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Role of Lys 558 and Lys 869 in Substrate and Inhibitor Binding to the Murine Band 3 Protein: A Study of the Effects of Site-Directed Mutagenesis of the Band 3 Protein Expressed in the Oocytes of *Xenopus laevis*

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Summary. The effect of mutation of either Lys 558 or Lys 869 or both on mouse erythroid band 3 protein (AE1)-mediated $^{36}Cl^-$ efflux and its inhibition by pyridoxal 5-phosphate (P5-P), DNDS and H_2DIDS were studied. Regardless of the mutation, band 3 was always capable of executing Cl^- self-exchange. P5-P (5 mM, pH 7.6) produced irreversible inhibition in the wild type (*KK*) and in the mutant in which Lys 558 (*NK*) or Lys 869 (*KM*) had been replaced by asparagine (*N*) or methionine (*M*), respectively. However, when both residues were replaced, mutant (*NM*), irreversible inhibition could no longer be achieved. This shows that P5-P is capable of producing inhibition with either one of the lysine residues, 558 or 869.

Inhibition by DNDS changed dramatically upon mutation. The $K_{i\,app}$ increased from 6.0 μM in the wild type (*KK*) to 23 μM in the mutant *NK*, to 73 μM in the mutant *KM* and to 474 μM in the double mutant *NM*. The K_m value for activation of the transport system by varying the substrate concentration by isosmotic substitution of Cl^- with SO_4^{2-} decreased from 42 mM in the wild type (*KK*) to 11.3 mM in the mutant *NM*. The results show that both Lys 558 and Lys 869 are involved in the maintenance of the structure of the overlapping binding sites for stilbene disulfonates and the substrate Cl^-.

In the double mutant *NM*, H_2DIDS is no longer able to produce irreversible inhibition at pH 7.6. This is evidently related to the replacement of Lys 558 (pK 8.2) by Asn 558 in this mutant (*see* Bartel, D., Lepke, S., Layh-Schmitt, G., Legrum, B., Passow, H., 1989. *EMBO J.* **8**:3601–3609). However, at pH 9.5, some irreversible inhibition could still be observed. This suggests that the other lysine residue (pK 10.8) that is known to be involved in covalent binding with the second isothiocyanate group of H_2DIDS is still present, and hence, not identical to Lys 869, which had been substituted by a methionine residue. However, this result remains inconclusive since after mutagenesis, the H_2DIDS may produce inhibition at a site that is not normally involved in H_2DIDS binding.

Key Words band 3 · expression · anion transport · red cell · *Xenopus* oocytes · mutagenesis · stilbene disulfonates

Introduction

Stilbene disulfonates belong to the most specific inhibitors of erythroid band 3-mediated inorganic anion transport (Cabantchik & Rothstein, 1974). They produce inhibition at a stoichiometric ratio of one mole inhibitor per mole of band 3 (Jennings & Passow, 1979). Some of these inhibitors (e.g., DNDS, DAS, DBDS)[1] bind reversibly (Barzilay & Cabantchik, 1979). Others bind irreversibly after initial reversible binding (e.g., SITS, DIDS, H_2DIDS; Cabantchik & Rothstein, 1974; Lepke & Passow, 1976; Kampmann et al., 1982). Inhibition kinetics suggest competition with the substrate anion for the transfer site (Shami, Rothstein & Knauf, 1978; Fröhlich, 1982). Stilbene disulfonate binding to band 3 is associated with a line broadening of the $^{35}Cl^-$ NMR signal, which has been interpreted as a release of Cl^- from the transfer site (Shami et al., 1977; Falke, Pace & Chang, 1984; Glibowicka et al., 1988). Although the stilbene disulfonate binding site overlaps with the substrate binding site, the two sites are not necessarily identical. The binding of the large, negatively charged stilbene disulfonates possibly involves interactions with additional amino acid residues which are not necessary for substrate binding or transport. This is suggested by studies on the interactions between stilbene disulfonates and other inhibitors (e.g., Cousin & Motais, 1979, 1982*a*,*b*; Fröhlich & Gunn, 1982). They show that some of these other inhibitors interfere differently with the binding of the substrate and the stilbene disulfonates. This has been interpreted on the assumption that the stilbene disulfonate binding site partially overlaps with binding sites for other agents which

[1] *Abbreviations:* DNDS: 4,4′-dinitro stilbene-2,2′-disulfonate; DAS: 4,4′-diacetamido stilbene-2,2′-disulfonate; DBDS: 4,4′-dibenzoyl stilbene-2,2′-disulfonate; SITS: 4,-acetamido, 4′-isothiocyano stilbene-2,2′-disulfonate; H_2DIDS: 4,4′-diisothiocyano dihydro stilbene-2,2′-disulfonate; DIDS: 4,4′-diisothiocyano stilbene-2,2′-disulfonate; and P5-P: pyridoxal 5-phosphate.

do not overlap with the substrate binding site (reviewed in Passow, 1986).

The present paper is concerned with a further exploration of the nature of the stilbene disulfonate binding site, using as a tool site-directed mutagenesis of two specific lysine residues. Both of these residues are located in the hydrophobic anion-transporting domain of band 3. One of them, Lys 558, resides in the N-terminal chymotryptic fragment of band 3, the other, Lys 869, in the C-terminal fragment. In previous work it was shown that substitution of Lys 558 by an asparagine residue leaves intact the capacity of band 3 to accomplish anion transport (Bartel, Hans & Passow, 1989*a*; Bartel et al., 1989*b*). However, the covalent binding of H_2DIDS to the N-terminal chymotryptic fragment of band 3 was no longer possible and $K_{i\,app}$ values for reversible binding of both H_2DIDS and DNDS rose by a factor of about 4 (Kietz et al., 1991). Lys 869 has been identified by amino acid sequence analysis as a site of covalent binding of pyridoxal 5-phosphate (P5-P), another inhibitor of band 3-mediated anion transport (Kawano et al., 1988). The binding of P5-P to this lysine residue can be prevented by the stilbene disulfonates DNDS or H_2DIDS. This suggests that Lys 869, in addition to Lys 558, is involved in the formation of intramolecular crosslinks between the N-terminal and C-terminal chymotryptic fragments of band 3 by the diisothiocyano derivative, H_2DIDS.

In the present work a double mutant was formed in which Lys 558 was substituted by an asparagine residue and Lys 869 by a methionine residue. Single mutants where only Lys 558 or Lys 869 were substituted were also prepared. After expression in *Xenopus* oocytes, all mutants were able to execute anion transport, indicating that the operation of the transfer site remained basically unaffected. However, the half-saturation concentration for Cl^- was found to be reduced. In parallel experiments, the DNDS concentration for half-maximal inhibition for Cl^- transport was determined and found to increase considerably after mutation of the lysine residues. In addition the effect of the various mutations on the inhibition of transport by P5-P was studied. The experiments confirm that both Lys 558 and Lys 869 are important for the inhibition of Cl^- transport by stilbene disulfonates or P5-P but not for Cl^- binding to the transfer site. However, the interpretation of the experiments remained inconclusive with respect to the suggestion that Lys 869 is involved in the formation of the intramolecular crosslink by H_2DIDS.

Materials and Methods

The mutation K869M was generated in three different cDNA clones of band 3 (Bartel et al., 1989*a*,*b*): (i) wild type band 3 (*KK*), (ii) the mutant K558N (*NK*), and (iii) the mutant K558N, K561N (*NNK*). This yielded three new mutants: K869M (*KM*); K558N, K869M (*NM*); and K558N, K561N, K869M (*NNM*).

Mutagenesis was conducted as previously described (Bartel et al., 1989*a*) using the gapped duplex DNA method (Kramer & Fritz, 1987). For mutagenesis phage M13mp18/L6 containing a mouse band 3 cDNA insert coding for the last 141 C-terminal amino acids of mouse band 3 was used. Gapped-duplex DNA was formed after hybridization of the single-stranded M13mp18/L6 phage DNA with linearized double-stranded M13mp18rev DNA which was previously digested with EcoR1 and Hind III. After annealing a phosphorylated mutagenic primer to the gapped DNA, the complex was simultaneously filled-in and ligated with DNA polymerase/*Escherichia coli* DNA ligase. The DNA was used to transfect *E. coli* BMH 71–18 mut S competent cells for the growth of mixed phage progeny. This was followed by transfection and growth in MK 30–3 cells for enriching the minus strand progeny containing the mutation. Isolated clones were sequenced using the Sanger, Nicklen and Coulson (1977) dideoxy-chain-termination method.

A 0.6-kb region in the M13 vector containing the mutation was isolated after digestion with Hind III and BstX1 and subsequent electrophoresis on agarose. The analogous region of the band 3-encoding cDNA in the previously used wild type (*KK*) expression vector, pSPT19-Bd 3, or the previously reported mutant expression vectors *NK* or *NNK* was similarly removed, and the remaining vector was isolated. The two segments were ligated, and the DNA was used to transform competent HB101 or XL-1 *E. coli* cells. Clones were isolated and the presence of the mutation K869M was verified by dideoxy sequencing of the plasmid DNA.

The capped cRNAs used for microinjection into the oocytes were transcribed in vitro with SP6 RNA polymerase from the mutated cDNA clones as previously described by Bartel et al. (1989*b*). The vectors encode the full length of mouse band 3, flanked at the 5′ end by the first 30 untranslated nucleotides proximal to the start codon, and at the 3′ end by 204 untranslated nucleotides. Transcription buffers and SP6 RNA polymerase were obtained from Promega, Madison, WI.

All other procedures were as described previously (Bartel et al., 1989*b*). In particular, the flux measurements were performed in single *Xenopus* oocytes (Grygorczyk et al., 1989). Two to four days after microinjection of wild type or mutant band 3 cRNA, 75 nl of $^{36}Cl^-$ (0.11 mCi/ml) were microinjected. The oocyte was placed onto a perfusion chamber, the bottom of which was formed by the mica window of a Geiger-Müller tube. The radioactivity escaping from the oocyte was continuously washed away, and the remaining radioactivity was recorded as a function of time. DNDS was obtained from Pfaltz & Bauer, Waterbury, CT. The DNDS solutions were carefully protected against light. The rate constants of efflux were determined after digitization and fitting a single exponential to the data by a nonlinear curve-fitting procedure. For the determination of apparent K_i or K_m values only one single inhibitor or substrate concentration was used per oocyte and at each of the measured concentrations. The results obtained with 8–12 different oocytes were averaged.

Results

Band 3 mutants were prepared in which either Lys 558 (K558) or Lys 869 (K869) or both were replaced by asparagine (K558N) or methionine (K869M), respectively. The various band 3 derivatives obtained

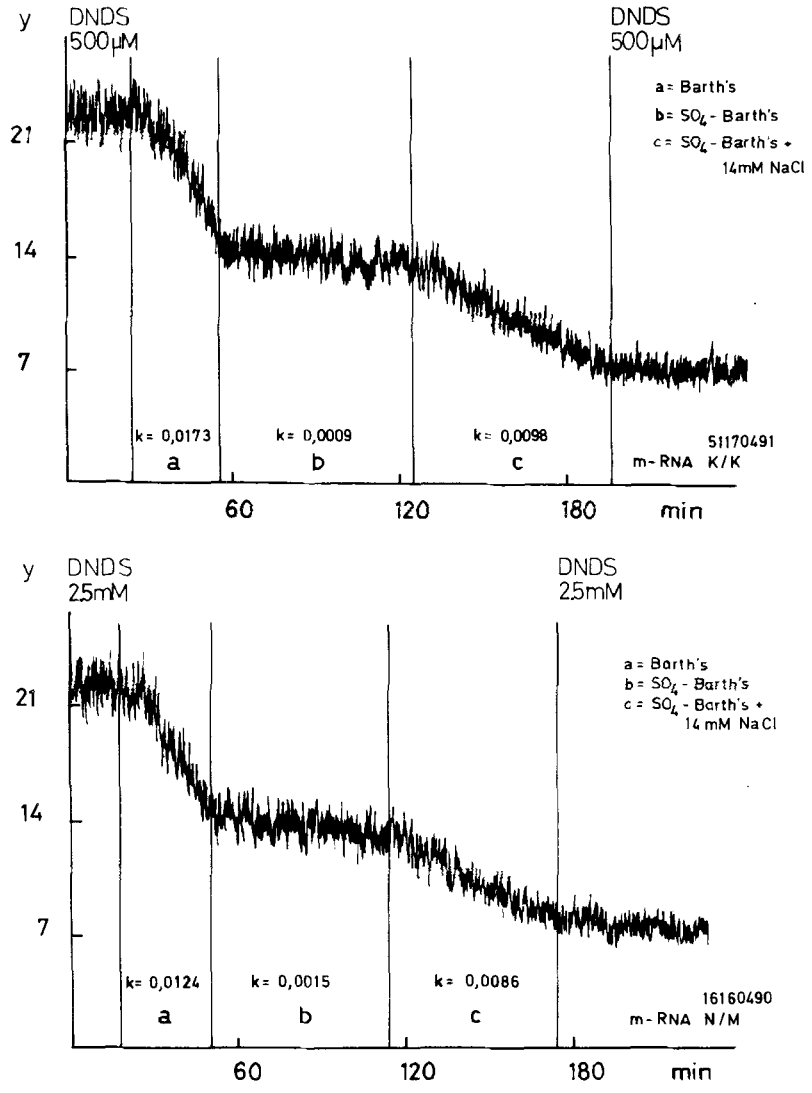

Fig. 1. The $^{36}Cl^-$ content of a single oocyte as a function of time. The oocyte contained either the wild type (*KK*) band 3 (upper panel) or the band 3 double mutant *NM* (lower panel) in which Lys 558 had been replaced by Asn and Lys 869 by Met. The medium used to wash out the radioactivity was either (*a*) Barth's solution (Cl^-) or (*b*) Barth's in which Cl^- was substituted by an osmotically equivalent concentration of SO_4^{2-} or (*c*) Barth's solution in which all except 14 mM Cl^- were substituted by SO_4^{2-}. In the time periods preceding and following the periods of efflux measurements, the medium contained DNDS at the concentrations indicated in the respective panels. k = rate constants of Cl^- efflux, in 1/min. Ordinate: $^{36}Cl^-$ in (cpm/oocyte) · 10^{-2}. Abscissa: time in min.

will be designated as *KK* (wild type), *NK* (K558N), and *KM* (K869M) and *NM* (K558N, K869M). After expression in *Xenopus* oocytes and microinjection of $^{36}Cl^-$, a single oocyte is mounted in a perfusion chamber which is continuously flushed with Barth's solution. The $^{36}Cl^-$ released from the oocyte is carried away, and the radioactivity remaining in the oocyte is determined by a Geiger-Müller tube and recorded as a function of time. The data are digitized and used for the calculation of band 3-mediated Cl^- efflux.

All mutants were able to mediate a stilbene disulfonate-inhibitable efflux. The efflux essentially represents an anion exchange, not an anion net movement. This is shown in Fig. 1 for the wild type and the double mutant *NM*: $^{36}Cl^-$ efflux was drastically reduced when the Cl^- in the external medium was replaced by SO_4^{2-}. Although band 3 is capable of transporting SO_4^{2-}, and hence, of mediating a Cl^-/SO_4^{2-} exchange, this exchange is barely detectable on the time scale of our experiments since SO_4^{2-} is exchanged against Cl^- at a rate which is several orders of magnitude slower than the rate of Cl^-/Cl^- exchange (reviewed by Knauf, 1979; Passow, 1986; Salhany, 1990). Thus the transport process studied in the mutant still exhibits one of the most typical features of band 3-mediated anion transport.[2]

[2] At high Cl^- concentration in the medium (e.g., in Barth's medium where $[Cl^-]$ = 90 mM), in oocytes without microinjected band 3-encoding RNA, $^{36}Cl^-$ efflux is negligibly small as compared to the efflux observed in oocytes in which band 3 had been successfully expressed. However, when the NaCl in Barth's medium is substituted by sodium sulfate, $^{36}Cl^-$ leakage from the oocytes via some endogenous pathway may occur. This leakage is variable amongst the oocytes of a given population and amongst the oocytes of different populations. The residual $^{36}Cl^-$ efflux from band 3-containing oocytes observed in the Cl^--free media (*see also* Fig. 4) is due to this type of leakage and not mediated by band 3.

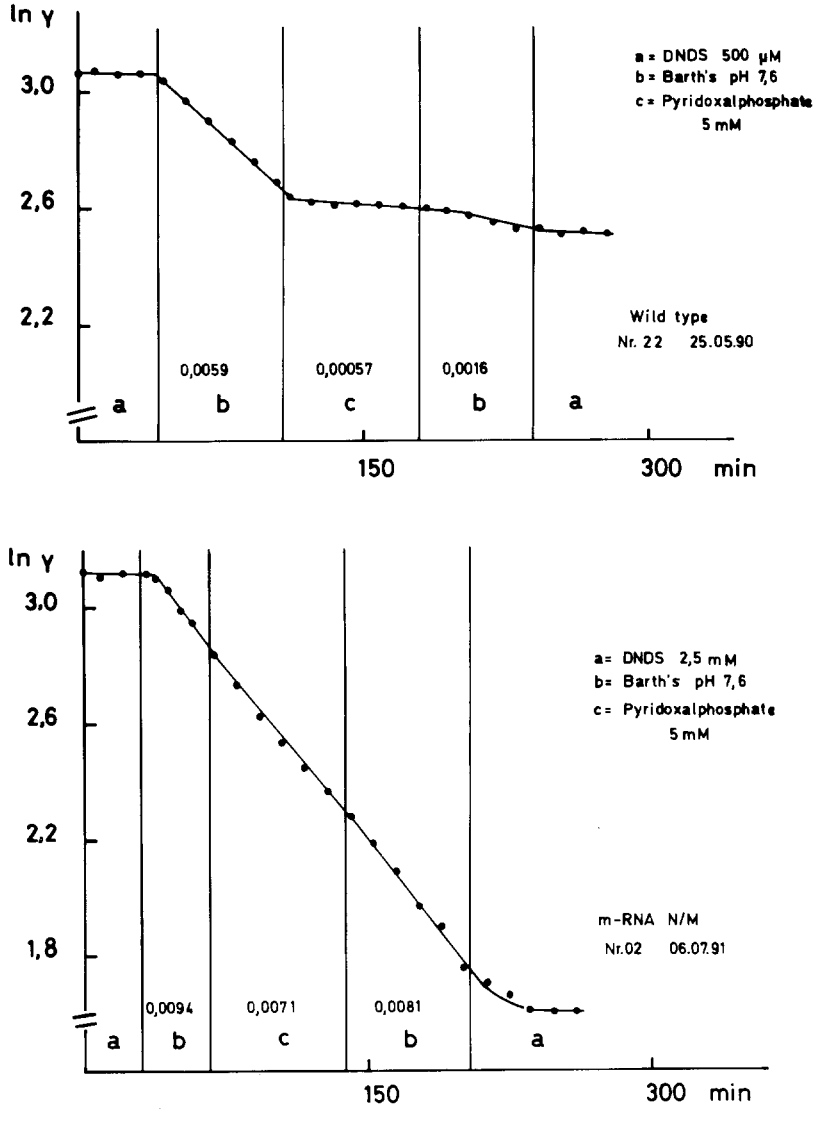

Fig. 2. Effect of pyridoxal 5-phosphate (5 mM) on band 3-mediated $^{36}Cl^-$ efflux from single oocytes containing either wild type (*KK*) or doubly mutated (*NM*) band 3. Semilog plot of the digitized data. The experimental conditions are indicated in the figure.

In a second set of experiments, the action of pyridoxal 5-phosphate (P5-P) was studied. The experiments serve to establish that the mutations lead to effects which are interpretable in terms of what is known from work in the red blood cells. In the wild type (*KK*), P5-P (5 mM, pH 7.6, 20°C) produced 91% inhibition of Cl^- efflux (Fig. 2, Table 1). 65% inhibition persists when the medium containing P5-P is replaced by a medium without P5-P, and hence is irreversible. After substitution of Lys 558 by an asparagine residue (*NK*), P5-P still causes 92% inhibition. 76% inhibition persists after exposure of the oocyte to a P5-P-free medium, indicating that again most of the effect is irreversible. Inhibition is reduced to 68% when Lys 869 is replaced by a methionine residue (*KM*). This inhibition is also irreversible. Finally, in the double mutant in which Lys 558 was replaced by Asn and Lys 869 by Met (*NM*), inhibition in the presence of P5-P was reduced to only 19–26%, all of which was reversible, i.e., after removal of P5-P from the medium, $^{36}Cl^-$ efflux continued at the same rate as prior to the exposure to the agent (Fig. 2).

As will be shown in the discussion, the results are essentially compatible with those obtained in red blood cells. We feel confident, therefore, that the study of stilbene disulfonate binding to the mutants should yield results that are pertinent for an understanding of the role of Lys 558 and Lys 869 in the wild type.

In wild type (*KK*) and mutants (*NK*, *KM*, *NM*), the reduction of $^{36}Cl^-$ efflux by DNDS could be described by the equation:

$$^0k_{Cl} = {}^0k_{Cl(o)} \cdot \frac{K_{i\,app}}{K_{i\,app} + [DNDS]} \quad (1)$$

Table 1. Effect of substitution of Lys 558 and/or Lys 869 by Asn or Met, respectively, on inhibition by pyridoxal 5-phosphate (P5-P) of mouse erythroid band 3-mediated $^{36}Cl^-$ efflux from *Xenopus* oocytes[a]

Amino acid residue no.		Inhibition (percent) ± SEM		
558	869	Total[b]	Irreversible[c]	*n*
K	*K*	91.0 ± 2.8	65.0 ± 4.7	3
N	*K*	91.8 ± 1.6	76.0 ± 4.6	8
K	*M*	67.8 ± 2.8	63.8 ± 2.1	6
N	*M*	19.3 ± 4.6	ND	6
N	*M*	25.9 ± 5.8[d]	0.6 ± 3.6	7

[a] The experiments were carried out at 5 mM P5-P, pH 7.6, 20°C.
[b] Total inhibition indicates the sum of reversible and irreversible inhibition, as determined during the exposure to P5-P.
[c] Determined after 1 hr of exposure to P5-P, in inhibitor-free, bovine serum albumin-containing medium (0.2%).
[d] pH 8.5.
ND = not determined.
SEM = SD/$\sqrt{n}$, where SD = standard deviation and n = number of oocytes used per data point.

where ${}^0k_{Cl}$, ${}^0k_{Cl(o)}$ represent, the rate constants for Cl^- efflux as measured at the inhibitor concentrations [DNDS] and [DNDS] = 0, respectively. $K_{i\ app}$ indicates the DNDS concentration at which the inhibition of efflux is half-maximal (Fig. 3). The $K_{i\ app}$ values obtained with the various mutants are compiled in Table 2.

In order to explore whether or not the extraordinary decrease of the susceptibility to inhibition by DNDS is associated with changes of substrate binding, the concentration required for half-maximal activation of the transport system with external Cl^- (K_m) was measured. Substituting external Cl^- by osmotically equivalent concentrations of SO_4^{2-} leads to a reduction of $^{36}Cl^-$ efflux. The relationship between efflux and external Cl^- concentration follows saturation kinetics:

$$ {}^0k_{Cl} = {}^0k_{Clmax} \cdot \frac{[Cl_o]}{K_m + [Cl_o]}. \quad (2) $$

${}^0k_{Cl}$ and ${}^0k_{Clmax}$ represent rate constants of $^{36}Cl^-$ efflux at the Cl^- concentrations $[Cl_o]$ and $[Cl_o] = \infty$, respectively.

We find that the K_m value for the double mutant *NM* in which both Lys 558 and Lys 869 had been replaced, was 11 mM instead of 42 mM as observed in the wild type (Fig. 4). The latter value is close to observations in mouse red blood cells (Passow et al., 1989, Fig. 4).

The experiments described so far demonstrate that Lys 869 has a profound influence on the binding of DNDS to band 3. They also show an involvement of this residue in the binding of P5-P, and hence, support the view that the binding sites for P5-P and DNDS overlap. Thus, it would seem plausible to assume that Lys 869 is the lysine residue involved in covalent H_2DIDS binding to the N-terminal chymotryptic fragment of band 3 and the establishment of the crosslink with the C-terminal chymotryptic fragment. If this were true, one would expect that in the double mutant *NM* covalent H_2DIDS binding to band 3 would no longer be feasible. From previous work, it is known that the reactivities of Lys 558 and the lysine residue in the C-terminal fragment are quite different, being much greater in the former than in the latter (Kietz et al., 1991). In accord with this conclusion, we find that in the mutant *NM*, exposure to 250 μM H_2DIDS at pH 7.6 produces little if any irreversible inhibition, although at that elevated concentration, more than 85% reversible inhibition still occurs (Table 3). One would expect that at pH 9.5 the covalent reaction with the chymotryptic C-terminal fragment should also no longer be possible. Contrary to this expectation, some irreversible inhibition persists after 1-hr incubation at that pH (Table 3), suggesting that Lys 869 is not responsible for covalent bond formation with the second -NCS group of H_2DIDS, and hence, not involved in the intramolecular crosslinking by this agent. The interpretation of this result remains, however, somewhat ambiguous since at the extremely high H_2DIDS concentration and pH used, irreversible binding to band 3 may also have taken place at other sites which are not involved in covalent binding to the crosslinking site that is normally participating in H_2DIDS binding to the C-terminal chymotryptic fragment.

We considered the possibility that at extremely high H_2DIDS concentration, in addition to Lys 558, Lys 561 might also become involved in covalent H_2DIDS binding and that this may be responsible for the ensuing irreversible inhibition. Thus a triple mutant was made where in addition to the substitution at Lys 869 with a methionine residue, both Lys 558 and Lys 561 were substituted with asparagine residues (i.e., by amino acid residues that are incapable of reacting covalently with the -NCS groups of H_2DIDS). In the triple mutant K558N/K561N/K869M (*NNM*), we observed an essentially similar fraction of irreversible inhibition by H_2DIDS as in the K558N/K869M (*NM*) double mutant. Thus, if an additional site for covalent H_2DIDS binding and irreversible inhibition of Cl^- transport exists, it is unlikely to be Lys 561 (Table 3).

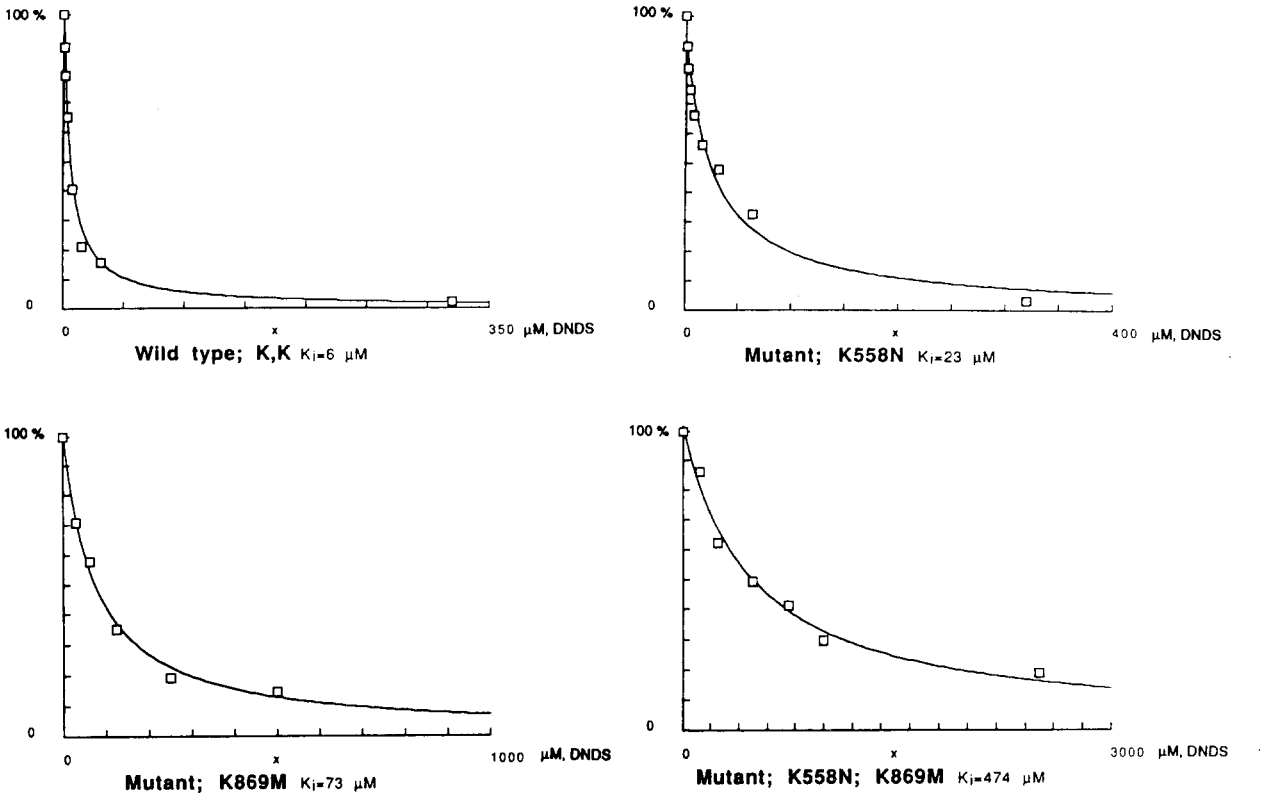

Fig. 3. Concentration dependence of effect of DNDS on band 3-mediated $^{36}Cl^-$ efflux into Barth's medium as measured in the various mutants described in the text. Ordinate: Rate of efflux as a percentage of control. Abscissa: DNDS concentration, in μM. The K_i values were obtained by a nonlinear, least-square curve-fitting procedure. Each data point represents the average of efflux measurements in 9–12 single oocytes. The errors (SEM) are in the range of ±10–±15%.

Table 2. Effect of substitution of Lys 558 and/or Lys 869 of mouse erythroid band 3-mediated Cl^- efflux on the $K_{i\,app}$ of DNDS and K_m of Cl^-*

Amino acid residue no.		DNDS	Chloride
558	869	$K_{i\,app}$, µM ± SD	K_m, mM ± SD
K	*K*	6 ± 0.3	42.0 ± 4.4
N	*K*	23 ± 1.0	ND
K	*M*	73 ± 3.1	ND
N	*M*	474 ± 3.9	11.3 ± 3.8

* Barth's medium, pH 7.6.
SD = standard deviation.
ND = not determined.

Discussion

Our experiments indicate that both Lys 558 and Lys 869 play a role in the inhibition of band 3-mediated Cl^-/Cl^- exchange by P5-P and stilbene disulfonates. They suggest that the epsilon-amino groups of these lysine residues are the sites of binding and action of P5-P on the C-terminal and N-terminal chymotryptic fragments of the hydrophobic domain of band 3. They show that the two lysine residues are essential for the maintenance of the tertiary structure of the stilbene disulfonate binding site in a state on which the extraordinary affinity and selectivity of the reversible binding of the inhibitor DNDS depends. Finally, in accord with the finding derived from kinetic studies in red cells that substrate binding and the binding of stilbene disulfonates are mutually interdependent (reviewed by Knauf, 1979; Passow, 1986; Jennings, 1989; Salhany, 1990), the structural changes produced by the mutations affect parameters related to both substrate and inhibitor binding.

Most of the previous work on band 3-mediated anion exchange was performed with human red blood cells while the present experiments were done with erythroid band 3 of the mouse. The sequence homology of the anion-transporting hydrophobic domains of band 3 of mouse and man amounts to about 90%. Moreover, previous work from our laboratory has shown that the pK values of the two lysine residues involved in covalent H_2DIDS binding to murine band 3 are similar to the pK values of the corresponding lysine residues in the human band 3 (Kietz

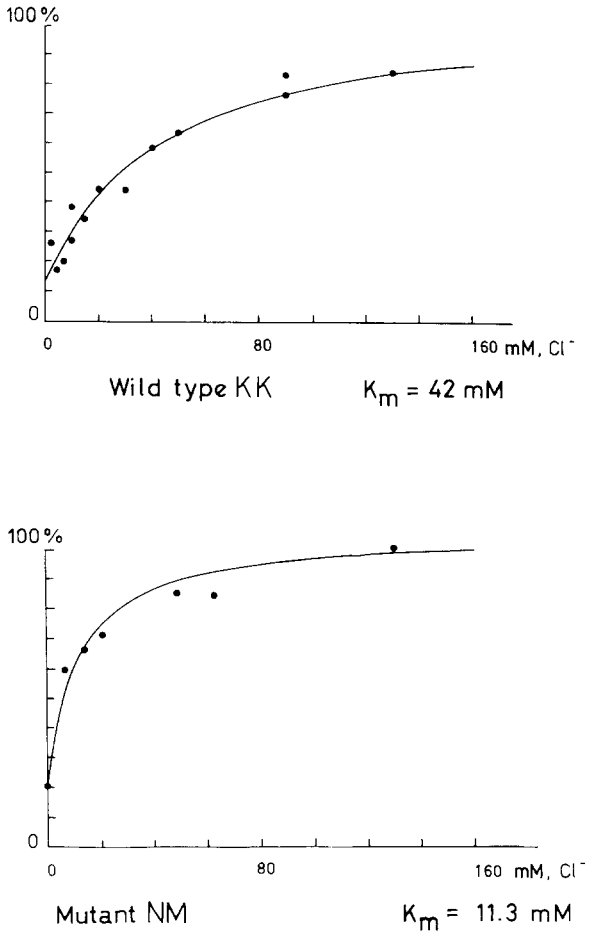

Fig. 4. Relationship between the rate of band 3-mediated $^{36}Cl^-$ efflux and Cl^- concentration in the medium for wild type (*KK*) and double mutant (*NM*). Ordinate: Rate of efflux as a percentage of control. Abscissa: Cl^- concentration, in mM. The Cl^- concentration was varied by isotonic replacement of Cl^- by SO_4^{2-}. K_m = chloride concentration at half-maximal activation. The drawn curves represent a fit of Eq. (2) to the data by a nonlinear curve-fitting procedure. Each data point represents the average of 8–12 measurements with single oocytes. The errors (SEM) amount to about ±10%.

et al., 1991). Since the pK values are sensitive indicators of the dielectric constant and the charge density in the vicinity of the dissociable groups, it is clear that the tertiary structure around the two lysine residues involved in covalent H_2DIDS binding must be rather similar in human and murine band 3. Hence, it seems permissible to discuss our work with mouse band 3 in relation to previous work with human band 3.

In the human red cell, pyridoxal phosphate (P5-P) binding can be demonstrated both in the C-terminal and N-terminal chymotryptic fragments of Band 3 (Salhany, Rauenbuehler & Sloan, 1987; BarNoy & Cabantchik, 1990; Salhany, 1990). Combination with either one of these fragments causes inhibition of anion exchange. When binding to the C-terminal fragment is prevented, a linear relationship between binding to the N-terminal fragment and inhibition is seen. When binding to both fragments is allowed to take place, some negative cooperativity is observed (Salhany, 1990). Our data are compatible with these previously published findings: They show that irreversible inhibition can still be observed if either Lys 558 (corresponding to Lys 539 on the N-terminal fragment of human band 3) or Lys 869 (corresponding to Lys 851 on the C-terminal fragment of human band 3) are substituted by unreactive amino acid residues. Only when both residues are substituted, is the irreversible inhibition by P5-P prevented. Although the compatibility of our results with previous findings in the red blood cell is obvious, due to their limitation to the use of one single nearly maximally inhibitory P5-P concentration, they do not permit comparison of the details of the reaction, such as the presence or absence of the cooperative effects between the two pyridoxal binding sites as described by Salhany et al. (1987). Nevertheless, the similarities observed leave little doubt that the two lysine residues involved in P5-P inhibition are in fact Lys 558 and Lys 869 in the mouse band 3 or the homologous Lys 539 and Lys 851 in the human band 3. Concerning Lys 851, our conclusion would support the previous findings of Hamasaki and co-workers (Kawano & Hamasaki, 1986; Kawano et al., 1988) who showed by chemical amino acid sequence analysis the covalent binding of P5-P to that residue.

Our experiments with mutant *NM* show that even in the absence of both Lys 558 and Lys 869 reversible inhibition is still possible. This indicates that prior to the covalent bond formation, P5-P does not produce reversible inhibition exclusively by the noncovalent binding to these lysine residues. Since P5-P is an anion which is known to be transported by band 3 (Cabantchik et al., 1975), it is evident that P5-P must also be able to combine with the substrate binding site and produce a competitive inhibition of Cl^- transport. Since Cl^- transport takes place even after mutation of the two lysine residues, it is clear that they are not directly involved in substrate binding to the transfer site. Thus, reversible binding represents the superimposition of the effects of reversible binding to either one or both of the lysine residues 558 and 869 and a combination with the transfer site. The small and possibly significant decrease of K_i in the mutant *KM* as compared to wild type *KK* and mutant *NK*, may be due to a change of K_m for P5-P binding to the substrate binding site. However, we do not wish to stress this interpretation

Table 3. Effect of substitution of (i) Lys 558 and Lys 869 by Asn and Met, respectively (double mutant *NM*), or of (ii) both Lys 558 and Lys 561 by Asn and of Lys 869 by Met (triple mutant *NNK*), on inhibition by H_2DIDS of mouse erythroid band 3-mediated $^{36}Cl^-$ efflux from *Xenopus* oocytes[a]

Mutant	pH	H_2DIDS (μM)	Residual efflux in presence of H_2DIDS	Residual efflux after exposure to H_2DIDS and BSA	*n*
			(percent of control, ± SEM)		
NM	7.6	25	32.7 ± 1.0	92.2 ± 6.9	3
	7.6	50	32.0 ± 9.8	106.8 ± 43.4	3
	7.6	125	18.0 ± 3.0	107.7 ± 6.8	3
	7.6	250	14.0 ± 8.8	126.1 ± 12.1	4
	7.6	500	16.1 ± 7.1	108.6 ± 19.1	6
NM	9.5	25	60.5 ± 5.5	106.8 ± 2.2	3
	9.5	50	30.1 ± 5.3	67.3 ± 14.0	5
	9.5	125	28.4 ± 7.1	89.8 ± 26.9	11
	9.5	250	15.5 ± 7.2	47.9 ± 11.9	11
	9.5	500	10.4 ± 7.4	62.2 ± 24.6	17
NNM	9.5	50	48.0 ± 14.2	47.9 ± 39.0	3
	9.5	250	11.5 ± 0.7	53.2 ± 13.4	11

[a] The experiments were carried out at the indicated pHs and concentrations of H_2DIDS at 20°C. Control flux = efflux measured prior to exposure to H_2DIDS. After 1 hr of exposure to H_2DIDS, the efflux was measured in the presence of 0.2% bovine serum albumin (BSA) to remove reversibly bound H_2DIDS from the plasma membrane.

since other explanations are also possible and the differences of the K_i values may not necessarily be significant.

Although neither one of the two lysine residues is necessary for the functioning of anion transport, nevertheless, both of them are essential for the maintenance of the tertiary structure of the binding sites for both the stilbene disulfonates and the penetrating substrate anions. The effects of substitution of Lys 558 and Lys 869 on DNDS binding are more than additive: Substitution of Lys 558 by Asn increases $K_{i\ app}$ for DNDS about fourfold. Substitution of Lys 869 by Met increases $K_{i\ app}$ about 12-fold. Both substitutions together lead to about an 80-fold increase. The dramatic reduction of the affinity for stilbene disulfonate binding is associated with a considerable change of substrate binding. The K_m value for Cl^- binding decreases to $\frac{1}{4}$ of the original value. This decrease may lead to a more effective competitive displacement of DNDS by Cl^-, and hence, may contribute to some extent to the increased K_i for DNDS binding. Our data do not permit, however, a quantitative assessment of this contribution. To minimize damage of the oocytes by nonphysiological conditions, K_m values were determined by measuring Cl^- fluxes at Cl^- concentrations that had been established by isosmotic substitution of NaCl by Na_2SO_4. Since Cl^- and SO_4^{2-} compete for a common substrate binding site, the change of K_m reflects a change of the relative affinity of Cl^- with respect to SO_4^{2-}, and hence, does not allow one to obtain a quantitative estimate of the influence on DNDS binding.

It has been shown previously that the rate of dinitrophenylation of Lys *a* (i.e., Lys 558 in mouse erythroid band 3 or Lys 539 in human erythroid band 3) is enhanced when the Cl^- concentration in the medium is increased, indicating a mutual interrelationship between substrate binding and the susceptibility of Lys *a* to dinitrophenylation (Passow et al., 1980). This led to the suggestion that Lys *a* may exist in two states, buried and exposed, and that the fraction of Lys *a* residues in the exposed state increases in proportion to the fractional occupancy of the transfer site with Cl^-. These inferences were supported by a detailed analysis of the time course of the intramolecular crosslinking of band 3 by the covalent binding H_2DIDS (Kampmann et al., 1982). The present experiments are compatible with the previous suggestion that Lys *a* is acting as a reporter group, the susceptibility of which to chemical modification, is directly related to the occupancy of the substrate binding site.

The lysine residues 558 and 869 are separated from each other by 311 amino acid residues in the amino acid sequence of band 3 (*see* Fig. 5). Their cooperation in the maintenance of the binding sites for substrate anions and stilbene disulfonates would suggest that in the properly folded transport protein they are located in close proximity to each other. It is known that the two isothiocyanate groups of the stilbene disulfonate H_2DIDS react irreversibly with two lysine residues located in two distinct

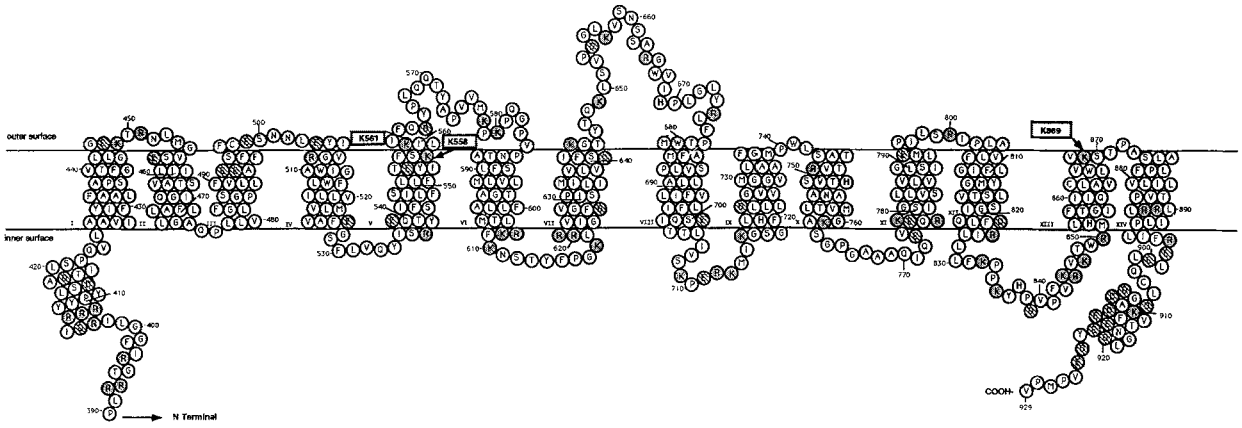

Fig. 5. Tentative schematic representation of the organization of the band 3 protein. The two horizontal lines represent the thickness of the lipid bilayer, assumed to be 30 Å. They are not intended to create the impression that each of the transmembrane helices penetrates the lipid bilayer separately. We assume that the protein forms a compact structure where the helices are in immediate contact with each other.

chymotryptic fragments of band 3. This leads to the formation of an intramolecular crosslink, suggesting that the distance between the two groups is not much different from the size of a H_2DIDS molecule, i.e., about 16 Å. Although there is no doubt that Lys 558 is one of the residues participating in crosslink formation (Bartel et al., 1989*a*,*b*; Kietz et al., 1991) our attempts to find out whether or not Lys 869 represents the other residue involved, remained inconclusive. Although it cannot be ruled out that the mutation of this latter lysine residue is transmitted allosterically to a distant site, it would seem plausible to assume tentatively that, whatever the role of Lys 869 in the crosslinking reaction, it is located somewhere in the vicinity of Lys 558. This would imply that the transmembrane helices 5 and 13 reside in close juxtaposition and participate in the formation of the anion transport pathway and the substrate binding site. Another lysine residue that may also be close to the transport region could be Lys 449. This residue is homologous to Lys 430 in the human erythroid band 3. It has been shown to constitute the binding site of the anion transport inhibitor eosin maleimide (Cobb & Beth, 1990). This binding site seems to overlap with the binding site for stilbene disulfonates. Lys 449 is located near the outer membrane surface at the interconnection between helices 1 and 2. This leads to the suggestion that either helix 1 or helix 2 and the helices 5 and 13 are possible candidates for the formation of the anion transport pathway in band 3.

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Note Added in Proof

Since the paper had been accepted for publication, we have determined the apparent K_m values for Cl^- and of the band 3 mutant NM (K558 and K869 substituted by N558 and M869) by isosmotic substitution of Cl^- with gluconate$^-$ rather than SO_4^{2-}. We obtained for KK K_m = 10.2 mM, for NM K_m = 11.9 mM. This suggests that the absolute values for Cl^- binding to the transfer site of band 3 remains unaffected by the mutations, even though they lead to an 80-fold increase of the apparent K_I value for the inhibition of Cl^- exchange by the stilbene disulfonate DNDS. Thus, binding of substrate and inhibitor is controlled by essentially distinct structural elements of band 3. This contrasts with the inhibition kinetics observed in red blood cells, which suggests competition between Cl^- and DNDS for a common binding site (for references, *see* the Introduction). The origins of the differences of the K_m values determined by Cl^-/SO_4^{2-} substitution as compared to Cl^-/gluconate$^-$ substitution remain obscure. The error of the K_m determinations in the oocytes is rather large and hence speculations seem to be premature.