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pH-dependence of inhibition by H_2DIDS of mouse erythroid band 3-mediated Cl^- transport in *Xenopus* oocytes. The effect of oligonucleotide-directed replacement of Lys-558 by an Asn residue

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The rapid reversible inhibition of band 3-mediated inorganic anion transport by 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate (H_2DIDS) turns slowly into irreversible inhibition. This is due to covalent bond formation of the two isothiocyanate groups of the inhibitor with two lysine residues on band 3, called Lys *a* and Lys *b*. In the red cell membrane, the p*K* value of Lys *a* is about 2.5 p*K* units lower than the p*K* value of Lys *b*. Hence the susceptibility of Lys *a* to irreversible modification by H_2DIDS far exceeds the susceptibility to Lys *b*. In the present paper, we have expressed in *Xenopus* oocytes cRNA's derived from cDNA clones encoding wild-type mouse band 3 and mouse band 3 in which Lys *a* (Lys-558) had been replaced by an Asn residue by oligonucleotide-directed mutagenesis. In accord with previous findings, in the oocytes both wild-type and mutated band 3 mediate Cl^- exchange. After determining the uninhibited exchange rate the oocytes were exposed for a fixed length of time to H_2DIDS at a concentration (20 μM) which saturates all H_2DIDS binding sites with reversibly bound H_2DIDS (K_i = 0.3 μM and 1.1 μM, respectively, for wild-type and mutant). Exposure was terminated by washing with a medium in which H_2DIDS was replaced by bovine serum albumin to remove free and reversibly bound H_2DIDS from the extracellular phase. Subsequent measurements of Cl^- efflux yielded a measure for the irreversible inhibition that persisted. Since the transition from reversible to irreversible H_2DIDS binding was found to follow first-order kinetics it was possible to calculate rate constants. From the pH dependence of the rate constants, p*K* values were calculated. These calculations could be made since in the wild-type, in which Lys *a* and Lys *b* are present, the exposure to H_2DIDS could be confined to a pH range in which little if any covalent binding to Lys *b* takes place. The data could be represented by a single p*K* value of 8.3. In the mutant, Lys *a* is missing. Hence, covalent reaction can only take place with Lys *b*. Measurements over the appropriate pH range could be described by a single p*K* of 10.8. These values are 0.8–0.9 p*K* units higher than those previously obtained in experiments with band 3 in the red cell membrane (Kampmann et al. (1982) J. Membr. Biol. 70, 199–216). The difference can be accounted for by the difference of temperature which amounted to 20°C in the present experiments with the oocytes and to 30°C in the previous work with red blood cells. The results suggest that location and function of Lys *a* and Lys *b* are essentially the same after expression in the lipid bilayer of the red blood cell and the oocyte.

Introduction

Amongst the various inhibitors of erythroid band 3-mediated anion transport the stilbenedisulfonate analog 4,4′-diisothiocyano dihydrostilbene-2,2′-disulfonate (H_2DIDS) plays a particularly conspicuous role: It combines with the band 3 protein with much higher selectivity and affinity than with other membrane proteins and thus is suitable for highly specific labelling [2]. One mole of band 3 protein binds one mole of H_2DIDS [3]. The relationship between inhibition of anion exchange and H_2DIDS binding is linear and inhibition becomes maximal when all band 3 molecules are occupied with the inhibitor [4].

The interest in the mode of action of H_2DIDS in the inhibition by band 3-mediated anion transport was

Abbreviations: H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; BSA, bovine serum albumin.

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stimulated by observations which suggest that the H_2DIDS binding site overlaps with or is close to the binding site for the substrate anions (reviewed in Ref. 5). Hence information about the nature of the H_2DIDS binding site may contribute to the characterization of the site that is responsible for substrate translocation across the membrane.

The reaction of H_2DIDS with the band 3 protein proceeds in two steps. The first leads almost instantaneously to reversible, non-covalent binding and is associated with inhibition. The second results in a slow irreversible fixation by covalent bond formation of the two isothiocyanate groups of the inhibitor with the ϵ-amino groups of two adjacent lysine residues called Lys *a* and Lys *b*, which may or may not be involved in reversible binding. This leads to intramolecular cross-linking [3]. The kinetics of H_2DIDS binding have been worked out by Kampmann, Lepke, Fasold, Fritzsch, Passow [1] on the basis of the following reaction sequence:

1. Fast, reversible reaction:

$$s + a\,b = a\,s\,b$$

2. Slow, irreversible reactions of the reversibly bound H_2DIDS:

$$\begin{array}{ccc} a\ s\ b & \rightarrow & a\ s\text{–}b \\ \downarrow & & \downarrow \\ a\text{–}s\ b & \rightarrow & a\text{–}s\text{–}b \end{array}$$

a, *b* = Lys *a* and Lys *b*, respectively; s = H_2DIDS; – = covalent bond.

Covalent bond formation between amino and isothiocyanate groups can only take place when the amino groups are deprotonated. Consequently, the rate of covalent bond formation between H_2DIDS and band 3 should vary with pH. This has actually been observed [3]. From the pH dependence of the kinetics of covalent H_2DIDS binding the p*K* values for the dissociation of Lys *a* and Lys *b* were determined to be 7.3 and 10.0, respectively [1].

Recently, the localisation of Lys *a* in the known amino acid sequence of mouse band 3 was achieved by means of site-directed mutagenesis. After replacement of Lys-558 by Asn-558 both the transport function and the inhibitory effect of reversible H_2DIDS binding were preserved. However, covalent H_2DIDS binding to Lys *a* was no longer possible. This suggests that Lys *a* is identical to Lys-558 [6].

In the present paper we report about the determination of the p*K* values of Lys *a* and Lys *b* after expression in the lipid bilayer of the *Xenopus* oocyte of mouse erythroid band 3 encoded by wild-type cRNA and a cRNA encoding a mutant band 3 in which Lys-558 was exchanged by Asn-558. It will be shown that this makes it possible to determine separately the p*K* values of Lys *a* and Lys *b*. The results agree well with those obtained with red blood cells and lend strong support to our previous finding that Lys *a* is identical with Lys-558 in the amino acid sequence of mouse band 3 [6,7].

Methods

Wild-type and mutant (Lys-558 replaced by Asn-558) cRNA of mouse erythroid band 3 protein were obtained as described by Bartel et al. [6,7].

Microinjection of cRNA into *Xenopus* oocytes and efflux measurements were performed as follows: After filtration of the cRNA through polycarbonate filters (pore size 0.2 μm), a volume of 50 nl containing approx. 30 ng cRNA was injected into defolliculated oocytes as described previously [8]. Incubation at 18°C for 48 h or more in a standard medium (Barth's solution (pH 7.6), a modified amphibian Ringer's solution; for composition see Ref. 8) ensured adequate expression of the transport protein in a functional state. Each oocyte was subsequently microinjected with 75 nl of a $^{36}Cl^-$ solution (0.129 mCi/ml) and placed into a hairloop in a perfusion chamber the bottom of which consisted of a mica window of a Geiger-Müller tube. As previously described, band 3-mediated Cl^- efflux could be followed by measuring the decrease of radioactivity in the microinjected oocyte while the released radioactivity was continuously washed away by flushing the perfusion chamber with Barth's solution. In the experiments in which the concentration dependence of the reversible inhibition of Cl^- efflux by DNDS and H_2DIDS was measured for wild-type and mutant band 3, inhibition was calculated by forming the ratio of the rate constants for flux in Barth's solution without inhibitor (= 100%) and with various concentrations of the inhibitors. The experiments with H_2DIDS were performed at pH values of 6.75 and 8.0 for wild-type band 3 and mutant, respectively, i.e. at values at which covalent bond formation can be neglected.

The time dependence of covalent bond formation with H_2DIDS was assumed to be reflected by the irreversible inhibition of anion transport. Rate constants were measured after various lengths of exposure to H_2DIDS. Exposure was terminated, reversibly bound H_2DIDS removed, and efflux initiated by switching from perfusion with H_2DIDS- containing medium to standard medium in which H_2DIDS was replaced by 0.5% BSA.

Results and Discussion

(1) Principles of experimental approach

Wild-type mouse band 3 and band 3 in which Lys *a* (= Lys-558) had been mutated to an aspargine residue

were expressed in *Xenopus* oocytes. The band 3-mediated Cl^- flux was measured prior to and after exposure of the oocytes to H_2DIDS at a concentration that suffices to achieve maximal reversible inhibition. The percentage change of the flux that persisted after the exposure to the H_2DIDS for a fixed length of time was used as a measure of the rate of irreversible inhibition of band 3. Exposure to H_2DIDS over a range of different pH values made it possible to construct curves which related the rate of irreversible inhibition to pH. These curves served as a basis for the estimation of the pK values of the lysine residues involved in covalent bond formation.

In the wild-type band 3 both Lys *a* and Lys *b* are present and, in principle at least, should be able to react with H_2DIDS. However, the pK value of Lys *a* is more than two pK units lower than the pK of Lys *b*. Hence, a pH range exists at which the irreversible modification by H_2DIDS binding to Lys *a* can be measured without significant interference by irreversible binding to Lys *b*. In the mutant, Lys *a* has been replaced by an amino acid residue that is incapable of covalent bond formation with H_2DIDS. Nevertheless, a transition from reversible to irreversible H_2DIDS binding still takes place. This is attributable to covalent bond formation with Lys *b*. Hence, the pH dependence of the rate of irreversible inhibition in the mutant yields the pH dependence of the rate of reaction of H_2DIDS with Lys *b*.

Mutation of band 3 at Lys *a* may lead to a change of the affinity for reversible H_2DIDS binding. Since complete saturation with H_2DIDS is a necessary prerequisite for the appropriate determination for the rate of irreversible inhibition, it was necessary to demonstrate that a change of the apparent K_I value for the reversible inhibition does not reduce the saturation of the H_2DIDS binding site with reversibly bound H_2DIDS. For this reason, the K_I values for reversible H_2DIDS binding were measured in wild-type and mutant, using pH values at which the rate of transition from reversible to irreversible binding is negligibly small. Knowing the K_I values, it was possible to chose an H_2DIDS concentration high enough to ensure complete saturation of band 3 with reversibly bound H_2DIDS in both wild-type and mutant.

For the quantitative description of the pH dependence of the transition from reversible to irreversible inhibition it is also necessary to know the reaction order. This was determined in separate experiments, in which the time of exposure to H_2DIDS prior to the flux measurements was varied. The decrease of the efflux as a function of time showed first order kinetics.

Knowing the reaction order and the pH dependence of the rate of transition from reversible to irreversible inhibition at an H_2DIDS concentration that suffices to saturate all H_2DIDS binding sites with reversibly bound H_2DIDS, it is possible to calculate rate constants from the irreversible inhibition measured after a fixed time of exposure to H_2DIDS at various pH values. Since it is only the deprotonated form of a lysine residue that is capable of covalent bond formation with an isothiocyanate group of H_2DIDS, it is possible to deduce from the pH dependence of the rate constants the pertinent pK values. For the reasons given above, the pK values obtained for the wild-type and the mutant refer to Lys *a* and Lys *b*, respectively.

(2) The protocol followed in the experiments

The protocol followed in the experiments on the action of H_2DIDS on Cl^- efflux from the oocyte is illustrated in Fig. 1. Two days after microinjection of wild-type cRNA, the oocyte was microinjected with $^{36}Cl^-$, and placed on the mica window of a Geiger-Müller tube which formed the bottom of a perfusion chamber [8,6]. Perfusion of the chamber was initiated with Barth's solution (pH 7.6), containing 500 μM DNDS, a reversibly acting stilbene disulfonate that combines with the same site on band 3 as H_2DIDS [9]. The record shows that there is little loss of $^{36}Cl^-$ from the oocyte, indicating that no leakage of $^{36}Cl^-$ through pathways outside band 3 takes place. After the establishment of this base line, perfusion was changed from perfusion with DNDS to perfusion without DNDS, again at pH 7.6. The ensuing decrease of radioactivity in the oocyte shows that a band 3-mediated Cl^- efflux takes place. This efflux is inhibited when perfusion is continued with Barth's solution containing H_2DIDS at

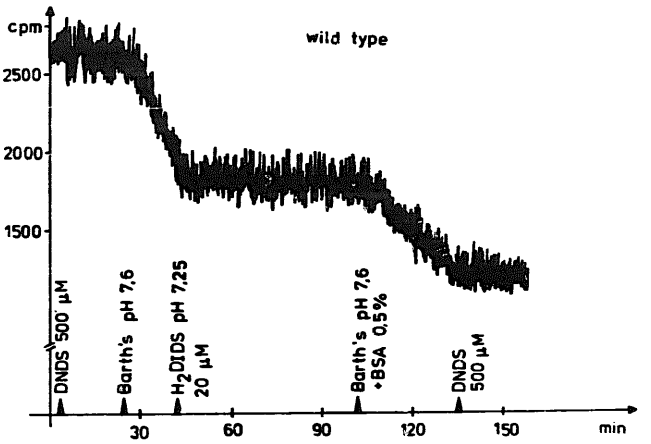

Fig. 1. Release of $^{36}Cl^-$ from a single oocyte in the experimental arrangement described in the text [8]. After the time period alloted for translation of microinjected wild-type cRNA into band 3 protein, the oocyte was injected with $^{36}Cl^-$, and mounted in the perfusion chamber on top of a Geiger-Müller counter. The curve indicates the time course of release of radioactivity into the non-radioactive perfusion medium. At the times indicated in the figure, the medium was changed from Barth's solution (pH 7.6) containing 500 μM DNDS to Barth's solution (pH 7.6) without additions; to Barth's solution (pH 7.25) containing 20 μM H_2DIDS; and finally to Barth's solution (pH 7.6) containing 0.5% BSA to remove reversibly bound H_2DIDS. In this and all subsequent experiments the temperature was 20°C. Ordinate: radioactivity in the oocyte (cpm). Abscissa: time (min).

maximally inhibitory concentration (20 μM). The Barth's solution containing the H_2DIDS had been adjusted to the 'experimental pH', i.e. to that pH at which the covalent reaction with the H_2DIDS was to take place. As a rule this pH was different from the 'standard pH' of 7.6, which was used to measure $^{36}Cl^-$ efflux before and after the exposure to H_2DIDS. In other words, the control fluxes before exposure to H_2DIDS and the flux after exposure were always measured at pH 7.6 while the exposure to H_2DIDS was performed at the experimental pH values indicated on the abscissa in Fig. 3. After perfusion with H_2DIDS at the experimental pH of 7.25 the change of the perfusate to Barth's solution at standard pH containing 0.5% BSA leads to a continuation of $^{36}Cl^-$ efflux at a rate about 30% lower than the rate observed prior to exposure to H_2DIDS (Fig. 1). This indicates a partially irreversible inhibition. Efflux can again be brought to a halt, when the perfusate is changed to Barth's solution containing 500 μM DNDS at standard pH. This confirms that during the exposure to H_2DIDS and the flux measurements preceding and following the exposure, the oocyte did not become leaky.

In Fig. 2, the experiment represented in Fig. 1 has been replotted on a semilog scale. On this scale the time course of release of $^{36}Cl^-$, as observed by continuously monitoring the radioactivity remaining in the oocyte, follows a straight line, the slope of which (0.021 min^{-1}) corresponds to the rate constant of $^{36}Cl^-$ efflux. The data in Fig. 2 show that during treatment with 20 μM H_2DIDS at pH 7.25 the slope of the straight line is reduced to 0.0006 min^{-1} corresponding to 97% inhibition. Most of this reflects reversible inhibition. After removal of the reversibly bound H_2DIDS by BSA, the rate constant for $^{36}Cl^-$ efflux does not return to the original value of 0.021 min^{-1}, but to the lower value of

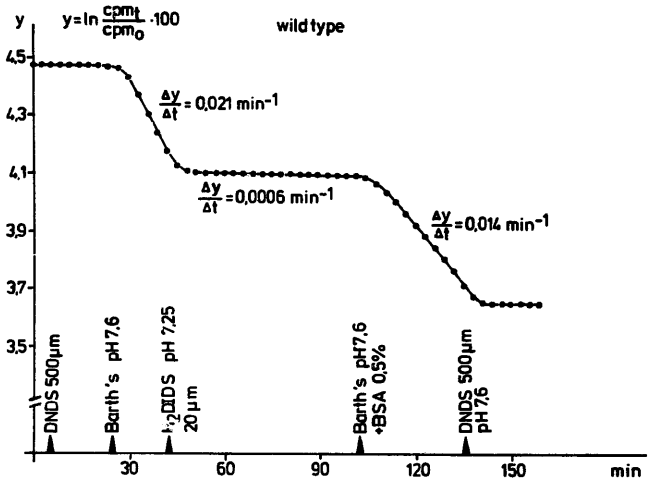

Fig. 2. The same experiment as in Fig. 1. Data were digitalized and replotted on a semilog scale. The figure illustrates the determination of rate constants ($^{\circ}k_{Cl} = \Delta y/\Delta t$). y = natural log of radioactivity in the oocyte as a percent of initial value. Abscissa: time (min).

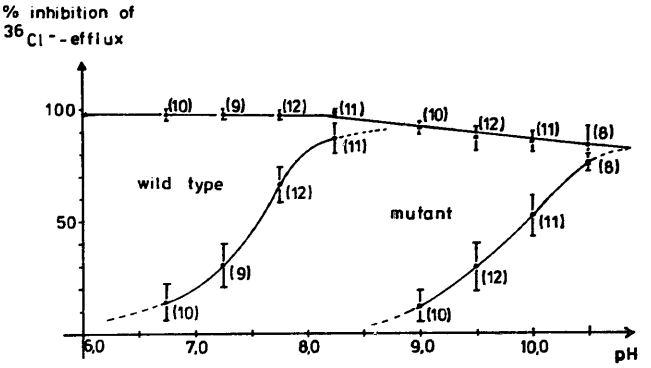

Fig. 3. Irreversible inhibition of $^{36}Cl^-$ efflux mediated by wild-type and mutant band 3 protein after exposure of the oocytes to H_2DIDS for 60 min at the pH indicated in the abscissa. Also shown reversible plus irreversible inhibition of $^{36}Cl^-$ efflux during exposure to H_2DIDS in the perfusate (upper curve). The data points were derived from experiments of the type shown in Figs. 1 and 2. The data points in the upper curve were calculated from the ratio of the $\Delta y/\Delta t$ values during and prior to the exposure to H_2DIDS. The data points in the two lower curves were calculated from the ratio of the $\Delta y/\Delta t$ values obtained after and prior to the exposure to H_2DIDS. The numbers at the error bars (S.D.) indicate the numbers of single oocytes subjected to flux measurements as shown in Figs. 1 and 2.

0.014 min^{-1} which indicates an irreversible inhibition of 33%. At sufficiently low experimental pH, no irreversible inhibition and hence presumably no covalent bond formation takes place at the stilbenedisulfonate binding site. At higher experimental pH values, irreversible inhibition measured after wash-out with BSA-containing Barth's solution is higher (see Ref. 7).

Fig. 3 summarizes the results obtained in experiments of the type described and represented in Fig. 2 at a range of experimental pH values using oocytes in which either wild-type or mutated (Lys-558 → Asn-558) band 3 had been expressed prior to the flux measurements. The upper curve indicates the inhibition observed during exposure to 20 μM H_2DIDS. At the experimental pH values the inhibition was calculated from the ratio of the Cl^- efflux measured during and prior to the exposure to H_2DIDS. The two s-shaped curves represent the pH dependence of the irreversible inhibition that persists *after* removal of reversibly bound H_2DIDS. The curve for the mutant is shifted by about 2.2 pH units to higher pH values, indicating that in the wild-type and in the mutant two distinct amino groups are titrated. There is little if any overlap, suggesting that the curve for the wild-type is nearly exclusively related to the titration of Lys *a* (= Lys-558) while the curve for the mutant in which Lys *a* had been replaced by a non-dissociating amino acid residue pertains to Lys *b* (see Introduction).

Since the reaction of H_2DIDS with Lys *a* and Lys *b* is irreversible, the curves do not represent simple titration curves. Their shapes depend on the time alloted for the reaction to proceed and would, after sufficiently long periods of exposure, lead to 100% inhibition irre-

spective of the pH employed. For further analysis, we calculated the rate constant for covalent H_2DIDS binding from the inhibition achieved after 60 min of exposure. This requires the knowledge of (A) the degree of saturation of the H_2DIDS binding site with reversibly bound H_2DIDS at the beginning of the perfusion period at the experimental pH, and (B) of the time course of the establishment of the covalent bond.

(A) Saturation of band 3 with reversibly bound H_2DIDS. This saturation was studied in the experiments shown in Fig. 4 which represent the relationship between H_2DIDS concentration in the medium and $^{36}Cl^-$efflux from the oocytes, as measured after expression of wild-type and mutant band 3. The data were obtained at pH values where little if any covalent bond formation takes place during the time required for the flux measurements. We find half maximal inhibition at 0.32 μM and 1.1 μM, respectively, for reversible H_2DIDS inhibition in wild-type and mutant band 3. The former value is close to what had been observed previously in human red cells [10]. The latter value suggests that the mutation at Lys *a* leads to some reorganisation of the stilbene disulfonate binding site, which decreases the apparent dissociation constant *.

The upper curve in Fig. 3 shows that over the entire pH range covered in our experiments with the wild-type saturation of the protein with reversibly bound H_2DIDS is achieved when an H_2DIDS concentration of 20 μM is used. Above pH 8.0, at the pH range used for the measurements with the mutant, a slight decrease of inhibition by H_2DIDS binding becomes noticeable. The effect can not be explained by a decrease of saturation as a consequence of the altered K_I value. No attempt was made to explore its nature. For the evaluation of the data it was assumed that for each pH maximal reversible inhibition also corresponds to maximal irreversible inhibition achieved after infinite reaction time. Although this assumption was not further verified, it is unlikely to affect significantly the evaluation of the data presented below since the differences between maximal and complete inhibition did not exceed 15% at the highest experimental pH examined.

(B) The time course of irreversible (covalent) bond formation. This time course was studied in separate experiments where the period of exposure to a saturating concentration of H_2DIDS was interrupted by intervals of efflux measurements at standard pH in the absence of H_2DIDS. The experiments represented in Fig. 5 are based on perfusion periods with 20 μM H_2DIDS at an experimental pH value of 8.25 for wild-type and 10.5 for the mutant for 10, 20, 30, 45 and 60

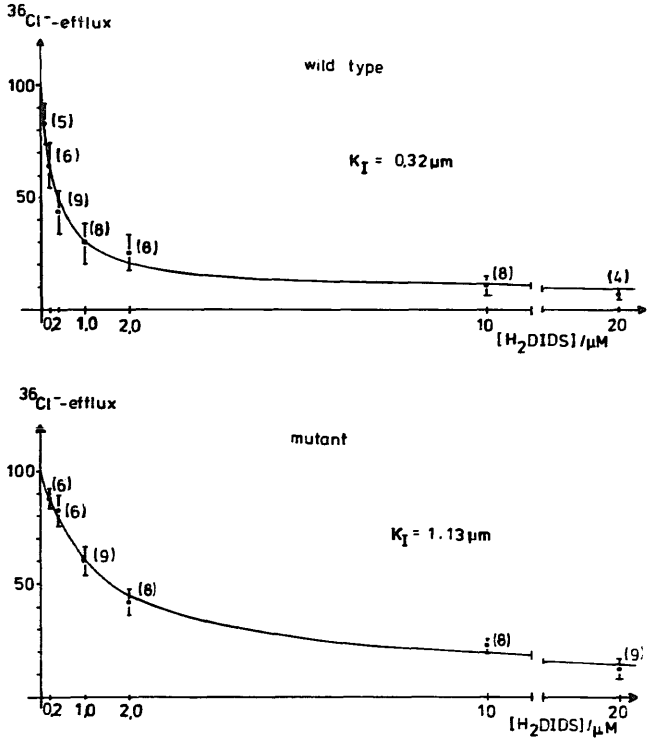

Fig. 4. Reversible inhibition by H_2DIDS of $^{36}Cl^-$ efflux mediated by wild-type and mutated (Lys-558 → Asn-558) band 3 protein, as measured at pH 6.8 (upper panel) and pH 8.0 (lower panel), respectively. Under the conditions chosen, the covalent binding of H_2DIDS is negligibly low. 20°C. Ordinate: Cl^-efflux as a percent of control without H_2DIDS. Abscissa: H_2DIDS concentration, μM.

min, where after each of these time periods the efflux was measured at standard pH (7.6) in the presence of BSA, which removes reversibly bound H_2DIDS from its binding site on band 3. With increasing time of exposure to H_2DIDS, the efflux measured at standard pH after removal of reversibly bound H_2DIDS becomes smaller. This is due to the decrease of the fraction of H_2DIDS binding sites at which H_2DIDS binding remains reversible. The time course follows a single exponential as required for a first-order reaction:

$$^{\circ}k_{Cl(t)} = {}^{\circ}k_{Cl(o)} \cdot e^{-k_{SCN}t}$$

where $^{\circ}k_{Cl(o)}$ and $^{\circ}k_{Cl(t)}$ represent, respectively, the rate constants for Cl^- efflux as measured at pH 7.6 prior to and after exposure to H_2DIDS at the experimental pH. The rate constant k_{SCN} refers to the transition from reversible to irreversible H_2DIDS binding at the experimental pH in the time interval t between the two flux measurements at the standard pH of 7.6.

The Cl^- efflux observed at standard pH after removal of reversibly bound H_2DIDS is proportional to the number of band 3 molecules which are not irreversibly blocked by covalently bound H_2DIDS. Hence k_{SCN} is a measure of the rate of conversion of band 3 molecules occupied with reversibly bound H_2DIDS into band 3 molecules with irreversibly bound H_2DIDS. It

* Similar observations were made with DNDS. The K_i values in wild type (5.8 μM) and mutant (23 μM) differed by about the same factor as for H_2DIDS (not documented).

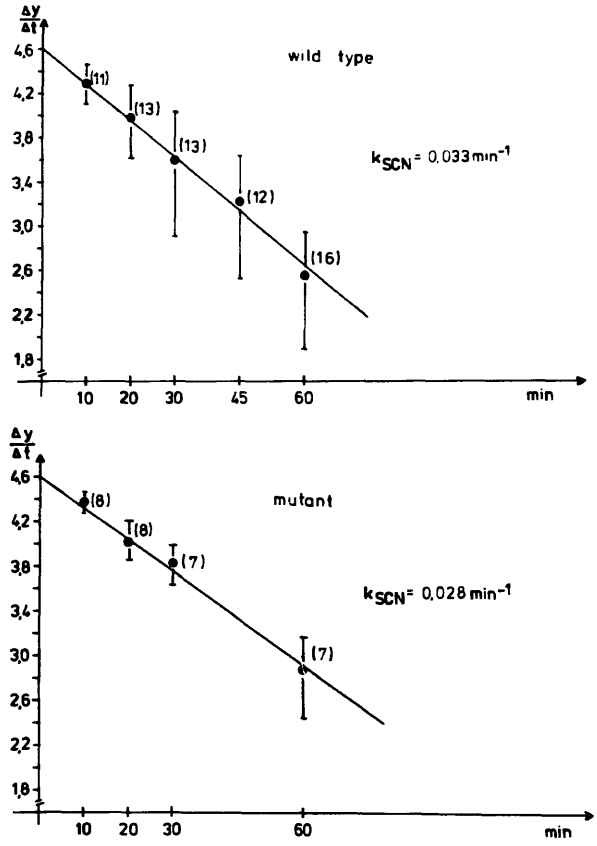

Fig. 5. Time course of irreversible inhibition of wild-type (upper panel) and mutant (lower panel) band 3-mediated $^{36}Cl^-$ efflux by H_2DIDS. Each data point was determined in a separate experiment following the protocol represented in Fig. 1. Rate constants were determined before and after exposure to H_2DIDS for the length of time indicated on the abscissa. Ordinate: $\Delta y/\Delta t$ = natural log of $^{36}Cl^-$ efflux after exposure to H_2DIDS as a percent of the efflux measured in the same oocyte before exposure to H_2DIDS. pH: 8.25 and 10.5 for wild-type and mutant, respectively.

pertains to the experimental pH maintained during exposure to H_2DIDS.

(3) The pH dependence of irreversible inhibition by H_2DIDS

The pH dependence of irreversible inhibition by H_2DIDS shown in Fig. 3 requires a conversion of the inhibition observed after a fixed length of exposure to H_2DIDS into rate constants, k_{SCN}, for covalent bond formation. For this purpose it is necessary to calculate

$$k_{SCN} = \frac{+\ln({}^{\circ}k_{Cl(o)}/{}^{\circ}k_{Cl(t)})}{\Delta t} \qquad (1)$$

where $k_{Cl(o)}$ and $k_{Cl(t)}$ represent, respectively, the rate constants for Cl^- efflux as measured before exposure to H_2DIDS and after removal of non-covalently bound H_2DIDS at standard pH at the end of the period of exposure to H_2DIDS at the experimental pH. Δt designates the time period of exposure to H_2DIDS which was always 60 min. The first order rate constants for

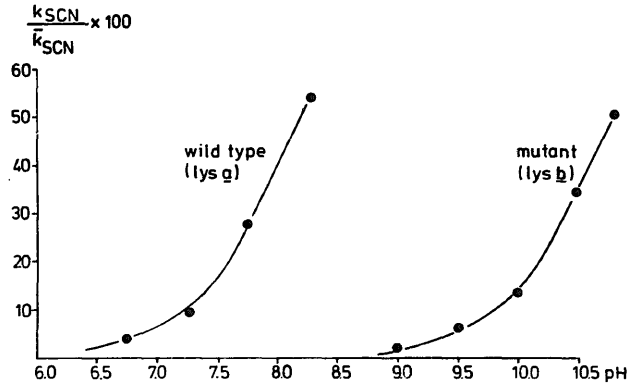

Fig. 6. Rate constants calculated from the date in Fig. 3, using Eqn. 1. The drawn lines represent non-linear least-squares fits to Eqn. 2. Ordinate: rate constant k_{SCN} for thiocyanylation of Lys *a* or Lys *b*, as a percent of maximal rate $\bar{k}_{SCN}$. Abscissa: pH.

irreversible H_2DIDS binding are calculated from the data in Fig. 3 and replotted in Fig. 6.

The thiourea bond formation between the ϵ-amino group of a lysine and one of the isothiocyanate groups of H_2DIDS takes place only when the ϵ-amino group is deprotonated:

$$R_1\text{-}NCS + H_2N\text{-}R_2 \rightarrow R_1\text{-}N\text{-}\underset{\underset{S}{\|}}{C}\text{-}R_2 \qquad (i)$$

where

$$H^+ + H_2N\text{-}R_2 \rightleftharpoons H_3\overset{+}{N}\text{-}R_2 \qquad (ii)$$

Hence k_{SCN} varies with the hydrogen ion concentration. Application of the mass law to (ii) yields the fraction of deprotonated lysine residues. For the pH dependence of k_{SCN} one obtains (see Eqn. 1)

$$k_{SCN} = \bar{k}_{SCN}\frac{K}{K + [H^+]} \qquad (2)$$

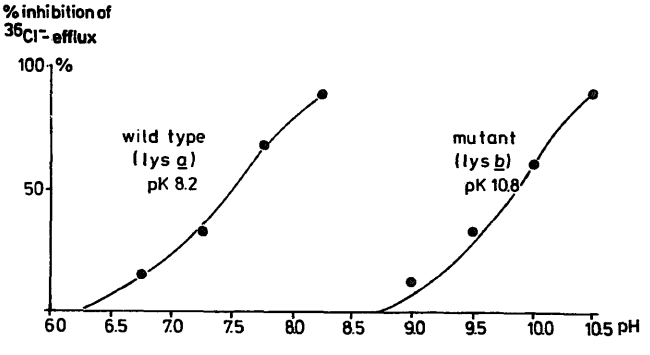

Fig. 7. Predicted pH dependence of irreversible inhibition by H_2DIDS of Cl^- efflux from oocytes containing mouse band 3. The predictions were made by means of Eqns. 1 and 2. Inhibition during exposure to 20 μM H_2DIDS in the perfusion medium (Barth's solution, upper curve in Fig. 3) at the respective experimental pH values was equated to 100 percent. Drawn line: predicted inhibition. Data points recalculated form the experiment shown in Fig. 3.

TABLE I

pH dependence of the reaction of H_2DIDS with Lys a and Lys b after expression of mouse band 3 in Xenopus oocytes and in the human red blood cell membrane

The values for band 3 expressed in the oocytes were calculated from the data in Figs. 6 and 7 using Eqn. 2. The values for human band 3 in the human red cell membrane were taken from the work of Kampmann et al. [1]. The last column represents pK values of Lys *a* and Lys *b* in the human red cell membrane at 20°C calculated from the original data (obtained at 30°C) on the assumption that the heat of dissociation of the ϵ-amino group of lysine amounts to 12.5 kcal/mol [11]. These calculated pK values should be compared with the pK values observed in the oocyte.

Lys	Mouse band 3 in oocytes		Human band 3 in human red cells	
	$\bar{k}_{SCN}$ (min^{-1})	$pK_{20°C}$	$pK_{30°C}$	$pK_{20°C}$
a	0.070	8.2	7.3	8.0
b	0.12	10.8	10.0	10.7

where K represents the dissociation constant pertaining to the equilibrium (ii) and $\bar{k}_{SCN}$ the (pH-independent) rate constant for the reaction with a deprotonated lysine residue.

Numerical values for $\bar{k}_{SCN}$ and pK for both wild-type and mutant can be calculated by means of Eqn. 2 using the data in Fig. 6 and a suitable non-linear least-squares curve fitting procedure. The values are listed in Table I, together with values previously determined [1] in studies of the cross-linking reaction in human red cells. On the basis of these values, it was possible to predict the pH dependence of irreversible inhibition by reaction of H_2DIDS with Lys *a* and Lys *b* as observed after 60 min of exposure to a saturating concentration of the agent. Fig. 7 shows that the data points taken from Fig. 3 are reasonably close to the predicted curves.

(4) Comments

The pK values for both Lys *a* and Lys *b* as determined in the present experiments are shifted to values which are about 0.8–0.9 units higher than determined in our previous work [1]. This is most likely related to the fact that in the previous work with red blood cells the measurements were done at 30°C while in the present work with the oocytes the temperature was 20°C. If one assumes that the heat of dissociation of the amino group of ethanolamine (12.5 kcal/mol [11]) is close to the heat of dissociation of the ϵ-amino group of a lysine residue in an electrically neutral environment (for a detailed discussion see Ref. 1) one can calculate from the pK values measured at 30°C the pK values expected to pertain to 20°C. These calculated values are not inconsistent with the observations made in band 3 expressed in the oocyte (Table I). We may conclude, therefore, that the present measurements on band 3 expressed in the plasma membrane of the oocyte support our previous work with red blood cells. They show that results obtained after exposure of the band 3 protein to H_2DIDS in the oocytes are comparable to results obtained in the red cell membrane, and that this conclusion is true for both wild-type and mutant.

The pK values of amino acid residues in a protein depend on the environment in which the residue is located. Dielectric constant, neighbouring charges, hydrogen bonds, exert more or less pronounced effects. Hence, the pK value of a specific amino acid residue represents a characteristic indicator for the tertiary structure of a specific region of the protein. Lys-558, although not directly involved in substrate binding, is located at or near the substrate binding site of the transport protein (for review see Ref. 5). Its pK value is much lower than expected for a lysine residue in an electrically neutral environment with the dielectric constant of water, where one would expect a pK of about 10.5 [11]. The pK values deduced from the rate of reaction of H_2DIDS are influenced by the two negatively charged sulfonyl groups introduced by the inhibitor into the neighbourhood of Lys *a* and Lys *b*. These charges as well as the conformational change of the protein associated with the reversible H_2DIDS binding alter the protein structure around Lys *a* and Lys *b*. Hence, the pK values deduced from our work of 8.2 and 10.8, respectively, are unlikely to be identical to the values found in the absence of the inhibitor. Nevertheless, the similarity of these values observed in band 3 expressed in the lipid bilayer of the red cell and the plasma membrane of the oocyte provides a fairly sensitive indicator for the essential similarity of the tertiary structure of the transport protein in both environments.

The agreement between our present results and those previously obtained in the red cell is all the more remarkable since the present work was done with mouse band 3 while the work with red cells refers to human band 3. Although the hydrophobic (i.e. anion transport) domains of the band 3 proteins of both species show more than 90% sequence homology, this result was not self-evident. In fact, a report by García and Lodish [12] indicates that after expression of human band 3 cRNA in oocytes of *Xenopus* even after replacement of Lys *a* (corresponding to Lys-539 in the human band 3) by a Gln residue, covalent bond formation is still possible as in the wild-type. This discrepancy is unexplained. A repetition of the work with human and mouse band 3 after expression in the oocytes and flux measurements under strictly identical conditions is under way and is expected to provide an explanation.

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