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Band 3, the anion exchanger of the erythrocyte membrane, is also a flippase

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Abstract

The transbilayer reorientation (flip-flop) of the long-chain amphiphilic anion DENSA (5-(*N*-decyl)aminonaphthalene-2-sulfonic acid) in the erythrocyte membrane was studied by fluorescence spectroscopy. DENSA intercalates into the membrane at a high membrane/water partition coefficient ($3.2 \cdot 10^5$) and rapidly reorients from the outer to the inner layer in a first order process ($k = 0.11$ min^{-1}, 37°C, pH 7.4) leading to a steady-state distribution inner:outer layer of about 30:70. The activation energy of the fully reversible and symmetric flip process is about 110 kJ/mol. DIDS and various other established covalent and non-covalent inhibitors of anion transport via the erythrocyte anion exchanger, band 3 (AE 1), suppress the flip to a minimum of about 30–35% of the control. The flip is also inhibited by Cl^- with a half maximal inhibitory concentration equal to that required for the inhibition of the exchange flux of ordinary anions via band 3. These findings indicate the involvement of a band 3 mediated (DIDS-sensitive) component of the flip and a DIDS-insensitive one, possibly involving, at least to some extent, simple transbilayer 'diffusion'. This latter component is stimulated by diamide, an SH oxidant known to increase the permeability of the membrane lipid domain of the erythrocyte. Alcohols (butanol, hexanol) accelerate both flip components. Papain treatment, known to inhibit 'ordinary' anion exchange, accelerates both flip and flop. The results suggest that band 3 protein, besides being a conventional transporter of anions, can act as a flippase translocating anionic, membrane-intercalated amphiphiles approaching the transporter from the lipid domain. The flippase mode of operation of band 3 must, however, differ in its mechanism from the conventional exchange mode.

Key words: Erythrocyte membrane; Flip-flop; Amphiphile; Anion exchange; Band 3; Flippase

1. Introduction

Physiology and biophysics of membrane transport have traditionally been concerned with the translocation of hydrophilic, polar or even ionic substrates by passive non-mediated diffusion or various modes of active or passive carrier-mediated transport. All these types of translocation have in common that the (apparent) membrane concentration of the transported species is very low in relation to its bulk aqueous concentration.

More recently, the transport of hydrophobic and in particular amphiphilic compounds has attracted increasing interest [1–4]. In the case of such compounds, a high membrane/water partition coefficient accounts for their preferential or essentially exclusive localization in the membrane. Under biological conditions, the handicap – for transmembrane transport – of the low solubility of such compounds in the aqueous phase is overcome by the contribution of binding proteins [5,6] serving as donor and/or acceptor. In the case of amphiphilic compounds with a polar or even ionic headgroup, this headgroup limits the rate of reorientation of the membrane-intercalated molecule from one to the other leaflet of the bilayer. Consequently, the simple 'flip-flop' of ionic or zwitterionic amphiphiles like phospholipids or their lyso-derivatives is usually a very slow process [7–9], in contrast to the fast flip-flop of uncharged nonpolar amphiphiles like alcohols, nonionized fatty acids, etc. [10–12].

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Abbreviations: DNDS, 4,4'-dinitrostilbene-2,2'-disulfonic acid; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; WWK, Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3'-sulfonate); DENSA, 5-(*N*-decyl)aminonaphthalene-2-sulfonic acid; TNBS, 2,4,6-trinitrobenzene sulfonate; DMSO, dimethylsulfoxide.

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Unexpectedly high rates of reorientation of anionic phospholipids (phosphatidylethanolamine, phosphatidylserine, etc.) observed in various membrane systems have led to the discovery and characterization of 'flippases' as a new type of transporters [13]. The data presented in the following provide evidence that a conventional transport system for hydrophilic ions, the anion exchanger (AE 1 [14]) of the erythrocyte membrane [15], can also serve as a flippase. Preliminary data were reported elsewhere [16].

2. Materials and methods

Materials

Human blood anticoagulated by citrate was obtained from the local blood bank and used within 10 days. After centrifugation, supernatant and buffy coat were carefully removed and the erythrocytes washed three times with isotonic saline.

DNDS and 5-aminonaphthalene-2-sulfonic acid were obtained from Aldrich, DIDS and Woodward's reagent K (WWK) from Fluka AG, TNBS from ICN, dipyridamole and papain (9.5 units/mg solid) from Sigma, diamide from Calbiochem, phloretin from Carl Roth, Karlsruhe.

5-(*N*-Decyl)aminonaphthalene-2-sulfonic acid (DENSA) (Fig. 1) was synthesized with slight modifications according to Waggoner and Stryer [17]. The Bucherer reaction [18] was used for coupling decylamine to 5-aminonaphthalene-2-sulfonic acid. The product was purified by thin-layer chromatography on silica plates (Merck, Darmstadt, No. 5745) using chloroform/ethanol/ethyl acetate/acetic acid (70:30:20:10, v/v). The fluorescent product ($R_f = 0.34$) was scraped from the plate and extracted from the silica with ethanol. The identity of the product was established by NMR spectroscopy.

Methods

The time-dependent transbilayer reorientation (flip) of DENSA after its primary insertion into the outer membrane layer of erythrocytes at 0°C was quantified using an albumin extraction procedure [7]. This method is based on the finding that long-chain amphiphiles can be removed quantitatively and selectively from the outer layer of the erythrocyte membrane by saline containing 1 $g\,dl^{-1}$ albumin, provided their rate of transmembrane reorientation is sufficiently lower than the rate of removal from the outer layer ('kinetic trapping'). Briefly, cells were suspended (Hct 25%) in medium A, containing (mM) NaCl (4), KCl (90), Na_2HPO_4/NaH_2PO_4 (12.5) (pH 7.4), sucrose (44 mM) at 0°C. The fluorescent probe was added from a stock solution in DMSO at a final concentration of about 60 μM. DMSO levels did not exceed 0.14 mM. After 15 min the cells were sedimented by centrifugation and washed three times in medium A at 0°C. Transbilayer reorientation, which is negligible at 0°C, was initiated by suspending cells (Hct 10%) in medium A at higher temperatures. After appropriate time intervals samples were removed, the cells sedimented ($8500 \times g$, 10 s), washed once in medium A, and extracted twice with bovine serum albumin (1 $g\,dl^{-1}$ in medium A) at 0°C. Subsequently, remaining probe was extracted from the cells with 7 volumes isopropanol at room temperature and quantified by fluorescence spectroscopy. In order to obtain the fractional reorientation of the probe to the inner layer, this residual fluorescence was related to the total fluorescence of cell samples extracted with isopropanol without preceding treatment with albumin. Rates of reorientation were calculated from those numbers according to Ref. [19], taking into account the final steady-state distribution of the probe between both leaflets of the bilayer.

Resealed ghosts were prepared by lysing cells in 10 volumes 5 mM K^+-Hepes (pH 7.4, 0°C) and reconstitution with one volume of a concentrated mixture of K^+-glutamate and KCl in different proportions as well as Na-succinate (final concentration 2 mM) in preparations used for flux measurements. After 45 min resealing at 37°C these ghosts were either used for measuring flip rates of DENSA as described above or for measuring exchange fluxes of succinate at 35°C after loading the ghosts with 2,3-[^{14}C]succinate (NEN-DuPont) following previously described procedures [20].

Pretreatment of the cells with DIDS [21], TNBS [22], WWK with subsequent reductive cleavage by $NaBH_4$ [23], papain [24] and diamide [19] was carried out essentially as described in the references quoted.

The membrane/water partition coefficient of the flip probe was determined by first 'loading' the cells with the probe at 22–24°C, washing and subsequent equilibration with medium (Hct 10%, 90 min). Concentrations of the probe determined in the membrane and in the supernatant after centrifugation served to calculate the dimensionless partition coefficient.

3. Results and discussion

Erythrocytes exposed to the long-chain alkylarylsulfonate DENSA (60 nmol/ml cells) at 0°C incorporate the probe almost completely. Using appropriate techniques, and assuming a membrane volume of 10 μl per ml cells, a membrane/water partition coefficient of about $3.2 \cdot 10^5$ was obtained. Thus, the bulk aqueous concentration of the probe during flip measurements was in the low nanomolar range.

DENSA reorients to the inner leaflet of the membrane after its primary insertion into the outer leaflet

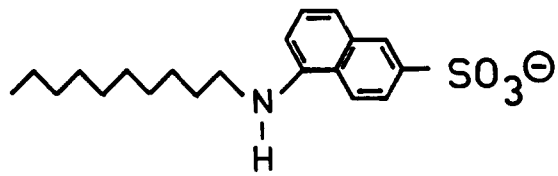

Fig. 1. Structure of the amphiphilic anionic probe 5-(*N*-decyl)amino-naphthalene-2-sulfonic acid (DENSA).

(Fig. 2). From the kinetics at 37°C and pH 7.4 a first-order rate coefficient (k) of 0.11 min^{-1}, equivalent to a halftime of 4.7 min, was derived. This k value is much higher than those obtained at the same temperature for structurally related long-chain zwitterionic amphiphiles such as palmitoyllysophosphatidylcholine ($k = 0.0003$ min^{-1} at 37°C) [7] or palmitoylcarnitine ($k = 0.002$ min^{-1} at 37°C) [25], which have a comparable length of the hydrophobic domain of the molecule. The flip process is fully reversible. Inward oriented probe returns to the outer layer ('flop') at the same rate ($k_{out} = 0.014$ min^{-1}, $k_{in} = 0.015$ min^{-1} at 22°C, pH 7.4). Transbilayer reorientation in both directions can be described by first-order kinetics and leads to a steady-state distribution of the probe at a ratio inner to outer layer of about 30:70.

Flip rates increase linearly with the membrane concentration of the probe in the range studied of 30–300 nmol per ml cells, equivalent to ratios probe/membrane phospholipid ranging from 1:140 to 1:14

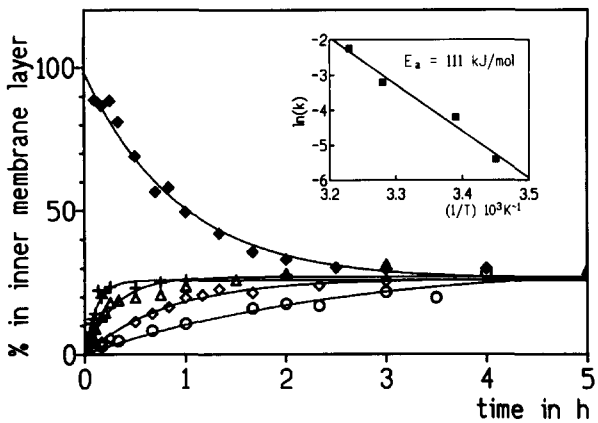

Fig. 2. Transbilayer inward ('flip') and outward ('flop') reorientation of DENSA at different temperatures: (+) 37°C, (△) 32°C, (◇, ◆) 22°C, and (○) 17°C. Flip: open symbols, flop: closed symbols. Erythrocytes were suspended (Hct = 25%) at 0°C in medium A (pH 7.4) containing the fluorescent probe DENSA. After its primary insertion into the outer layer, the time-dependent reorientation of the probe to the inner layer of the membrane was studied by fluorescence spectroscopy as described in Methods. For measuring the 'flop', the cells were equilibrated with the probe at 37°C. The probe in the outer layer was then extracted at 0°C with albumin and the time-dependent reorientation of inner layer probe to the outer layer was measured by the albumin extraction procedure. (Inset) Arrhenius plot derived from the first order rate coefficients calculated from the curves. Data from a representative experiment.

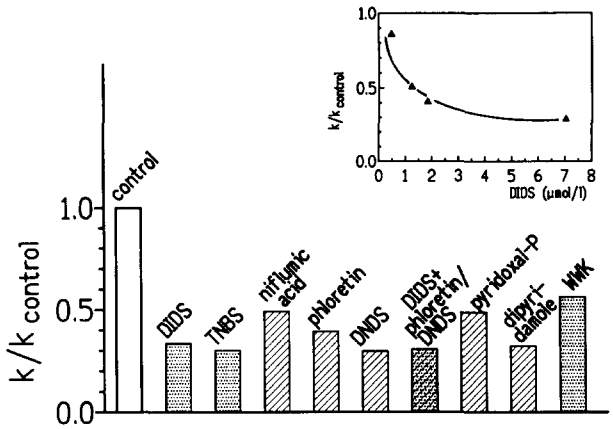

Fig. 3. Inhibition of the flip of DENSA by compounds known as inhibitors of anion exchange via band 3 protein. Dotted columns: covalent inhibitors, pretreatment at 10% Hct: DIDS (50 μM, 30 min, 37°C, pH 7.4), TNBS (5 mM, 30 min, 37°C, pH 8.0), WWK (2 mM, 10 min, 0°C. pH 7.0) followed by $NaBH_4$ (2 mM, 10 min, 0°C, pH 7.0). Hatched columns: noncovalent inhibitors present during flip measurements: niflumic acid (20 μM), phloretin (250 μM), DNDS (500 μM), pyridoxal phosphate (5 mM), dipyridamole (15 μM). (Inset) Dose–response curve for DIDS.

(data not shown). The apparent activation energy derived from Arrhenius diagrams is constant between 17°C and 37°C (Fig. 2, inset) and amounts to about 110 kJ/mol. This number corresponds to values obtained for the simple non-mediated flip of other amphiphiles [7]. On the other hand, activation energies of this magnitude are also characteristic for many carrier-mediated transport processes. The involvement of such a process is strongly suggested by data presented in the following paragraphs.

(1) The flip of DENSA is markedly suppressed by a number of compounds having in common the potency to inhibit anion transport via band 3 protein (AE 1 [14]), the anion exchanger of the erythrocyte membrane. As shown in Fig. 3, treatment of the cells with covalent amino- or carboxyl-reactive modifiers of band 3 (DIDS [21], TNBS [26], and WWK/$NaBH_4$ [23]) as well as flip measurements in the presence of a number of reversible inhibitors of anion exchange (see Ref. [27] for detailed references) cause a marked, concentration-dependent (Fig. 3, inset) decrease of the flip rate of DENSA. The inhibition is not complete, however, but levels off at a residual flip rate of about 30–35% of the control. The I_{50} value for DIDS (0.9 μM) is not too different from I_{50} values reported for the 'conventional' transport of hydrophilic anions [28]. The flop (outward reorientation) of the probe was also suppressed by the inhibitors. Although the specifity of the tested inhibitors is not absolute, the finding that the flip rate of DENSA, an anionic amphiphile, could be altered by such a great variety of compounds known to affect anion transport clearly points to an involvement of the anion exchange protein in the flip of DENSA.

(2) A characteristic, specific feature of anion transport via band 3 is the anion dependence of its inhibition by dipyridamole [29]. There is essentially no inhibition when chloride is replaced by sulfate, while other transporters (lactate, monosaccharides) are inhibited by dipyridamole regardless of the anion milieu (Deuticke, B., unpublished results). The marked inhibition of the flip rate of DENSA by dipyridamole observed in chloride (I_{50} about 2 μM) but not in sulfate medium (Fig. 4) provides strong evidence for an involvement of the anion exchanger.

(3) Proteolytic digestion of erythrocytes by papain cleaves band 3 protein exofacially, producing an N-terminal 60 kDa fragment, a C-terminal fragment of about 28 kDa and two small fragments of about 1.3 and 7.5 kDa [30]. This cleavage goes along with a marked inhibition of anion exchange [31,32]. Papain treatment also affects the flip of DENSA. Both flip and flop were accelerated (Fig. 5) to an extent increasing progressively with the concentration of the proteinase. The enhancement did not saturate even after extensive cleavage of band 3 according to the pattern outlined above.

Two further observations are also compatible with an involvement of band 3 in this flip process:

(a) The DIDS-sensitive component of the transfer of DENSA is affected qualitatively by changes of the anion milieu like anion exchange, though to a lesser extent. Normalized to chloride medium flip rates increase as follows: NO_3^-: 0.46, Br^-: 0.88, Cl^-: 1.0, acetate: 1.2, SO_4^{2-}: 1.6. In the case of phosphate exchange the sequence of anion effects is [33]: NO_3^-: 0.28, Br^-: 1.0, Cl^-: 1.0, acetate: 1.9, SO_4^{2-}: 2.7.

(b) The DIDS-sensitive component of the flip of DENSA exhibits the species differences established earlier [34] for anion exchange. In rat and rabbit erythrocytes the flip of the probe is much faster and in sheep erythrocytes considerably slower than in human erythrocytes (data not shown).

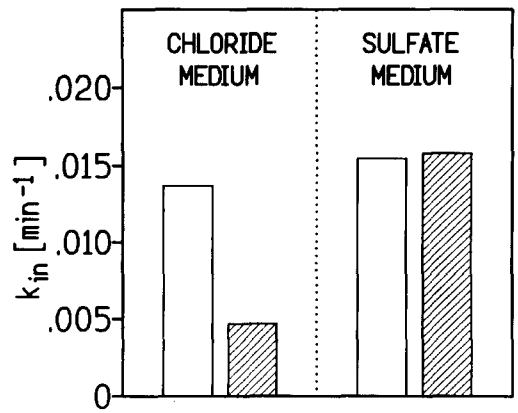

Fig. 4. Influence of dipyridamole (15 μM) on the flip of DENSA as a function of the anion milieu. Note that a strong inhibition of the flip rate is only observed in chloride medium.

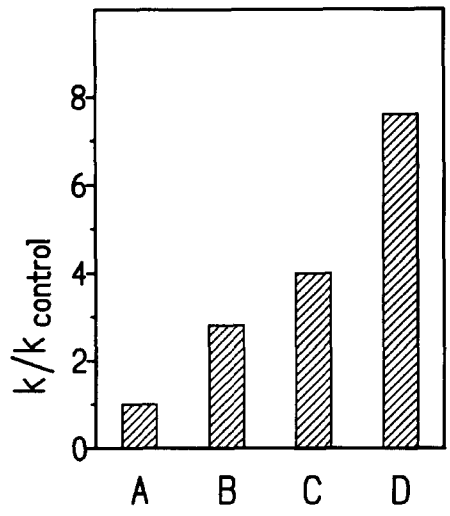

Fig. 5. Effect of a proteolytic digestion of the extracellular surface of erythrocytes by papain on the flip rate of DENSA: control (A), 0.1 mg papain/ml cells (B), 0.2 mg papain/ml cells (C), 0.5 mg papain/ml cells (D). Cells were treated with the cysteine-activated proteinase for 60 min, washed twice with medium A containing bovine serum albumin (0.2 g dl^{-1}) and used for flip measurements.

On the other hand, the characteristic variations, with pH, of the exchange of mono- and divalent anions [15,30], are not observed in the case of the flip of DENSA. Rates are essentially constant between pH 6 and 9.3 (data not shown). Moreover, the probe proved to be a rather poor inhibitor of the exchange flux of 'normal' anions (oxalate). 50% inhibition required about 60–80 μM, in contrast to less hydrophobic sulfonates such as 8-anilinonaphthalene-1-sulfonate or 6-toluidinylnaphthalene-2-sulfonate ($I_{50} \leq 15$ μM).

The incomplete inhibition of the flip of DENSA by noncovalent and covalent inhibitors of band-3-mediated anion transport might suggest a component of the flip of the probe occurring by a DIDS-insensitive 'non-mediated' process comparable to the flip of certain lyso- and diacylphospholipids involving the lipid domain of the membrane [13]. This pathway of flip is characterized by an enhancement after a pretreatment of the cells with the mild SH oxidant, diamide, which converts SH-groups into disulfides, produces cross-linking of membrane skeletal proteins and thereby somehow perturbs the lipid phase of the membrane [19]. Diamide pretreatment indeed enhances the flip rate of DENSA (Fig. 6), though only much less than that of phospholipids or palmitoyl carnitine [7,25]. Interestingly, the steady-state distribution of the probe becomes nearly symmetric after diamide. The stimulating effect of diamide only concerns the DIDS-insensitive component of the flip of DENSA, which was enhanced by a factor of 5.1 ± 1.0 ($n = 3$). In contrast, the DIDS-sensitive component of the flip was inhibited

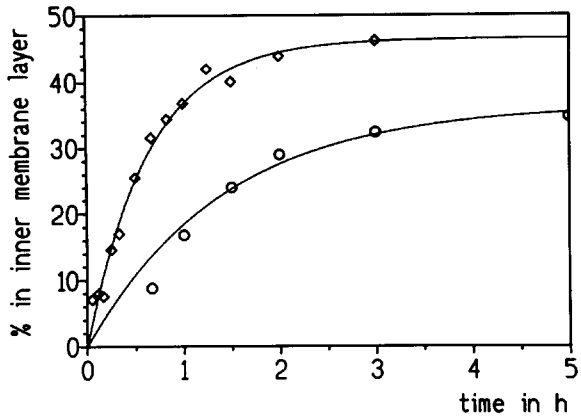

Fig. 6. Influence of pretreatment of erythrocytes with diamide on the flip of DENSA. Erythrocytes were incubated with diamide (5 mM, 45 min, 37°C, pH 8.0) prior to flip measurements. (○) control, (◇) diamide.

up to 50% after diamide treatment, a finding not fully unexpected in the light of slight inhibitory effects of diamide on the transport of ordinary anions via band 3 [35,36]. These results suggest an involvement of the lipid domain in the DIDS-insensitive component of the flip-flop of DENSA.

Hexanol (Table 1) and butanol (data not shown), alcohols known to enhance flip processes across the lipid domain of the erythrocyte membrane [37], also accelerate the flip of DENSA. Since alcohols are known to be inhibitors of the transport of 'hydrophilic' anions via band 3 [38], we analyzed the effects of the alcohols on both, the DIDS-sensitive (band 3-mediated) and the DIDS-insensitive component of the flip of DENSA. Surprisingly, both components were enhanced in the presence of hexanol.

Thus, there may be in fact two components of the flip-flop of DENSA. A major one is probably mediated somehow by band 3, which would therefore have the capability to act as a flippase, i.e., a carrier accepting and translocating substrates which approach the transporter not from the aqueous phase but from the lipid bilayer subsequent to a partitioning step [39].

It might be objected that the transport of DENSA via band 3 is in fact not different from that of 'ordinary' hydrophilic anions and occurs by normal anion exchange of that fraction of the amphiphile that is present in the bulk water phase. Although this argument can not be fully discarded at present, it seems not very likely that nanomolar levels of an anion would be transported at the rate observed in the presence of high millimolar levels of chloride. Moreover, the discrepancies between flip and anion exchange in the response to papain and alcohols are hard to reconcile with a strictly conventional mode of operation of the anion transporter.

Table 1
Influence of hexanol on the flip of DENSA

	k (min^{-1})		
	total	DIDS-sensitive	DIDS-insensitive
Control	0.080	0.054	0.026
Hexanol (10 mM)	0.147	0.091	0.056
k_{hex}/k_{con}	1.8	1.7	2.1

Flip was measured in normal and DIDS pretreated (50 μM, 37°C, 30 min) cells in the absence (controls) and presence of hexanol.

The minor, DIDS-insensitive component might involve the lipid domain and could thus be comparable to the flip of other zwitterionic or anionic long-chain amphiphiles. The stimulation of this component by alcohols and by diamide is in line with such a concept. On the other hand, the rate obtained for this putatively non-mediated component of the flip of a fully ionized anionic probe is surprisingly high ($k = 0.032\ min^{-1}$ at 37°C) as compared to that of zwitterionic amphiphiles known to penetrate through the lipid domain of the membrane (palmitoyllysophosphatidylcholine 0.0003 min^{-1} [7], palmitoylcarnitine 0.002 min^{-1} at 37°C [25]). In the light of this discrepancy one has to consider the possibility that the flip of DENSA after maximal inhibition might still comprise a component mediated by band 3, due to an incomplete inhibition of the flippase mode of operation of band 3.

The alternative that a flippase similar to that catalyzing the fast, ATP-dependent, transbilayer reorientation of aminophospholipids [13] mediates the DIDS-insensitive flip of DENSA can be dismissed, since neither ATP depletion nor vanadate inhibit the flip of the probe (data not shown).

Transport, via band 3, of anionic amphiphiles is not restricted to the fully ionized sulfonate studied here. Recent data from our laboratory indicate that the weakly anionic lysophospholipid, 1-palmitoyl-*sn*-glycero-3-phosphomethanol is also translocated to a considerable extent by this flippase mode of operation of band 3 [40].

The mechanism of operation of band 3 in mediating a flip of DENSA that is sensitive to DIDS and other inhibitors of band 3 remains to be clarified. In view of its stimulation by papain treatment and by alkanols, which both inhibit anion exchange, this mode of operation of band 3 is most likely different from the exchange mode. An incomplete inhibition by agents known to produce an essentially complete suppression of 'conventional' anion exchange via band 3 (see above) would also support such a concept.

A major issue to be addressed will be whether the binding site that is required for the transport of hydrophilic ions approaching the barrier via an 'access channel' [41,42] is also involved in the flippase mode of operation. We have undertaken a first attempt to clarify this problem: Using resealed ghosts, prepared as

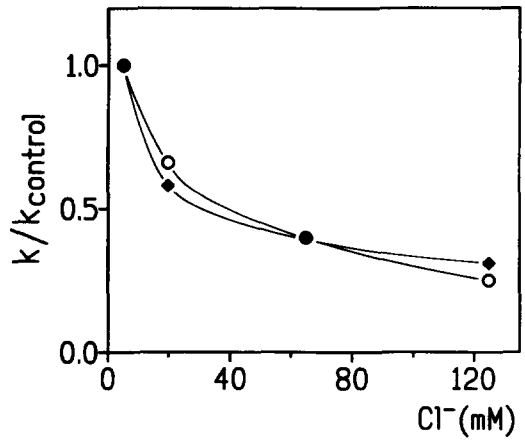

Fig. 7. Inhibition, by chloride, of the flip of DENSA (27°C, pH 7.4) (♦) and the exchange flux of succinate (35°C, pH 7.4) (○) in resealed ghosts. Ghosts were prepared as described in Methods. Chloride levels were set by reconstituting the open ghosts after hypotonic lysis to isotonicity in media containing the desired chloride concentrations. Isotonicity was established by adding K^+-glutamate. After resealing, flip rates and succinate fluxes were measured in suspension media having the same ionic composition as the ghost interior.

described in Methods and reconstituted with media containing various levels of chloride as a substrate of the anion transporter and glutamate as a monovalent 'spectator anion' not transported by band 3 [43], we could demonstrate inhibition of the flip of DENSA by chloride with an I_{50} (about 45 mM) of the same magnitude found for the inhibition, by chloride, of the self exchange of succinate via band 3 under comparable conditions (Fig. 7). Although a competitive nature of this chloride inhibition of the flip of DENSA can not be directly demonstrated in this experimental set-up, the agreement of the I_{50} values for flip and anion self exchange indicates that the chloride binding site responsible for the inhibition of succinate transport, which is almost certainly competitive, also accounts for the inhibition of the flip of DENSA.

On the other hand, the lack of any pH dependence of the flip of DENSA clearly suggests that the titration of the groups that render the exchange of small anions pH-sensitive [30] does not affect the flip process. In this respect, the flippase mode of operation of band 3 resembles its conductive mode of operation, which allows net anion movements. Anion conductance via band 3 differs from the exchange mode of operation of band 3 but lacks significant pH dependence [44]. Experiments are presently under way to clarify whether the flip of DENSA is electrogenic, i.e., can be affected by changes of membrane potential, as expected for a conductive mode of transport.

As a preliminary interpretation of the finding that small alkanols but also papain treatment enhance the DIDS-sensitive component of the flip one might speculate, that in the flippase mode of operation of band 3 the rate-limiting step consists in the transmembrane movement of the bulky alkyl-aryl moiety of our probe between the aggregated, immobilized intramembrane helices [45,46] of band 3. Acceleration of the flip of DENSA in the presence of alkanols or after cleavage of interhelical junctions by papain might in this model be attributed to an increased mobility of the helices and thus a lowered 'resistance' of the intramembrane domain of band 3. This could facilitate the movement of the long lipophilic chain of DENSA between adjacent helices [47].

Acknowledgements

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