Inhibition of Mouse Erythroid Band 3-Mediated Chloride Transport by Site-Directed Mutagenesis of Histidine Residues and Its Reversal by Second Site Mutation of Lys 558, the Locus of Covalent H_2DIDS Binding†

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ABSTRACT: Substitution by site-directed mutagenesis of any one of the histidine residues H721, H837, and H852 by glutamine, or of H752 by serine, inhibits Cl^- flux mediated by band 3 expressed in Xenopus oocytes. Mutation of Lys 558 (K558N), the site of covalent binding of H_2DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonate) in the outer membrane surface, in combination with any one of the His/Gln mutations leads to partial (H721Q; H837Q) or complete (H852Q) restoration of Cl^- flux. In contrast, inhibition of Cl^- flux by mutation of proline or lysine residues in the vicinity of His 837 at the inner membrane surface cannot be reversed by the second-site mutation K558N, indicating specificity of interaction between Lys 558 and His 837. The histidine-specific reagent diethyl pyrocarbonate (DEPC) is known to inhibit band 3-mediated anion exchange in red blood cells [Izuhara, K., Okubo, K., & Hamasaki, N. (1989) *Biochemistry 28,* 4725−4728]. It was also found to inhibit transport after expression in the oocyte of wild-type band 3, of the double mutants of the histidines listed above, and of the single mutant H752S. The effects on the wild type and the double mutants were indistinguishable, while the mutant H752S exhibited a considerably reduced sensitivity to inhibition, suggesting that His 752 is the most prominent site of action of DEPC. According to a hydrophobicity plot of band 3 and further independent evidence, Lys 558, the mutated histidines, and Glu 699, the mutation of which was also found to inhibit Cl^- flux [Müller-Berger, S., Karbach, D., Kang, D., Aranibar, N., Wood, P. G., Rüterjans, H., & Passow, H. (1995) *Biochemistry 34,* 9325−9332], are most likely located in five different transmembrane helices. The interactions between Lys 558 and the various histidines suggest that these helices reside in close proximity. Together with the helix carrying Glu 699, they could form an access channel lined with an array of alternating histidine and glutamate residues. Together with a chloride ion bridging the gap between His 852 and His 837, they could have the potential to form, at low pH, a transmembrane chain of hydrogen bonds. The possible functional significance of such channel is discussed.

Previous attempts to explore the molecular mechanism of band 3-mediated anion exchange included the use of a variety of group specific chemical reagents. They suggested that lysines, arginines, histidines, and carboxylate groups are required for the normal functioning of the transport process (cf. Jennings, 1989; Knauf, 1989; Passow, 1986, 1992; Salhany, 1990; Tanner, 1993). The use of site-directed mutagenesis is a suitable tool to support these previous results and to add more detailed information concerning the location of the target sites and the nature of the effects produced.

The present paper deals with the analysis of the mutation of four histidine residues (H721, 752, 837, 852) in the hydrophobic, anion transporting domain of the erythroid band 3 protein (AE1) of the mouse. These residues are conserved in most of the 14 members of the band 3 family cloned and sequenced so far.

A possible role of at least one histidine residue was previously postulated by Hamasaki and co-workers (Izuhara et al., 1989; Hamasaki et al., 1990, 1992). They inferred from their findings with the histidine-specific reagent diethyl pyrocarbonate (DEPC)[1] that a histidine residue is responsible for the control of the pH dependence of anion exchange. In the present paper we show that mutation of any one of the four histidine residues examined leads to inhibition of chloride transport. The inhibition is partially or completely reversed when the mutation of each one of at least three of these histidine residues is accompanied by the additional mutation of Lys 558, the site of covalent binding of the anion transport inhibitor H_2DIDS (Bartel et al., 1989b; Kietz et al., 1991; Okubo et al., 1994). Although not required for the execution of anion transport, the lysine residue is allosterically linked to the transfer site (Wood et al., 1992; Passow et al., 1992). An attempt is made to explain the interactions among the transfer site, Lys 558, and the various

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[1] Abbreviations: H_2DIDS, 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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histidines on the assumption that, together with a number of glutamate residues and a substrate anion, they reside in an access channel to the substrate binding site formed by the helices 5, 8, 9, 10, and 13. We speculate that the formation of hydrogen bonds in this channel could explain the inhibition of anion equilibrium exchange with decreasing pH first described by Gunn et al. (1973) and, perhaps, the parallel appearance of chloride−proton cotransport postulated by Jennings (1978). The usefulness of some of these speculations is discussed in an accompanying paper by Müller-Berger et al. (1995).

MATERIALS AND METHODS

Transport Assay. The flux measurements were performed in single defolliculated Xenopus oocytes of stage 5 or 6 (Grygorczyk et al., 1989; Bartel et al., 1989a). Two to four days after microinjection of wild type or mutant band 3-cRNA (~30 ng per oocyte), 75 nL of $^{36}Cl^-$ (0.11 mCi/mL) was microinjected. A single oocyte was placed into a hairloop in a perfusion chamber, the bottom of which was formed by the mica window of a Geiger−Müller tube. The radioactivity released from the oocyte by band 3-mediated transport was continuously washed away with Barth's solution, pH 7.6, and the remaining radioactivity was recorded as a function of time. At the beginning and the end of each experiment, there were washing periods with Barth's medium containing the reversibly acting, band 3-specific inhibitor 4,4′-dinitrostilbene-2.2′- disulfonate (DNDS, 500 μM). They served to make sure that there was no $^{36}Cl^-$ released other than that originating from band 3.

The rate constants of efflux were calculated after digitalization and fitting a single exponential to the data by a nonlinear curve fitting procedure (Kietz et al., 1991).

Mutagenesis. All mutants were prepared from an expression plasmid containing wild type mouse band 3 [pSPT19-Bd3; see Bartel et al. (1989a)] or a derivative thereof (pSPT19-Bd3-5xmut) which contained five silent mutations introducing additional restriction sites into the wild type cDNA (S. Lepke and H. Passow, unpublished results).

For the preparation of the mutant H752S, a 43 bp *Mlu*I/*Sty*I segment was replaced by a phosphorylated DNA cassette in which the codon for H752 (CAC) had been converted to the codon for a serine residue (TCC). For the mutations H837Q or H837R, a 90 bp *Bam*HI/*Sph*I segment of band 3 in pUC 18 was replaced by cassettes in which the codon of H837 (CAC) was converted to codons for R (CGG) or Q (CAG), respectively. Since the 3′ end of band 3 contains an additional restriction site for *Sph*I, a 523 bp segment encoding the C-terminal region of band 3 had to be added at the *Sph*I restriction site. Finally, the full length band 3 cDNA was inserted into the plasmid pSPT19 using the restriction sites *Eco*RI and *Hin*dIII.

The mutants H721Q and H852Q were obtained using PCR methodology (Higuchi, 1990). For the former mutation the plus strand mutagenesis primer oMB1 and the minus strand primer oMB2, complementary to each other, were used (for primer sequences and positions, see Table 1). In both oligonucleotides the H721-encoding triplet (CAC) and its complementary sequence (GTG) is mutated to the codon CAG, coding for glutamine. To introduce the mutation into the wild type sequence, mutagenesis primer oMB1 was used in combination with the downstream vector primer T7 and

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

	+2156	+2175	strand
oMB1	5′-GCTTCCAGCTGGACCTGTTG-3′		plus
	+2173	+2154	
oMB2	5′-ACAGGTCCAGCTGGAAGCCA-3′		minus
	+1372	+1390	
oMB3	5′-GAGCTGCTCATCTCCACAG-3′		plus
	+2548	+2566	
oMB5	5′-CGCATGCAGCTCTTCGCGGC-3′		plus
	+2565	+2546	
oMB6	5′-CGTGAAGAGCTGCATGCGCC-3′		minus
	+2106	+2086	
T7 vector primer	5′-TAATACGACTCACTATAGGGAGA-3′		

[a] Mutated sites are underlined.

oMB2 with the upstream primer oMB3. The resulting amplified fragments had lengths of 870 (oMB1-T7 primer) and 803 bp (oMB3-oMB2). They were purified by gel electrophoresis and used as templates for a further PCR reaction. After denaturation, the plus strand of fragment oMB3-oMB2 and the minus strand of the fragment oMB1-T7 primer were allowed to hybridize within the short complementary sequence encoding the mutation H721Q and the flanking amino acids and amplified using the far 5′ and 3′ primers oMB3 and T7 vector primer. The fragment obtained had a length of 1673 bp.

The mutation H852Q was performed analogously. The primer combinations in the first amplification reaction were oMB5-T7 vector primer and oMB3-oMB6. The generated fragments had lengths of 480 and 1193 bp, respectively. The final amplification product encoding the mutation H852Q was again obtained with the primers oMB3-T7 primer, resulting in a DNA fragment with a length of 1693 bp. Subsequently both fragments were subcloned into the *Sma*I site of pUC 18, and the mutations were verified by DNA sequencing. To insert the mutated fragments into wild type mouse band 3 cDNA cloned in the transcription vector pSPT19, the fragment encoding the mutation H721Q was cut out of the pUC18 plasmid with *Bsm*I and *Bst*XI and inserted into pSPT19-Bd3 pretreated with the same restriction enzymes. The H852Q fragment was cut out of the pUC18 with *Hin*dIII and *Bst*XI and inserted into pSPT19-Bd3 pretreated with the same two restriction enzymes.

The double mutants were prepared from the single mutants described above and the single band 3 mutant K558N described in a previous publication (Bartel et al., 1989a). To obtain {K558N,H721Q}, pSPT19 (K558N) and pSPT19 (H721Q) were restricted with *Xma*I and *Bst*XI, and the corresponding fragments were ligated. To obtain {K558N, H852Q}, the plasmids pSPT19 (K558N) and pSPT19 (H852Q) were cut with *Xma*I and *Hin*dIII. The corresponding fragments were ligated to form pSPT19 {K558N,

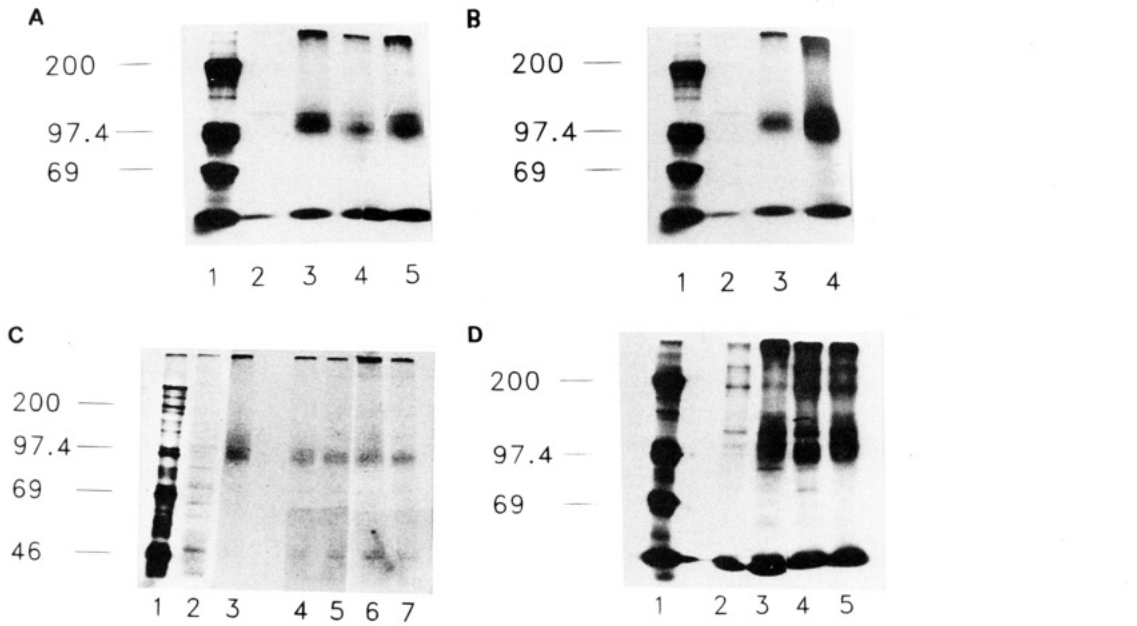

FIGURE 1: SDS−polyacrylamide gel electropherograms of immunoprecipitates of wild type erythroid band 3 and band 3 mutants expressed in *Xenopus* oocytes. (A) Wild type, H721Q, and {K558N, H721Q}. Lane 1: radioactive molecular weight markers (Rainbow marker, Amersham, Germany). Lane 2: control oocytes, no cRNA injected. Lane 3: oocytes with cRNA encoding wild type band 3. Lane 4: oocytes with cRNA encoding mutant H721Q. Lane 5: oocytes with cRNA encoding mutant {K558N,H721Q}. (B) Wild type and H752S. Lane 1: radioactive molecular weight markers. Lane 2: control oocytes, no cRNA injected. Lane 3: oocytes with cRNA encoding wild type. Lane 4: oocytes with cRNA encoding mutant H752S. (C) Wild type and mutants H837Q, H837R, {K558N,H837Q}, and {K558N, H837R}. Lane 1: radioactive molecular weight markers. Lane 2: control oocytes, no cRNA injected. Lane 3: oocytes with cRNA encoding wild type band 3. Lane 4: oocytes with cRNA encoding mutant H837R. Lane 5: oocytes with cRNA encoding mutant {K558N,H837R}. Lane 6: oocytes with cRNA encoding mutant H837Q. Lane 7: oocytes with cRNA encoding mutant {K558N,H837Q}. (D) Wild type, H852Q and {K558N, H852Q}. Lane 1: radioactive molecular weight markers. Lane 2: control oocytes, no cRNA injected. Lane 3: oocytes with cRNA encoding wild type. Lane 4: oocytes with cRNA encoding mutant H852Q. Lane 5: oocytes with cRNA encoding mutant {K558N,H852Q}.

H852Q}. Finally, to obtain {K558N,H837R} or {K558N, H837Q), the plasmids were restricted with *Bst*XI and *Hin*dIII, and the wild type sequence was replaced by the corresponding sequence of the mutant.

Protein Expression. All mutations were verified by sequencing, and it was shown that appropriate translation products were obtained in a cell free reticulocyte lysate. The expression of band 3 or its mutants in the oocytes was verified by immunoprecipitation by the method described previously (Grygorczyk et al., 1989). Briefly, each cRNA species was microinjected into 10 oocytes. Each batch of 10 oocytes was incubated in Barth's medium containing 50 μCi of ^{35}S-labeled methionine for at least 2 days. Subsequently each batch of oocytes was homogenized. After immunoprecipitation with polyclonal anti-mouse band 3 antibodies and purification of the precipitated protein, the total amount of recovered protein was subjected to SDS−polyacrylamide gel electrophoresis. The electropherograms were placed on X-ray films for 1−2 weeks. After microinjection of strictly standardized quantities of cRNA (accuracy about ±5%) and simultaneous incubation under identical conditions, we observed in all cases the appearance of wild type band 3 and of all the mutants used in this work (Figure 1).

Unfortunately, it was not possible to determine quantitatively the fraction of synthesized band 3 that appeared in the plasma membrane. For example, using a procedure similar to that described by Garcia and Lodish (1989) for human band 3, we did not succeed to raise antibodies against H_2DIDS-labeled mouse band 3 that reacted with sufficient specificity with H_2DIDS to permit the quantitative determination of the appearance of H_2DIDS binding sites in the outer surface of the plasma membrane. Moreover, in many of our experiments we used mutants in which the site of covalent H_2DIDS binding (Lys 558) was substituted by an asparagine residue and hence could no longer be used to generate anti-H_2DIDS antibodies. We also tried a method described by Tanner (1993) which involves the determination of the fraction of band 3 that can be hydrolyzed into two fragments by externally applied chymotrypsin. In a number of experiments with wild type band 3 we observed that about 20−30% of the total band 3 expressed in the oocyte appeared in the plasma membrane. However, during incubation with the high concentration of chymotrypsin required (1 mg/mL), in an unacceptably large fraction of the experiments, the oocytes became leaky during the incubation period (1 h). We finally designed band 3 mutants which contained in the interhelical loop between helices 5 and 6 an insert that consisted of the antigenic determinant for a powerful antibody against a synthetic peptide of an AE2 protein and which was flanked at each end by several glycine residues. Unfortunately, we obtained no measurable binding of the externally applied antibody. Detailed studies of Staub (Ph.D. Thesis, Frankfurt, 1994) showed that within 3 h after microinjection of cRNA encoding wild type band 3 or abnormally spliced band 3 mutants, the biosynthesis of the protein can be demonstrated in histological slices of the oocytes by immunofluorescence techniques. After that time, much of the wild type band 3 appears in the region of the plasma membrane and stays there for at least another 2 days. The abnormally spliced band 3 mutants are not exported to the plasma membrane. They remain in the vicinity of the nucleus and are degraded within

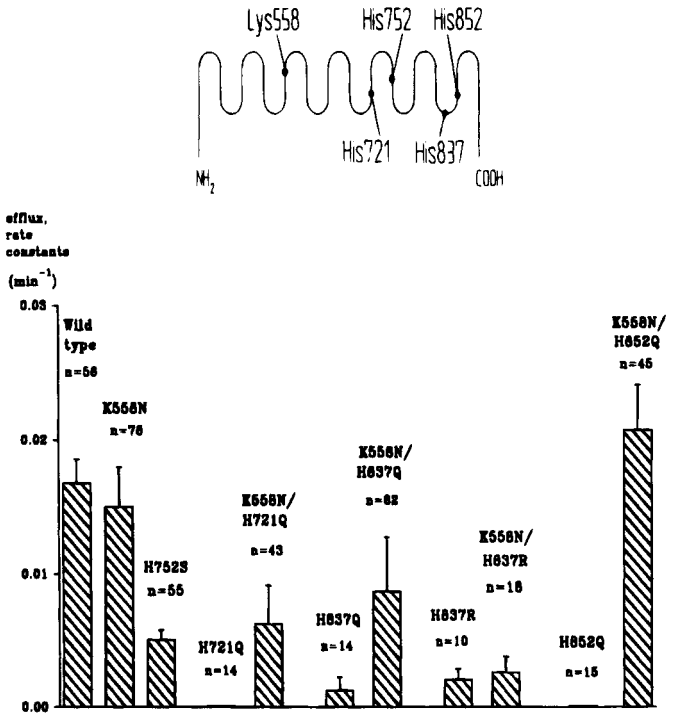

FIGURE 2: Effect of mutations of conserved histidine residues on band 3-mediated Cl^-/Cl^- exchange. Ordinate: rate constant of $^{36}Cl^-$-efflux (min^{-1}) mediated by wild type band 3 and mutants, Barth's medium, pH 7.6. The error bars indicate the standard deviation (SD). The insert depicts a schematical representation of the membrane spanning domain of the band 3 protein to indicate the location of the mutated histidine residues. The double mutant {K558N,H752S} was not prepared.

less than 1 day. In the experiments presented in this paper we incubated the oocytes after microinjection of the cRNA's for at least 2 days and then verified the survival of the protein by immunoprecipitation. In additional experiments (not shown) with mutants produced by substitution of one single amino acid residue (e.g., with the mutant R509T) we found that after 3 h the mutated protein was able to execute anion transport, similar to the wild type band 3 as measured after the same time period. However, in contrast to the wild type, the transport activity of the mutant disappeared within the next 20 h, indicating that even in the plasma membrane incorrectly folded band 3 protein is susceptible to premature proteolysis.

In view of the findings described, it is clear that those of our mutants in which transport is altered but not abolished (e.g., H752S and H837Q) are inserted into the plasma membrane. It is also quite likely that the other mutants, which show no transport activity but also survive for more than 2 days in the oocyte (e.g., H721Q and H852Q), are properly exported to the plasma membrane and inserted. In the discussion presented below we assume, therefore, that even in these mutants the inhibition and "revival" of the transporter by the second site mutation K558N are consequences of mutagenic replacement of the selected amino acid residues on band 3-mediated transport rather than on export or folding of the transport protein. Nevertheless, in these cases a caveat remains.

RESULTS

Mutation of either One of the Histidine Residues 721, 752, 837, and 852 Reduces Chloride Self-Exchange Flux

Figure 2 shows the effect of mutation of the histidine residues 721, 752, 837, and 852. In all instances, transport is reduced. In the mutants H721Q and H852Q little if any detectable transport is left, while in the mutants H752S and H837Q (or H837R) some residual transport persists.

Mutation of Lys 558 Enhances the Reduced Chloride Self-Exchange Flux Mediated by Band 3 with Mutated Histidine Residues

After mutation of Lys 558 to an asparagine residue, the capacity of band 3 for covalent binding of H_2DIDS is lost (Bartel et al., 1989a,b; Kietz et al., 1991). Nevertheless, the mutant is still able to accomplish anion exchange. Figure 2 demonstrates that the additional mutation of Lys 558 increases the anion exchange in the mutants H721Q and H837Q to easily measurable levels and in the mutant H852Q to at least the same high level as in the wild type. It should also be mentioned that the "revival" of the transport activity by the "second site mutation" K558N is not seen with the mutant {K558N, H837R}.

Mutation of Proline or Lysine Residues near H837 Leads to Inhibition of Anion Equilibrium Exchange: This Cannot Be Reversed by Second Site Mutation K558N

The endofacial loop connecting the putative transmembrane helices 12 and 13 contains, in addition to His 837, prolines and lysines (Figure 3) that are important for the maintenance of the transporter in a functional state (Kay et al., 1990; Hamasaki et al., 1992; Schnell, 1993 personal communication). This suggested that any distortion of the organization of this loop rather than the specific substitution of H837 by asparagine or arginine would be responsible for the alteration of anion exchange. The effects are, however, rather specific with respect to the nature of mutation. Only mutations of specific combinations of prolines or lysines in the near neighborhood of H837 are associated with inhibition of anion exchange (Table 2). For example, in the double

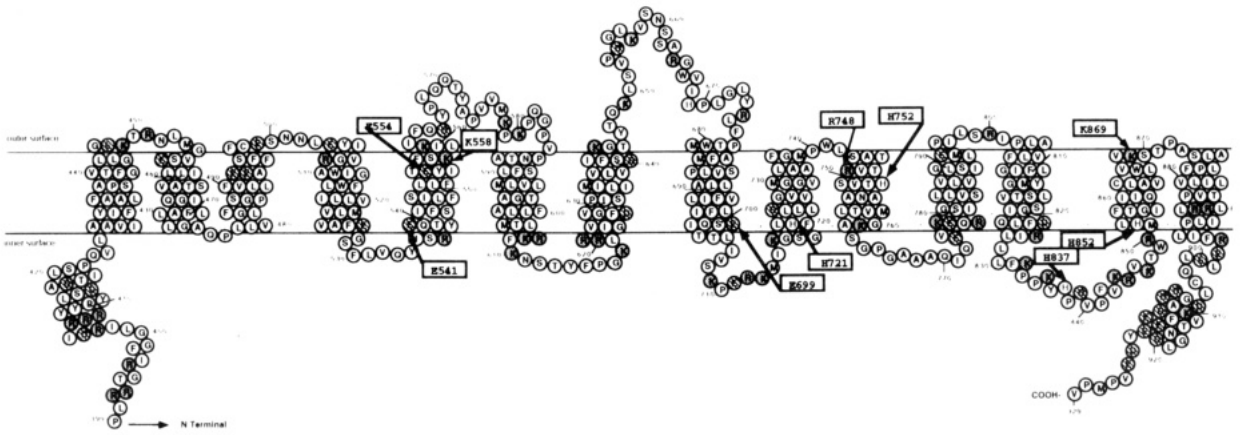

FIGURE 3: Amino acid sequence of the hydrophobic, anion-transporting domain of mouse band 3. Folding pattern essentially derived from hydrophobicity plot [according to Wood (1992)].

Table 2: Effect on Anion Transport of Mutations at the Endofacial Loop Connecting Transmembrane Helices 12 and 13

mutant	efflux, rate constant (min^{-1}) ± SEM	*n*
wild type	0.0128 ± 0.0005	6
{K832D,K835D,K844D,K847D}	<0.0001	8
{K832D,K835D}	<0.0001	7
{K844D,K847D}	0.0120 ± 0.0022	15
K832N	0.0054 ± 0.0006	10
K835D	0.0031 ± 0.0004	14
K835C	0.0113 ± 0.0014	13
K835G	0.0091 ± 0.0014	9
{P833V,P834V,P838V,P841V}	<0.0001	12
{P833V,P834V}	0.0048 ± 0.0008	5

mutant {K832D,K835D} anion transport is abolished, while in the double mutant {K844D,K847D} transport remains essentially unaltered. The single mutation of either K832 or K835 does not suffice to abolish transport. In the case of the prolines, mutation of P833 and P834 is not strongly inhibitory. The effect appears only if, in addition to P833 and P834, P838 and P841 are also mutated (Table 2).

Although these findings suggested already that the individual amino acid residues play rather specific roles, we explored whether or not an allosterically induced revival of anion transport by the second site mutation K558N can also be seen in the double mutant {K832D,K835D}. We produced, therefore, the triple mutant {K558N,K832D, K835D} and found a complete abolishment of anion exchange (not shown). Thus, if an interaction exists between K558 in helix 5 and the loop interconnecting the putative membrane helices 12 and 13, then the nature of this interaction is quite specific for the individual amino acid residues: the inhibition by the single mutation H837Q is partially relieved, while the inhibition by the double mutant {K832D,K835D} remains unaffected.

Identification of Sites of Action of Diethyl Pyrocarbonate

Determination of the Rate of Inactivation of Band 3-Mediated Chloride Transport by DEPC. DEPC is able to inhibit anion exchange reversibly as well as irreversibly. The latter effect can only be derived from measurement of the rates with which the agent produces irreversible inhibition. Using wild type band 3 and the mutants H752S and K558N, we determined the rate of inactivation at a DEPC concentration of 800 μM that causes maximal reversible inhibition (Table 3). In these experiments, the rate of irreversible inactivation

Table 3: Effect of Various Mutations of Band 3 on (i) Rate Constants (k_b) for the Rates of Irreversible Inactivation of the Transporter by DEPC and (ii) Residual Cl^- Efflux (ϕ_∞) after Maximal Irreversible Inhibition of the Transporter[a]

mutant	k_b (min^{-1}) ± SEM	ϕ_∞ (%) ± SEM
wild type	0.123 ± 0.01	5.2 ± 2.0
K558N	0.140 ± 0.02	24.1 ± 2.3
{K558N,H721Q}	0.133 ± 0.01	8.3 ± 2.1
{K558N,H837Q}	0.205 ± 0.04	21.1 ± 2.1
{K558N,H852Q}	0.229 ± 0.03	7.3 ± 1.4
H752S	0.041 ± 0.0046	21.8 ± 3.2

[a] The residual Cl^- effluxes are represented as a percent of the inhibited flux. 800 μM DEPC in Barth's solution, pH 7.6, 23 °C.

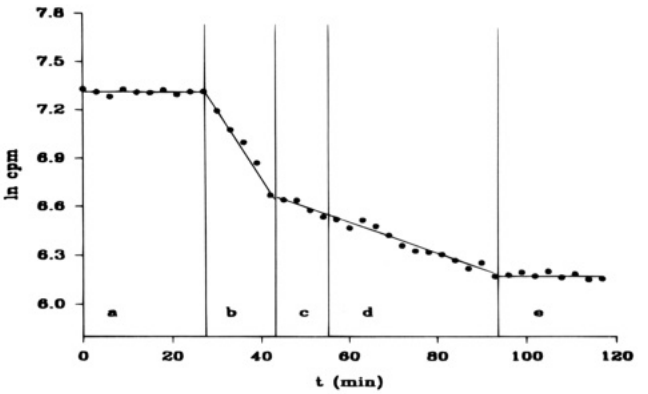

FIGURE 4: Protocol for the measurement of the inhibition by DEPC of band 3-mediated chloride efflux. After superfusion with Barth's medium containing DNDS (time period a), superfusion was continued in the same medium without DNDS (b). Subsequently there followed a superfusion period of variable length with DEPC present (c). In this example, the DEPC concentration was 800 μM, the time of superfusion 10 min. After this period, efflux was followed in the absence of DEPC (d). The experiment was terminated by a superfusion period in the absence of DEPC and in the presence of DNDS. The Barth's medium contained 90 mM NaCl, pH 7.6. The DNDS concentration was always 500 μM. The oocyte contained wild type band 3.

was determined by interrupting the exposure to DEPC after suitable time intervals and measuring the rate of $^{36}Cl^-$ efflux into a DEPC- free Barth's medium (Figure 4). To minimize the effects of spontaneous decomposition of DEPC (first order rate constant of 0.029 min^{-1} at pH 7.6) in the course of the reaction with band 3, the DEPC-containing Barth's solutions used for the incubation of the oocytes were replaced by Barth's solutions containing freshly dissolved DEPC every 15 min. Following this protocol, at the DEPC concentration

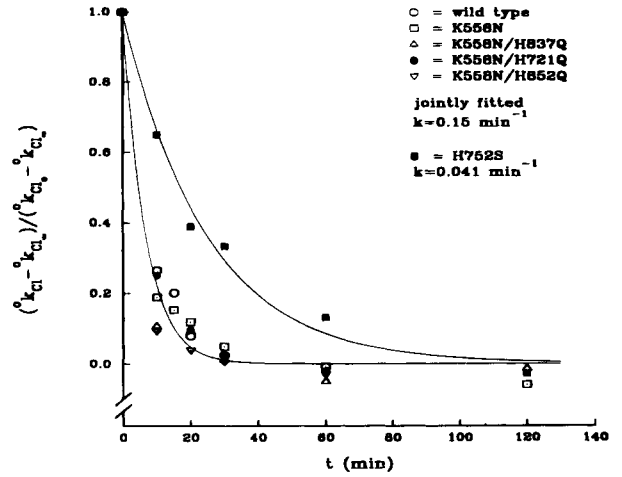

FIGURE 5: Rate of inactivation by 800 μM DEPC of wild type band 3 and the band 3 mutants indicated in the figure. The ordinate represents the progress of the change of the rate constants for efflux, the abscissa the time of exposure to DEPC prior to the efflux measurements in the absence of DEPC. The drawn curve depicts a nonlinear least-squares fit to the data of the equation $(k_{Cl,t} - k_{Cl\infty})/(k_{Cl0} - k_{Cl\infty}) = \exp(-k_b t)$, where $k_{Cl,t}$, k_{Cl0} and $k_{Cl\infty}$ indicate, respectively, rate constants of inactivation of band 3 as measured after exposure to 800 μM DEPC for times t, $t = 0$, and $t = \infty$.

used, the influence of spontaneous degradation of DEPC on the rate of inactivation of the transporter can be neglected. The results show that even after the longest time of exposure where the transporter is maximally inhibited, it is still able to execute some transport. The magnitude of this residual transport depends on the nature of the mutant used.

Susceptibility to Inhibition by DEPC of Cl^- Transport Mediated by Band 3 Mutants in Which the Original Transport Inhibition Had Been Relieved by a "Second Site Mutation" of the Transporter. Since the more or less complete inhibition of anion transport by the mutation of the single histidines H721, H837, and H852 precludes the study of their sensitivity to modification by DEPC, we have performed one series of experiments in which the transport activity of mutants in which the individual histidine residues had been replaced by glutamine residues was restored by the second site mutation K558N. They are summarized in Table 3 and Figure 5. In each case, DEPC was still capable of producing inhibition, although there were differences of the magnitudes of the efflux that survives at maximal inhibition. There were also differences with respect to the rate of inactivation of the transport activities. The rate of inactivation was either unaltered ({K558N,H721Q}) or somewhat enhanced ({K558N,H837Q} and {K558N,H852Q}). The only case where a significant reduction of the rate of inactivation of the transporter occurred was the single mutant H752S. This is most clearly seen in Figure 5, where all data obtained in these experiments were normalized with respect to the maximal inhibition at infinite time. The data points of the wild type and all mutants used follow essentially the same pattern, except those of the mutant H752S which fall on a curve pertaining to a rate constant that amounts to about one quarter of the value obtained for the composite of the other points. It seems therefore, that His 752, which is involved in the control of the pH dependence of anion transport [see the accompanying paper by Müller-Berger et al. (1995)], is also most susceptible to modification by DEPC, although an allosteric effect cannot be excluded entirely. This residue is, therefore, most likely responsible for the previous findings of Hamasaki and co-workers (1992). The data also leave no doubt that His 752 is not the only site of action of DEPC. It is most likely that the other histidines and/or possibly the serine residue itself that was used to substitute for His 752 are also involved.

Table 4: Influence of the Presence of the Reversibly Binding Anion Transport Inhibitor DNDS (500 μM) on the Inhibition by DEPC (800 μM) of Band 3-Mediated Cl^- Efflux[a]

	action of 800 μM DEPC in the absence of 500 μM DNDS		action of 800 μM DEPC in the presence of 500 μM DNDS	
mutant	% efflux ± SEM	n	% efflux ± SEM	n
wild type	4.4 ± 1.4	10	41.8 ± 1.1	11
K558N	23.5 ± 2.3	13	50.6 ± 4.5	8
{K55N,H837Q}	16.7 ± 2.1	12	37.2 ± 3.5	10
H752S	32.1 ± 4.4	11	69.7 ± 3.7	12

[a] The reaction between the inhibitor and the transporter was allowed to proceed in the absence or presence of DNDS at 23 °C. After a 60 min incubation, the oocytes were washed, and Cl^- efflux was measured in the absence of the inhibitors. Barth's solution, pH 7.6

Protection against Irreversible Inhibition of Anion Transport When the Transporter Is Exposed to DEPC in the Presence of the Reversibly Binding Anion Transport Inhibitor 4,4'-Dinitrostilbene-2,2'-disulfonate (DNDS). Using red blood cells, Izuhara et al. (1989) observed that the rate of irreversible modification of the band 3 protein by DEPC is considerably reduced when the exposure to the agent takes place in the presence of DNDS. This suggests that the stilbene disulfonate binding site controls the access of DEPC to its site(s) of action. Similar experiments with band 3 expressed in the oocytes confirm this result. They show more specifically that the action of DNDS does not require the presence of Lys 558 at the stilbene disulfonate binding site, and that the protection afforded by the stilbene derivative is not confined to H752 but also includes other histidines (Table 4).

DISCUSSION

Nature of the Effects of Mutagenesis on Anion Transport

In the discussion presented below, the effects of site-directed mutagenesis are attributed to local effects of the substitution of a normally occuring amino acid residue by an amino acid residue of our choice. This assumption is, of course, not self evident since the substitution could affect the folding and orientation of the mutated protein in the lipid double layer and the export to and insertion in the plasma membrane. In the present paper, we relied on observations (see Materials and Methods) which suggest that band 3 protein that is not exported to or properly inserted in the plasma membrane is degraded in the Xenopus oocytes within less than 1 day. We performed, therefore, all measurements of anion transport in oocytes in which the presence of the band 3 protein could be demonstrated by immunoprecipitation 2 or more days after microinjection of the cRNA. In spite of this precaution, in those cases where the mutation leads to a complete cessation of anion transport, it is impossible to tell with absolute certainty whether or not this interpretation is always correct.

Inhibition by Single Site Mutation of the Individual Histidine Residues and Reversal of Inhibition by Second Site Mutation of Lys 558

To elucidate the possible role of specific histidine residues in band 3-mediated anion transport, we used site-directed mutagenesis and substituted each one of four conserved histidines (out of five) in the hydrophobic, anion-transporting domain of band 3 by a serine (H752) or a glutamine (H721, H837, H852) residue. This resulted in a partial (H752, H837) or complete (H721, H852) inhibition of Cl^- transport. The inhibition in the mutants H721Q, H837Q, and H852Q was partially (H721, H837) or completely (H852) prevented when each one of the mutations was combined with the mutation of Lys 558. Obviously, the "second site mutation" [a term introduced by Nelson and Douglas (1993) in conjunction with their work on yeast mitochondrial ADP/ATP translocator] K558N leads to a rearrangement of the helices in which the various histidine residues are located such that the structural requirements for the successful execution of the transport process are restored. The specificity of the effect of the second site mutation K558N is suggested by the observation that it does not relieve the inhibition of anion transport produced by the simultaneous mutation of two lysine residues in the near neighborhood of His 837 (K832D and K835D).

The persistence of some anion transport in the mutants H752S and H837Q and the recovery of the transport function in the double mutants shows that none of the four histidines studied is necessary for anion transport, but that, under physiological conditions, each one of them is needed to maintain the transporter in a functional state. This is obviously the result of allosteric interactions between the histidines and the transfer site. The transfer site is also allosterically linked to Lys 558, a site that is involved in noncovalent (Wood et al., 1992; Passow et al., 1992) and covalent (Bartel et al., 1989a,b; Kietz et al., 1991; Okubo et al., 1994) binding of inhibitory stilbene disulfonates. Thus, at least each one of the three histidines, from which double mutants had been made and in which a restoration of transport activity had been observed, is not only allosterically linked to the transfer site but also to Lys 558.

Mutagenesis of Histidines and the Action of the Histidine Specific Reagent Diethyl Pyrocarbonate on Anion Transport

Our results show that, after restoration of the transport in the histidine mutants by the second site mutation K558N, anion transport by each one of the double mutants could still be inhibited by DEPC. This ascertains that the agent did not produce its effect by modification of Lys 558, which has an abnormally low pK of about 8 (20 °C) (Kietz et al., 1991) and hence an unusually high reactivity with respect to a number of potent irreversibly acting inhibitors of anion transport (Passow, 1986). This supports the inference of Hamasaki et al. (1992) that the action of the agent is due to the modification of a histidine residue. Our results indicate further that in the double mutants {K558N,H721Q}, {K558N, H837Q}, and {K558N,H852Q} the susceptibility to inhibition by DEPC is nearly indistinguishable from that of the wild type band 3. In the mutant H752S, however, the rate of inactivation of anion transport by DEPC is considerably reduced. This suggests that the effects seen by Hamasaki et al. are largely due to a modification of His 752. Nevertheless, even after the mutation H752S, some inhibition still occurs. This may be due to reaction of DEPC with the mutagenetically introduced serine residue but is more likely the result of modification of one or several of the other histidines in band 3.

Speculations about the Functional Organization of Band 3

As a point of departure for our speculations we considered the possible consequences of the assumption that the observed allosteric relationships reflect close proximity of the amino acid residues involved in the maintenance of the transport activity. Close proximity of these residues can be expected if the helices carrying Lys 558 (helix 5), His 721 (helix 9), His 752 (helix 10), and His 852 (helix 13) form a bundle of adjacent transmembrane helices that together with at least one further helix may constitute a channel. A good candidate for such an additional helix would be helix 8. It carries Glu 699, which is known to be required for anion–proton cotransport (Jennings, 1992; Jennings & Smith, 1992) and has been shown to be involved, together with His 752, in the control of the pH dependence of chloride equilibrium exchange (Müller-Berger et al., 1995).

The intuition that in the native band 3 protein the functionally significant helices 5, 8, 9, 10, and 13 are located in close juxtaposition is supported by several pieces of evidence. Most instructive are the observations on the site and mode of action of stilbene disulfonates on transport and binding of the anions and the influence of these agents on the irreversible inhibition of anion transport by DEPC and WRK.

The action of the reversibly binding stilbene disulfonate DNDS leads to several different effects at a number of distinct locations in the transport protein that are separated by large distances in the amino acid sequence. The binding of the inhibitor involves two outward-facing lysine residues, K558 and K869 in helices 5 and 13, respectively. Neither one of the two residues is directly involved in substrate binding or transport, although weak allosteric relationships exist between the binding site of the inhibitor and the substrate binding site (Wood et al., 1992; Passow et al., 1992). The inhibitor arrests the substrate binding site in an outwardly-directed orientation (Barzilay et al., 1979; Fröhlich, 1982) and interrupts the exchange of the substrate between its bound and free state (Shami et al., 1977; Falke et al., 1984). In addition, access of irreversibly binding modifiers of anion transport to their respective binding sites is inhibited: WRK is no longer able to react with Glu 699 in helix 8 (Jennings, 1992), and DEPC is no longer able to react with His 752 in helix 10 and with at least one of the histidines H721 (helix 9), H837 (interhelical loop 12/13), or H852 (helix 13). Independent evidence for a close juxtaposition of His 752 and Glu 699 will be provided in the accompanying paper (Müller-Berger et al., 1995).

DEPC penetrates easily across the red cell membrane and exerts its inhibitory effect by approaching the transport protein from the cell interior (Hamasaki et al., 1992). This indicates that the protective action of DNDS on the histidines listed above is the result of an allosteric effect that is transmitted all the way across the membrane. Since both the binding of DNDS and the revival of the transport activity in the various histidine mutants (H721Q in helix 9, H837Q

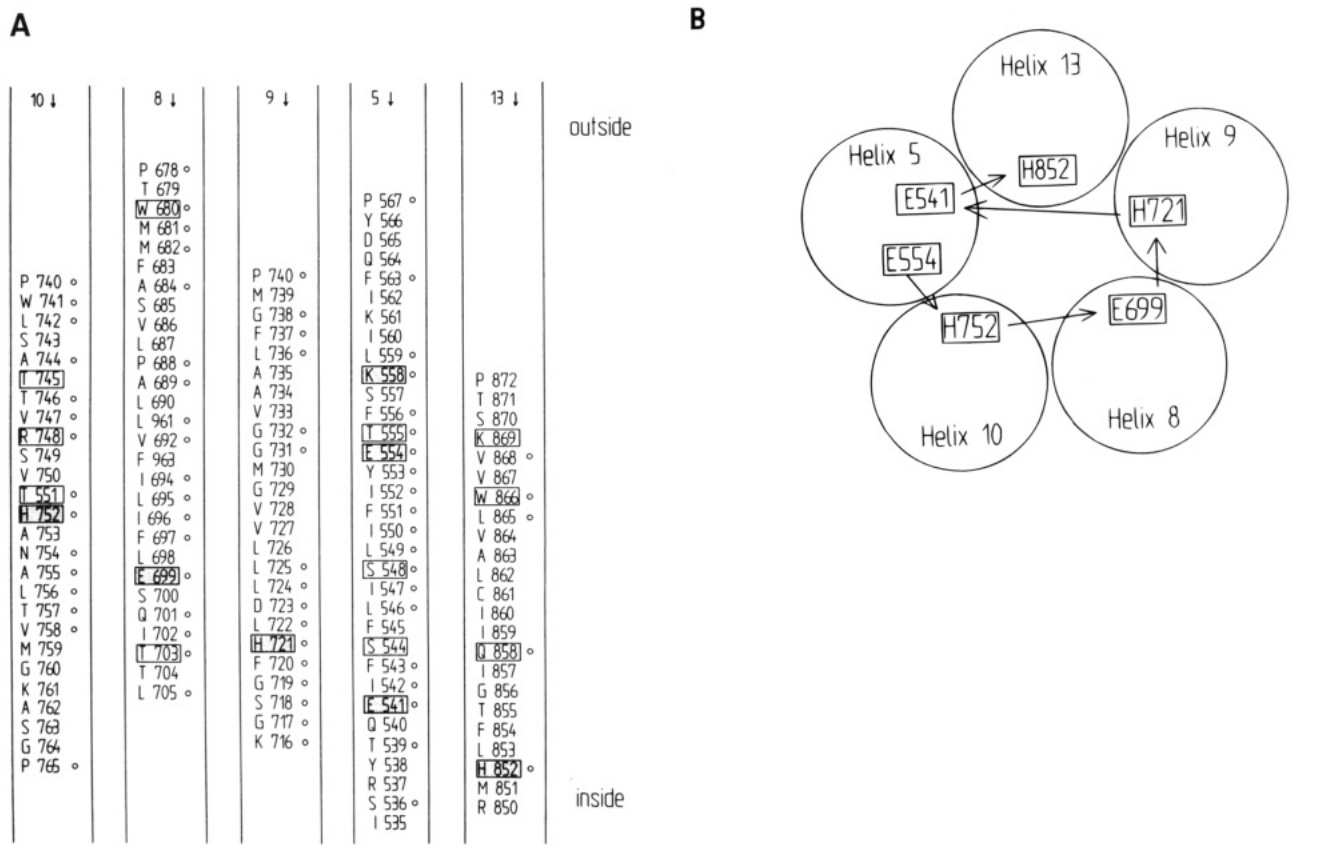

FIGURE 6: (A) Amino acid sequence of the five transmembrane helices containing the mutated amino acid residues K558, H721, H752, H852, and E699. The symbols for amino acid residues that, on account of their chemical nature and their position relative to each other should posses the *potential* to form hydrogen bonds are enclosed by rectangular boxes. (B) View from the top at the positions of the helices represented in panel A. When the amino acid sequences of the transmembrane helices 5, 8, 9, 10, and 13 are listed on separate strips of paper and shifted parallel to each other, it is feasible to align them in such a manner that there appears a transmembrane chain of alternating histidines and glutamates (A). In a helical wheel representation it appears that these residues should have the *potential* to form a chain of hydrogen bonds (B). This chain could be extended toward the outer membrane surface by inclusion of Lys 558 that should be located on top of E554. Near the inner membrane surface, H837 resides below H852. The gap between these two histidines could be bridged by a chloride ion which could have the *potential* to form hydrogen bonds with each one of them and with the neighboring lysine residues K832 and K835 (Figure 7). In panel A the side chains involved in forming the transmembrane array of hydrogen bonds are indicated by the shaded boxes. All further amino acid residues that are conserved in all band 3 molecules (designated by a small circle) and that are capable of hydrogen bond formation and oriented in the helices in the same direction as the histidines and glutamates are indicated by unshadowed boxes. One exception is H852, which is not fully conserved, but is replaced in 2 out of 14 members of the band 3 family by D or T, both of which have the *potential* to act, at sufficiently low pH, as H^+ donors to form hydrogen bonds with the bound anion (Figure 7). It should be noted that the arrangement of the helices in the figure (clockwise 13, 9, 8, 10, 5) is not the only one that would permit the establishment of the array of glutamate and histidine residues depicted in the figure.

in the loop between helices 12 and 13, and H852Q in helix 13) by the second site mutation K558N are associated with a change of the state of Lys 558, it would seem most likely that both cases are reflections of the same allosteric mechanism.

Although by no means conclusive, the observations summarized above support the contention that helices 5, 8, 9, 10, and 13 are constituents of a bundle of adjacent transmembrane helices with functionally important side chains. These helices could form an access channel to the transfer site and cause the observed allosteric effects with a minimum of disturbance of the protein structure.

Mutual interrelationships between the binding of eosin maleimide to Lys 449 in helix 1 and the stilbene disulfonates are well known [e.g., Cobb and Beth (1990) and Passow et al. (1992)]. This has been interpreted to indicate the overlap of the binding site for the two inhibitors. Thus, instead of five helices, the hypothetical access channel may consist of 6 helices. However, according to interesting speculations of Low and co-workers (1994), the first four transmembrane helices of band 3 may form a separate bundle. It could be located close to the bundle described here, perhaps with helix 1 and helix 5 in close juxtaposition, or with helix 1 as a common constituent of both bundles.

Putative Transmembrane Chain of Potentially Hydrogen Bond-Forming Amino Acid Side Chains

Using helical wheel representations, it can be demonstrated that, in a pore formed by the transmembrane helices 5, 8, 9, 10, and 13, an array of alternating lysines, histidines, and glutamic acid residues could exist. Together with a bound anion, these side chains should have the *potential* to form a chain of hydrogen bonds traversing the thickness of the plasma membrane (Figures 6 and 7). This observation invites speculations about the origins of (i) the pH dependence of anion transport, (ii) the proton−anion cotransport, and (iii) the "revival" of the inhibited anion transport in the various histidine mutants.

pH Dependence of Anion Transport. At physiological pH, the hydrogen bonds may be broken. This could be a requisite for anion exchange to take place. With decreasing pH, the

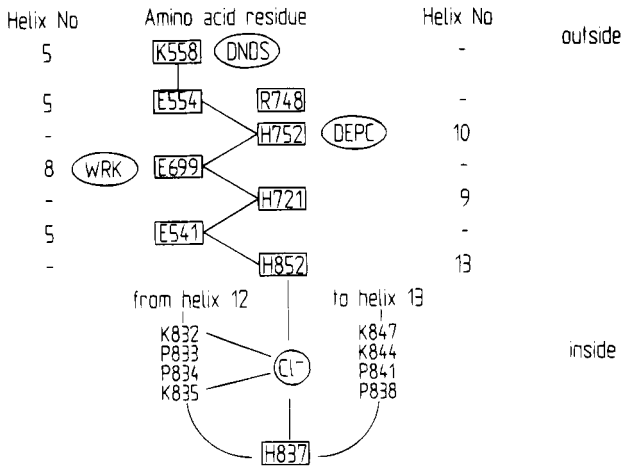

FIGURE 7: Schematic representation of the locations of the amino acid residues discussed in the text and in the legend to Figure 6. The location of the substrate binding site corresponds to more or less explicit suggestions made by Kay et al. (1990) and Hamasaki et al. (1992) and most detailed by Schnell (1994, personal communication). The drawn lines connect amino acid residues that may have the *potential* to form hydrogen bonds at sufficiently low pH.

formation of hydrogen bonds should become feasible with a concomitant inhibition of anion exchange. This hypothesis would provide a simple explanation of the pH dependence of monovalent anion transport in the pH range below pH 8.5. It could, perhaps, even explain the pH dependence of divalent anion transport if one takes into account that the divalent anions are cotransported with a proton, and that the inhibitory effect of hydrogen bond formation in the band 3 protein at low pH is superimposed on the accelerating effect of the cotransported hydrogen ions. In an accompanying paper, we provide some evidence that a hydrogen bond between His 752 in helix 10 and Glu 699 in helix 8 may in fact dominate the pH dependence of chloride transport (Müller-Berger et al., 1995).

Of course, the function of the histidines would not be confined to such regulatory role. The inhibition caused by their mutagenesis indicates that, *in the band 3 wild type*, each one of them is required for the maintenance of the chloride/chloride exchange over the range of high pH values, where hydrogen bonding is not likely to be of measurable significance.

Anion−Proton Cotransport. It has been suggested by Jennings (1978) that the band 3-mediated pH equilibration seen *in the absence of CO_2 and bicarbonate* may be the consequence of a H^+-Cl^- cotransport [which should not be confused with the $H^+-SO_4^{2-}$ cotransport described by Jennings (1976)] that takes place at a rate several orders of magnitude more slowly than the Cl^-/Cl^- equilibrium exchange. Perhaps the inhibition of the chloride self exchange by lowering the pH is associated with a conversion of the band 3 protein from a rapidly acting Cl^-/Cl^- exchanger to a much more slowly operating H^+-Cl^- cotransporter. This would be associated with an enhancement of the rate of band 3-mediated pH equilibration. However, even at its maximum rate, the H^+-Cl^- cotransport would still be very much smaller than the original Cl^-/Cl^- exchange and hence still be compatible with the observed inhibition of Cl^- exchange. In the case of H^+-Cl^- cotransport, the chloride ion should be firmly bound in the three- or four-coordinated state in the niche formed by Lys 832, Lys 835, His 837, and His 852. The occasional release of such bound Cl^- could destabilize proton binding to one of these residues and hence induce the release of a proton at the substrate binding site.

Revival of Anion Transport by the Second Site Mutation K558N. Above, the revival of the transport activity by the second site mutation K558N was simply taken as an useful indicator for the existence of functionally important relationships that suggest the close proximity of the amino acid residues involved. The nature of the realignment process that leads to functional recovery remains unknown. Nevertheless, the following observations may serve as a guide for further enquiry.

The mutations that lead to a partial inhibition of transport (H752S, H837Q) or to a partial or complete inhibition which could be reversed by the second site mutation K558N (H721Q, H837Q, H852Q) involve replacements of histidines by an amino acid residue that, like the original histidines, is able to participate in hydrogen bond formation. This is also true for the asparagine residue that substituted for Lys 558. If one assumes that each one of the histidines is localized in the transmembrane helices such that, at sufficiently low pH, it is able to form hydrogen bonds with suitable proton acceptors, one could speculate that as a consequence of the second site mutation K558N the small disarray caused by the substitution of the histidine by a glutamine would be corrected by minute movements of adjacent helices in the direction of and/or around their longitudinal axes until they are oriented like the histidines for which they substitute. The glutamines should now assume positions in which, like the original histidines that they replace, they gain the *potential* to form hydrogen bonds. This may be the structural requirement for the observed recovery of the Cl^-/Cl^- exchange by the second site mutation. In this context it seems interesting to recall that the inhibition seen in the mutant H837R cannot be reversed by the second site mutation K558N. Perhaps this is related to the fact that the hydrogen bonding capacity of the arginine residue differs considerably from that of the glutamine in the mutant H837Q, in which the reversal is easily seen (Figure 2).

Conclusions

Our experiments show that each one of the four conserved histidines in the hydrophobic domain of band 3 is necessary for the normal execution of the anion exchange. They seem to reside in three different helices (9, 10, 13) which, together with the helices that contain K558 (helix 5), the site of covalent H_2DIDS binding, and E699 (helix 8), the site of action of Woodward's reagent K, may form a channel which guides the anion to the substrate binding site. The channel would be lined with an array of alternating histidyl and glutamate residues with the potential to form, at appropriate pH, a transmembrane chain of hydrogen bonds (Figure 7). The chain would include Lys 558 and Lys 869 in helices 5 and 13, respectively, His 752 and Glu 699 in helices 10 and 8, respectively, and His 721 in helix 9. Near its outwardly-pointing orifice, the chain could end with the potential to form a hydrogen bond between Glu 554 and Lys 558. Near the inwardly-pointing orifice, it could terminate at the substrate binding site at which the potential exists for hydrogen bond formation between the substrate anion as an acceptor and three or four donors including His 852 in helix 13 and His 837 as well as the lysine residues 832 and 835 in the loop interconnecting helices 12 and 13.

The functional significance of such a transmembrane array of potentially hydrogen bond-forming amino acid residues is not yet clear. It would seem plausible to assume, however, that the actual formation of a hydrogen bond within the array (e.g., between His 752 and Glu 699) is responsible for the inhibition of the rapid chloride−chloride equilibrium exchange at low pH (Müller-Berger et al., 1995) and, perhaps, that the completion of the transmembrane chain of hydrogen bonds and the rise of configurational changes accounts for the appearance of the slow proton−chloride cotransport discussed by Jennings (1978).

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