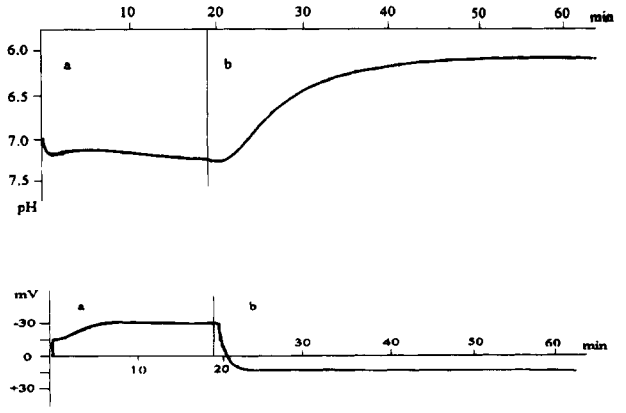


FIGURE 1: Effect of partial substitution of NaCl in Barth's medium by $NH_4Cl$ on membrane potential and intracellular pH of an oocyte as measured with intracellular electrodes. Ordinate: Intracellular pH (upper panel) or membrane potential in mV (lower panel). Abscissas: Time in min. (a) Up to the time marked by the vertical bar the oocyte was superfused with Barth's medium containing 90 mM NaCl. (b) Beginning at the bar, superfusion was changed to Barth's medium containing 30 mM NaCl and 60 mM $NH_4Cl$. Both media contained 500 $\mu$M DNDS. The external pH was 8.3 throughout.

the membrane potential drops rapidly to values close to 0 mV. In the depolarized state the membrane becomes slightly more permeable for protons. Transfer of the oocytes after depolarization in 110 mM KCl Barth's solution at pH 7.3 to 110 mM KCl Barth's solutions of pH 6.1 or 8.3 leads to a slow, continuous change of intracellular pH. In the former case it decreases by about 0.1 pH units per hour, in the latter case it increases by about 0.1−0.2 pH units per hour (not documented).

The depolarization in KCl Barth's medium leads to an effective clamping of the membrane potential near 0 mV, which can be used to eliminate the influence of variations of membrane potential on chloride transport. It has little effect on the rate of proton equilibration between oocyte and medium. In order to enhance this process, we followed up the observations of Lee and Steinhardt (1981), who reported that addition of ammonium chloride to the external medium leads to a reasonably rapid acidification of the cell interior. The effect is seen in Barth's medium prior to and after substitution of part of the NaCl by sufficient KCl to depolarize the membrane. The effects are virtually the same in the unsubstituted and the substituted media. The addition of ammonium, which acts as a proton carrier, produces a strong depolarization even in the absence of KCl (Figure 1).

The results of a number of experiments with ammonium chloride in unsubstituted and KCl-substituted Barth's solution are summarized in Table 3. The intracellular pH values obtained after equilibration with ammonium chloride are only

Table 3 [a]

| Barth's medium containing | $\Delta V_m$ (mV) ± SD | $pH_i \pm SD$ | $n$ |
|---|---|---|---|
| 90 mM NaCl + 0 mM $NH_4Cl$ | − | 7.33 ± 0.10 | 10 |
| 50 mM NaCl + 40 mM $NH_4Cl$ | 40 ± 8 | 6.78 ± 0.18 | 12 |
| 30 mM NaCl + 60 mM $NH_4Cl$ | 37 ± 8 | 6.12 ± 0.10 | 14 |
| 110 mM KCl + 0 mM $NH_4Cl$ | − | 7.35 ± 0.10 | 16 |
| 70 mM KCl + 40 mM $NH_4Cl$ | 0 ± 0 | 6.66 ± 0.10 | 13 |
| 50 mM KCl + 60 mM $NH_4Cl$ | 0 ± 0 | 6.10 ± 0.10 | 14 |

[a] (Upper part) Effect on intracellular pH ($pH_i$) of isosmotic substitution of NaCl in Barth's solution by ammonium chloride, pH 8.3, containing 500 $\mu$M DNDS. (Lower part) Essentially the same experimental arrangement, except that the medium consisted of KCl Barth's solution, pH 8.3, in which the NaCl had been replaced by 110 mM KCl and in which the KCl was isosmotically substituted by ammonium chloride. $\Delta V_m$ = change of membrane potential relative to the membrane potential measured in the absence of ammonium chloride (designated in column 2 by −).

maintained as long as the ammonium salt is present. After readmission of Barth's solution without ammonium chloride, the pH inside the oocytes increases and has the tendency to return slowly to the original level of pH 7.3. The results described are obtained irrespective of whether or not band 3 protein had been expressed in the oocytes.

The findings described above indicate that measurements of the pH dependence of band 3-mediated anion transport require a clamping of the membrane potential at a fixed value. Depolarization to 0 mV by replacement of the NaCl in the Barth's solution with 90 or 110 mM KCl is suitable to achieve this end. The intracellular pH can be efficiently decreased from its physiological level of 7.3 to lower levels by the addition of ammonium chloride to the external medium (Lee & Steinhardt, 1981). This decrease occurs in KCl or NaCl Barth's solution and is even in the latter associated with a virtually complete depolarization of the membrane.

*(ii) Measurements of $^{36}Cl^-$ Efflux.* It has been shown previously (Grygorczyk et al., 1987) that the time course of decrease of microinjected $^{36}Cl^-$ by band 3-mediated chloride efflux initially follows a single exponential. After some time, deviations become apparent. Their magnitude varies among different oocytes. The nature of these deviations is not yet completely understood. Figure 2 shows, however, that they are, at least in part, related to the retention of some chloride that does not exchange with the chloride in the external medium at the same rate as the bulk of the microinjected chloride (Figure 2A). This becomes apparent if, instead of the semilogarithmic representation of the cpm's recorded at times $t$ (Figure 2B), the logarithms of the differences between the cpm's at times $t$ and $t = 2$ h (assumed to be close to the cpm's at infinite time) are plotted against $t$ (Figure 2C). The straight line relationship that originally represented no more than about two-thirds of the total loss now extends over virtually the whole time interval covered by the measurements. Evidently, if one does not correct for the chloride retention at infinitely long times of observation, the slopes of the initial two-thirds of the straight lines obtained agree with the slopes that take this retention into account. Under most experimental conditions, it is not possible to determine the final chloride content that each oocyte would have reached if one could have permitted the oocyte to lose its $^{36}Cl^-$ up to the establishment of the final chloride retention. Under these conditions we omitted in the calculation of the rate constants the data pertaining to less than one-third of the original chloride content of the oocyte.

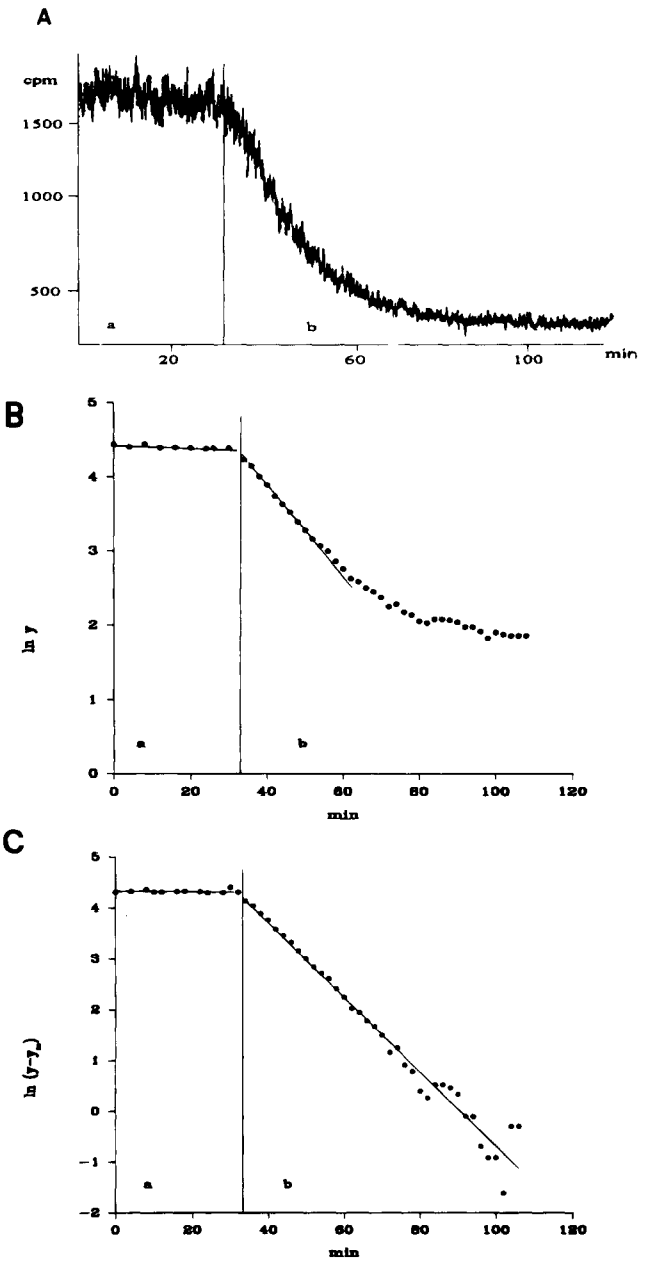


FIGURE 2: (A) Original record of the $^{36}Cl^-$ content of a single oocyte (ordinate) as a function of time (abscissa). The oocyte had been microinjected with the radioactive chloride 2 days after microinjection of band 3-encoding cRNA. Up to the end of time period a, the Barth's medium used for superfusion contained 500 $\mu$M DNDS to inhibit band 3-mediated chloride efflux. The Barth's medium with DNDS was then changed to Barth's medium without DNDS. Note that the ensuing decrease of intracellular $^{36}Cl^-$ does not tend toward zero. (B) The data recorded by the rate meter in panel A are electronically averaged and replotted on a semilog scale. The deviations from a straight line relationship (slope: ln $y/\Delta t$ = 0.069 $min^{-1}$) after longer times of observation are again apparent. (C) Replot of the data in panel B after forming the logarithms of the differences between the radioactivities in the oocyte at times $t$ and $t$ = 2 h (slope of the straight line: ln $(y - y\infty)/\Delta t$ = 0.073 $min^{-1}$).

For the quantitative evaluation of the recorded results, the data were electronically sampled, averaged, and replotted on a semilogarithmic scale (Figure 3). The slopes of the straight lines yield the rate constants of chloride efflux. In actual practice, the rate constants were calculated by a nonlinear least-squares curve fitting procedure.

Figure 3 provides an example of the protocol followed for the determination of each single data point of the pH

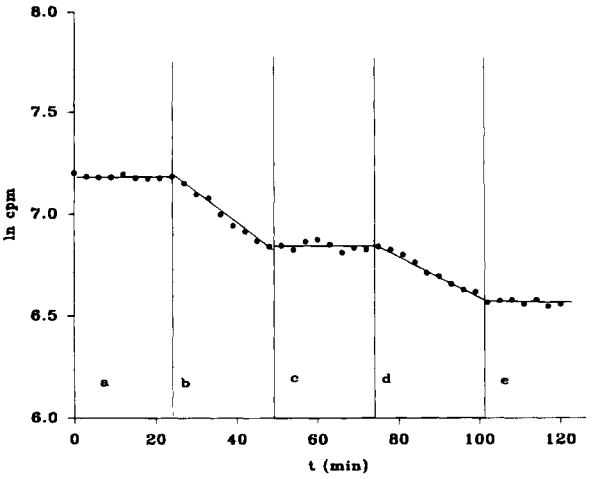


FIGURE 3: Protocol for measuring changes of wild type band 3-mediated $^{36}Cl^-$ release by changing the external pH. The letters between the vertical bars designate consecutive periods of superfusion with media of the following composition: (a) KCl Barth's medium, pH 7.3, containing DNDS, (b) KCl Barth's medium, pH 7.3, without DNDS, (c) same DNDS-containing KCl Barth's medium as in (a), (d) KCl Barth's without DNDS, pH 6.1, and (e) same DNDS-containing Barth's medium, pH 7.3, as in time period a. When DNDS was present, the concentration was 500 $\mu$M. The KCl concentration was 110 mM. Ordinate: Logarithm of radioactivity inside the oocyte. Abscissa: Time in min. From the slopes of the straight lines in time periods b and d, one obtains the rate constants for pH 7.3 and 6.1 in the external medium. In this example they amount to 0.0143 and 0.0108 $min^{-1}$, respectively. At pH 6.1 the $^{36}Cl^-$ leak flux from uninjected oocytes was indistinguishable from the $^{36}Cl^-$ leak flux at pH 7.3. For more details see text.

dependence of $Cl^-$ efflux from a single oocyte. The oocyte that rests in a hair loop at the bottom of the perfusion chamber is superfused with KCl Barth's medium, pH 7.3, containing DNDS at a concentration that produces a virtually complete inhibition of band 3-mediated chloride efflux. The intracellular $^{36}Cl^-$ content recorded by the Geiger–Müller tube that forms the bottom of the perfusion chamber remains essentially constant. This indicates that there are no leaks in the oocyte membrane. At the vertical straight line that separates the perfusion intervals a and b, we change to perfusion with KCl Barth's, pH 7.3, without DNDS. After washing out the reversibly binding inhibitor, band 3-mediated chloride efflux begins. The radioactivity that now escapes from the oocyte is carried away, and the intracellular radioactivity recorded by the Geiger-Müller tube decreases. Return to superfusion with DNDS-containing KCl Barth's, pH 7.3, (superfusion period c) stops the efflux and thus confirms that the recorded decrease of radioactivity was entirely due to the activity of band 3. During the subsequent time period d, superfusion was continued with KCl Barth's, pH 6.1. After wash out of the previously used medium, efflux is resumed, although at a rate different from that seen during superfusion with KCl Barth's, pH 7.3, in the time interval b. Finally, at the end of time interval d, we return to superfusion with KCl Barth's, pH 7.3, containing DNDS. The record of time interval e confirms that the experimental conditions remained stable throughout the experiment. It may be added that in control oocytes without microinjected band 3 cRNA, the rate of $Cl^-$ efflux at the lower pH values used during superfusion period c was not significantly different from that at pH 7.3.

The measurements of chloride efflux as a function of internal pH at constant external pH followed essentially the same pattern as in the experiments described above, except

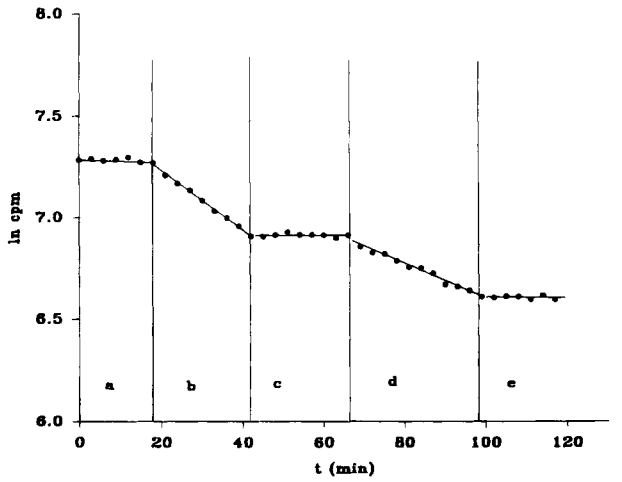


FIGURE 4: Protocol for measuring changes of wild type band 3-mediated $^{36}Cl^-$ release by changing the internal pH. The letters between the vertical bars designate consecutive periods of superfusion with media of the following composition: (a) KCl Barth's medium, pH 7.3, containing DNDS, (b) KCl Barth's medium, pH 8.3, without DNDS, (c) KCl Barth's medium, pH 8.3, in which 60 mM KCl had been isosmotically substituted by $NH_4Cl$, containing DNDS, (d) same medium as in time period c but without DNDS, and (e) same medium as in time period a, but with DNDS. When DNDS is present, its concentration amounts to 500 $\mu$M. During the superfusion periods a, b, and e, the pH inside the oocyte is 7.3; during the periods c and d the inside pH is 6.1 (see Table 3). Ordinate: Radioactivity in an oocyte obtained after electronic averaging plotted on a semilog scale. The rate constants derived from the slopes covering the superfusion periods b and d amounted to 0.0151 and 0.0091 $min^{-1}$, respectively.

that the intracellular pH was varied by partial substitution of the KCl in the medium by ammonium chloride, as described above (Figure 4).

## RESULTS

*pH Dependence of Chloride Efflux Mediated by Wild Type Band 3 and Mutants H752S and K558N.* Figure 5a summarizes the results of chloride efflux measurements performed as described above at a fixed internal pH of 7.3 and varying pH values in the KCl Barth's media used for the superfusion of the individual oocytes. The curve represents a fit of the equation $k_{Cl} = CK/(K + H^+)$ to the data. In this equation, $K$ indicates the hydrogen ion concentration in the external medium, $H^+$, at which the chloride efflux takes place at half the maximal rate $C$ measured in the absence of the inhibitor $H^+$. This equation is the standard expression for inhibition at a single site. For the wild type we find $K = 1.58 \times 10^{-6}$, corresponding to an apparent p$K$ value of 5.8. In the mutant H752S, the maximal rate of efflux amounts to only 35% the rate of maximal efflux in the wild type, and we find $K = 1.26 \times 10^{-7}$, equivalent to an apparent p$K$ of 6.9. A few data points were also obtained with the mutant K558N. The value of $C$ was close to that for the wild type. The apparent p$K$ value was, if anything, slightly higher than in the wild type. The number of measurements was too small to make sure that the difference is real.

Since the survival of the oocytes in the ammonium chloride-containing KCl media was rather poor, the effect of variation of intracellular pH at constant extracellular pH was only determined at three different intracellular pH values and only in the wild type and in the mutant K558N (Figure 5A). The results obtained with wild type and mutant were indistinguishable and showed that the apparent p$K$s are rather similar to the values found when the extracellular pH was

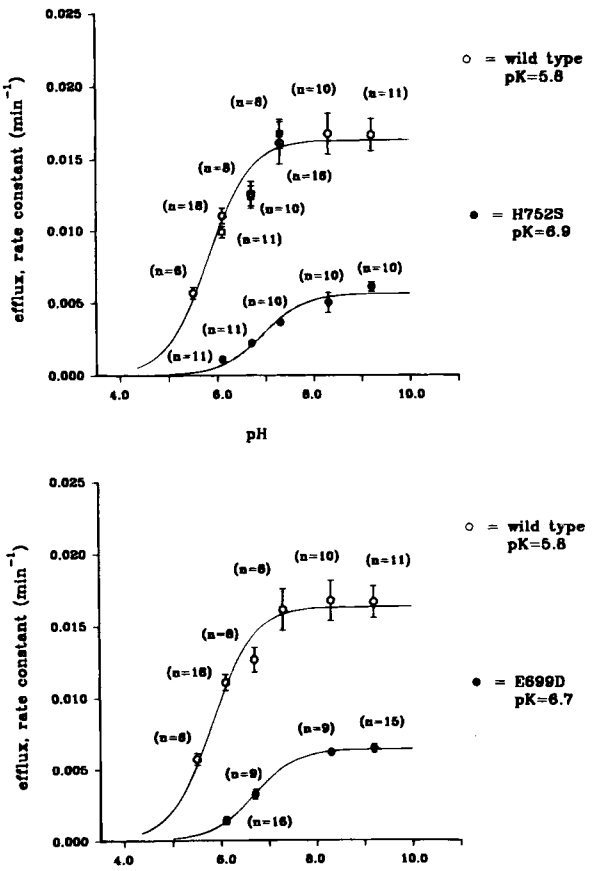


FIGURE 5: Dependence of band 3 protein-mediated chloride transport on extracellular pH. (A, upper panel) The intracellular pH was constant at pH 7.3, and the membrane potential was clamped to values close to 0 mV by the use of Barth's medium in which 90 mM NaCl had been replaced by 110 mM KCl. An example for the protocol for the individual measurements is provided in Figure 4. The drawn line represents a nonlinear curve fit of the equation $k_{Cl} = CK/(K + H^+)$ to the data. $C$ represents the rate constant $k_{Cl}$ for chloride efflux at the hydrogen ion concentration $H^+ = 0$. The open symbols refer to wild type band 3, the closed symbols to the band 3 mutant H752S. $n$ = number of oocytes used to obtain the data points. The error bars indicate SD's. Ordinate: Rate constant $k_{Cl}$, abscissa: pH in the external medium. For comparison, data points obtained at the fixed external pH of 8.3 and three different internal pHs (abscissa; see Table 4A) are included (open squares). The protocol for such measurements is represented in Figure 4. (B, lower panel) The open symbols refer to the wild type, the closed circles to the band 3 mutant E699D. The curve for the wild type is identical to the curve in the upper panel and serves to facilitate comparison. Same ordinate and abscissa as in the upper panel.

varied at constant intracellular pH (Figure 5A and Table 4). The Table also shows that the pH dependence of $Cl^-$ transport without potential clamping in NaCl Barth's differs from that measured with clamping in KCl Barth's. These findings explain why in a previous publication (Hanke-Beier et al., 1988) we found a pH dependence of band 3-mediated $Cl^-$ transport in the oocyte that differs from that reported here for a membrane potential of 0 mV.

*pH Dependence of Chloride Efflux Mediated by the Mutant E699D.* After substitution of Glu 699 by Asp, anion transport remains intact, although at a considerably reduced rate. Transport could still be inhibited by DNDS. However, the p$K$ value for chloride transport changed from 5.8 in the wild type to 6.7 in the mutant (Figure 5B).

*pH Dependence of Chloride Binding to Band 3 in the Red Blood Cell Membrane.* To interpret the nature of the effect due to the mutations H752S and E699D on band 3-mediated

Table 4: Effect of Varying the Intracellular pH at a Constant Extracellular pH of 8.3 on Chloride Efflux from (A) Wild Type and (B) Mutant K558N Mouse Band 3-Containing Oocytes

| Barth's medium containing | $\Delta V_m$ (mV) ± SD | $pH_i$ ± SEM | % residual flux ± SEM | *n* |
|---|---|---|---|---|
| | | (A) Wild Type | | |
| 90 mM NaCl + 0 mM $NH_4Cl$ | – | 7.33 ± 0.1 | 100.0 ± 0.0 | 10 |
| 50 mM NaCl + 40 mM $NH_4Cl$ | 40 ± 8 | 6.78 ± 0.1 | 58.8 ± 3.6 | 17 |
| 30 mM NaCl + 60 mM $NH_4Cl$ | 37 ± 8 | 6.12 ± 0.1 | 46.0 ± 4.1 | 12 |
| 110 mM KCl + 0 mM $NH_4Cl$ | – | 7.35 ± 0.1 | 100.0 ± 0.0 | 16 |
| 70 mM KCl + 40 mM $NH_4Cl$ | 0 ± 0 | 6.66 ± 0.1 | 73.9 ± 4.5 | 10 |
| 50 mM KCl + 60 mM $NH_4Cl$ | 0 ± 0 | 6.10 ± 0.1 | 59.1 ± 2.4 | 11 |
| | | (B) K558N | | |
| 90 mM NaCl + 0 mM $NH_4Cl$ | – | 7.30 ± 0.1 | 100.0 ± 0.0 | 12 |
| 50 mM NaCl + 40 mM $NH_4Cl$ | 40 ± 8 | 6.75 ± 0.1 | 61.5 ± 3.5 | 13 |
| 30 mM NaCl + 60 mM $NH_4Cl$ | 37 ± 8 | 6.20 ± 0.1 | 48.9 ± 4.5 | 12 |
| 110 mM KCl + 0 mM $NH_4Cl$ | – | 7.33 ± 0.1 | 100.0 ± 0.0 | 14 |
| 70 mM KCl + 40 mM $NH_4Cl$ | 0 ± 0 | 6.67 ± 0.1 | 69.1 ± 4.8 | 11 |
| 50 mM KCl + 60 mM $NH_4Cl$ | 0 ± 0 | 6.18 ± 0.1 | 58.8 ± 2.6 | 9 |

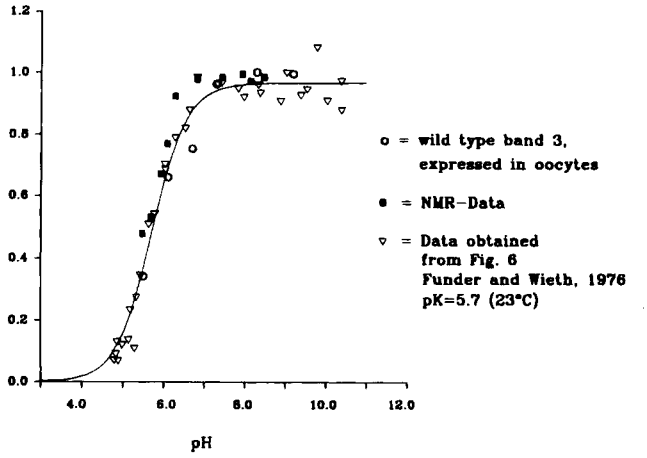


FIGURE 6: pH dependence of monovalent anion transport and anion binding to band 3. (a) Dependence of band 3 protein-mediated chloride transport in oocytes on extracellular pH (Figure 5A). (b) Chloride equilibrium exchange as measured at equal, nearly saturating chloride concentrations inside and outside the ghosts [data of Funder and Wieth (1976)]. (c) DNDS-sensitive line broadening of the $^{35}Cl$ NMR signal. The data indicate that the exchange of $^{35}Cl$ between band 3 in the plasma membrane and the medium varies with pH either because the DNDS prevents $Cl^-$ binding to the substrate binding site by competition (Falke et al., 1984) or because the inhibitor binding prevents the release of the bound anion by causing an obstruction of an access channel to the substrate binding site (Aranibar et al., 1994). Ordinate: Transport rates or NMR line broadining, normalized with respect to the plateau values reached at the highest pH values covered. Abscissa: pH. According to Funder and Wieth (1976) the pH dependence of chloride equilibrium exchange follows a titration curve with a single p*K* value of about 6.2. Since our measurements had been performed at about 23 °C, it is necessary to correct their data for the influence of temperature on the dissociation constant. The heat of dissociation should be somewhere between that of a carboxyl group (close to 0 kcal/mol) and a that of the imidazole group of a histidine (about 7.5 kcal/mol; Greenstein & Winitz, 1961). To determine the upper limit, we used the value for the histidine imidazole group and arrived at a p*K* of about 5.7 for chloride transport across the red cell membrane at 23 °C. The figure shows that, after this latter correction, the data from Funder and Wieth's work agree reasonably well with the data obtained after expression of band 3 in the oocyte.

anion transport, we studied chloride binding to band 3 in red cell ghosts. For this purpose we measured the DNDS sensitive line broadening of the $^{35}Cl$ NMR signal as described previously (Glibowicka et al., 1988) as a function of pH (Figure 6). Over the pH range pertinent for the present work, after appropriate correction for temperature differences (see figure legend), the chloride binding curve is virtually indistinguishable from the relationship between chloride transport and pH published by Funder and Wieth (1976). In terms of the conventional interpretation of the changes of the NMR signal, this suggests that the variation of pH causes the changes of anion transport essentially by changing chloride binding to the substrate binding site.

## DISCUSSION

*Role of His 752 and Glu 699 in Band 3-Mediated Anion Transport.* Our previous experiments have shown (Müller-Berger et al., 1995) that at least four out of the five histidines in the hydrophobic, anion transporting domain of mouse band 3 are involved in maintaining the band 3 protein in a functional state. At least two of them, but possibly all four, are susceptible to modification by the histidine specific reagent DEPC with consequent inhibition of chloride transport. One of them, His 752, is more susceptible than the others. Its mutagenic substitution by a serine residue reduces the rate of chloride transport and shifts the p*K* of the acidic branch of the relation between chloride transport and proton concentration in the medium from 5.8 to 6.9. Mutagenic replacement of Glu 699 by aspartate also leads to partial inhibition and shifts the p*K* for chloride transport from 5.8 to 6.7, i.e., to a value rather similar to that seen after mutagenesis of His 752.

Although our experiments show that two distinct amino acid residues, Glu 699 and His 752, participate in the control of the pH dependence of anion equilibrium exchange, the latter can be described by a single p*K* value. This suggests that the pH dependence is governed by the dissociation of a single proton. It seems reasonable to assume, therefore, that the two amino acid residues, although separated in the amino acid sequence by a large distance, reside in the three-dimensional structure of band 3 in close juxtaposition and are interconnected by a hydrogen bond. At sufficiently low pH, the formation of this bond could be responsible for the inhibition of transport with a single p*K* value. In accord with observation, this p*K* should be higher than that of the carboxyl group of the glutamate and lower than that of the imidazole group of the histidine. Within these limits, the exact magnitude of the p*K* value would depend on many additional, essentially unknown factors, such as the local dielectric constant, the neighborhood of charged amino acid residues, and the presence of other proton donors or acceptors, including water molecules.

Although it seems reasonable to stipulate that the capacity to establish a hydrogen bond between the histidine and the glutamate residue is essential for the maintenance of the

normal pH dependence of anion transport, it must remain open whether or not this same hydrogen bond, or some other dissociating group allosterically linked to it, is responsible for the effect on transport. This reservation with respect to the localization of the anion transport controlling site is emphasized if one considers the p$K$ value that dominates the pH dependence in the individual mutants. This newly appearing p$K$ value could hardly been explained solely by the changes of the conditions for hydrogen bonding between Glu 699 and His 752.

Most likely, after mutation of either Glu 699 or His 752, the conditions for hydrogen bond formation are sufficiently altered to enable the surviving and the mutated residue to get involved in hydrogen bonding with other side chains or even with the peptide backbone (Jeffrey & Saenger, 1992). This could give rise to structural changes that lead to a shift of the rate-controlling structure to other dissociable groups or to the hydrogen bonds existing amongst suitable donors and acceptors. Such changes may be responsible for the emergence of the new p$K$ value of $Cl^-$ transport of pH ~6.8, which is not apparent in the titration curve of the wild type. This p$K$ value that, after mutation of either the glutamate or the histidine residue, governs the transport rate suggests that in both mutants the new rate-limiting step involves the operation of the same as yet unidentified third amino acid residue or the same hydrogen-bonded pair(s) of residues. It would seem plausible to suspect that, in place of His 752, one of the other DEPC-sensitive histidines now assumes a decisive role.

The inferences drawn above are rather vague, but any further analysis of the nature of the stipulated hydrogen bonds raises more questions than can be answered without independent information on the three-dimensional structure of band 3. Nevertheless, without any claim for eternal validity, it may be useful to integrate the observations presented here into the simple, provisional model described in the accompanying paper. There, it was assumed that the helices 5, 8, 9, 10, and 13 form an access channel to the substrate binding site in the vicinity of the histidine residues 837 and 852, and the lysine residues 832 and 835, in the interhelical loop between helices 12 and 13. The reversibly binding DNDS combines with band 3 with a 1:1 stoichiometry (Barzilay et al., 1979) near the outward-facing lysine residues 558 and 869 and impedes the access of DEPC to His 752 (Hamasaki et al., 1992) and of WRK to Glu 699 (Jennings, 1992). This is accompanied by the arrest of the substrate binding site in an outwardly oriented position (Barzilay et al., 1979; Fröhlich, 1982) and an inhibition of the $Cl^-$ exchange between the bound and the free state (Figure 6). This picture would be entirely compatible with the idea that Glu 699 and His 752 are located close enough to each other to form a hydrogen bond that, like DNDS binding near the outer orifice of the access channel, could impede the mobility of the substrate binding site and inhibit the chloride exchange between that site and the surrounding medium by a steric or allosteric mechanism (Figure 7). It may be noted that the idea of the location of the substrate binding site close to the inward-pointing surface of band 3 has also been discussed by Schnell (personal communication), Kay et al. (1990), and Hamasaki et al. (1992).

The measurements of the line broadening of the $^{35}Cl$ NMR spectra as a function of pH support the latter assumption. Over the $Cl^-$ concentration range where no self-inhibition of $Cl^-$ transport (Dalmark, 1975) occurs, $Cl^-$ transport is

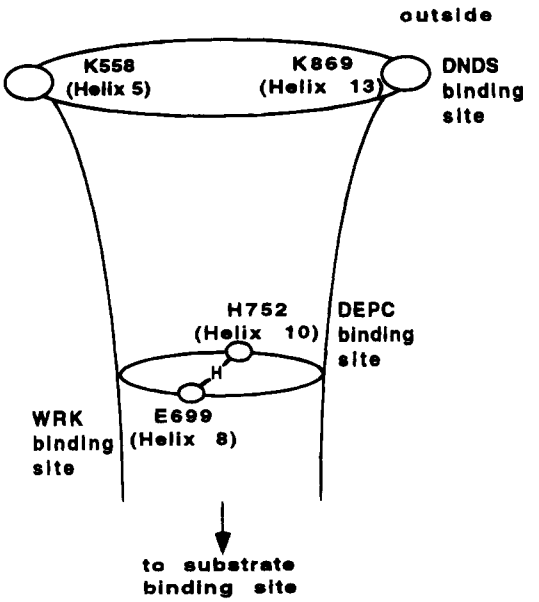


FIGURE 7: Schematic representation of the putative access channel with the relative positions of the binding sites for DNDS, DEPC, and WRK and the substrate. The figure indicates only the four helices which are stipulated to carry the amino acid residues involved in the binding of the various agents and the location of the hydrogen bond that controls the access to the substrate binding site by a steric or an allosteric effect. For simplicity's sake helix 9 has been omitted even though it is assumed to participate also in the channel formation [see also Figure 7 in the accompanying paper of Müller-Berger et al. (1995)]. DNDS binding blocks access of DEPC, WRK, and substrate to their respective binding sites probably by a combination of a steric and an allosteric effect. The figure represents a highly speculative attempt to integrate the various observations described in the text into a coherent picture. It claims no definitive truth.

linearly related to $Cl^-$ binding as derived from the line broadening of the NMR signal (Passow et al., 1988; Aranibar, Ph.D. Thesis, Frankfurt, 1992). Our comparison of the effects of pH on transport and line broadening shows that over the pH range 5.7−8.5 both $Cl^-$ transport across the membrane and $Cl^-$ exchange between the medium and the substrate binding site undergo strictly parallel changes. This suggests that the hydrogen bond formation between Glu 699 and His 752 does in fact, directly or allosterically, interrupt the exchange between bound and free $Cl^-$ and that this interruption is responsible for the inhibition of transport. Interestingly enough, modification of band 3 with DEPC also leads to an inhibition of line broadening (Hamasaki et al., 1990), which suggests that chemical modification of His 752 interrupts $Cl^-$ exchange with the substrate binding site like the formation of the hydrogen bond.

*Accessibility of Glu 699 and His 752 to Protons from the Inner and Outer Membrane Surface.* Work with red cells has shown that the pH dependence of anion transport is dependent on one or several sites in the band 3 protein that are accessible to hydrogen ions from either surface of the membrane (Jennings, 1992). The present work confirms this result for band 3 expressed in the plasma membrane of the oocyte. When the fixed pH inside the cell (pH 7.3) exceeds the variable outside pH, or if the fixed outside pH (pH 7.3) exceeds the variable inside pH, we find no significant difference of the effect on chloride transport of varying the pH at the inside or outside of the cell. These findings strongly suggest that the dissociation of His 752 is rather similarly affected by the pH inside or outside the cell and

thus in this respect behaves essentially similar to Glu 699 (Jennings, 1992), which shares this property with the histidine residue.

It has been discussed by Jennings (1992) that, perhaps, proton transport by Glu 699 due to this residue's stability may be able to alternate between two positions in which it is in contact either with the medium inside or outside the red cell. It would seem, however, equally likely that a proton may be transported to the location of the two amino acid residues from either membrane surface by a series of configurational changes in the transmembrane chain of potentially hydrogen-bonding histidines and glutamates discussed in the accompanying paper (Müller-Berger et al., 1995). This chain would include His 752 and Glu 699 inside the membrane and other amino acid residues closer to one or the other membrane surface, such as Lys 558 at the outer or His 837 at the inner surface. According to the model presented in Figure 7 of the accompanying paper, this configurational change could only occur if an anion is bound at the gap between His 852 and His 837 that completes the transmembrane chain of potentially hydrogen bonding groups. This would be in accord with a transport kinetics without slippage, which requires anion binding for the execution of the conformational change that leads to the reorientation of the substrate binding site from outward-facing to inward-facing as observed in band 3-mediated anion exchange [reviewed by Knauf (1989) and Passow (1986)].

*Conclusions.* The mutations H752S or E699D do not only lead to a partial inhibition but also to a change of the pH dependence of anion transport. The p$K$ value for chloride equilibrium exchange increases by a full p$K$ unit. Hence, in the unmodified membrane, there should be little overlap with the dissociation of other rate-controlling groups at pH values where His 752 or Glu 699 undergo the biggest changes of their state of protonation. Whatever overlap exists at more elevated pH values, it exerts only little influence on the physiological transport rate. This suggests that the physiological pH dependence at the acidic flank of the pH titration curve of band 3-mediated anion transport is dominated by the protonation of His 752 and Glu 699 with the single p$K$ value of 5.8, which most likely reflects the formation of a hydrogen bond between the two amino acid residues. It also shows that either one of the two mutations results in a change of the rate-limiting step, which is again pH dependent but with a single p$K$ of 6.9 instead of 5.8 as in the wild type. Such p$K$ value would not be inconsistent with the action of a histidine with a normal p$K$ that now assumes a rate-limiting role. The formation of the hydrogen bond in the wild type seems to take place inside the access channel to the substrate binding site and leads, parallel to the inhibition of anion transport, to a decrease of the line broadening of the $^{35}Cl$ NMR signal. This suggests that the stipulated hydrogen bond formation inhibits not only anion transport across the membrane but also anion exchange between the substrate binding site and the medium.

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