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Band 3-Mediated Flip-Flop and Phosphatase-Catalyzed Cleavage of a Long-Chain Alkyl Phosphate Anion in the Human Erythrocyte Membrane

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Abstract. In pursuit of the characterization of the recently discovered flippase mode of operation of the anion transporter (band 3, AE1) of the human erythrocyte membrane, the transbilayer translocation (flip) of a fluorescently labeled, membrane-intercalated long-chain alkyl phosphate, 10-(α-napthyl)-1-decyl-phosphate (NDP) was investigated. In contrast to the alkyl sulfonates and esters of phosphatidic acid studied as yet, NDP moves exclusively via band 3. NDP is, however, dephosphorylated at the inner membrane surface by a cytoplasmic phosphatase likely to interact specifically with endofacial membrane structures of the erythrocyte. This phosphatase shares characteristic inhibitor sensitivities with protein tyrosine phosphatases present in the erythrocyte interior. Vanadate as an inhibitor of NDP dephosphorylation provided a means to study the kinetic properties and patterns of inhibition (by inhibitors of anion exchange) and stimulation (by proteolysis of band 3 and aliphatic alcohols) of the flip of NDP. NDP is also an inhibitor of the exchange of hydrophilic anions via band 3, while hydrophilic anions interfere with the flip of NDP. The results are compared with the characteristics of the flip, via Band 3, of other amphiphilic anions and of the exchange of hydrophilic anions. Attempts are presented to understand the low flip rate of long-chain amphiphilic anions on the basis of their molecular properties and the thermodynamics of the ''transition state'' of the flip process.

Key words: Erythrocyte membrane — Flippases — Band 3 — Anion transport — Phosphatase

Introduction

Membrane transport physiology has traditionally been dealing with the characterization of passive and active movements of hydrophilic solutes (and water) between aqueous compartments separated by a membrane serving as a barrier. Only recently attention has also been paid to translocation processes occurring *within* biomembranes, i.e., between the two leaflets of the lipid bilayer [14, 67]. Such ''flip-flop'' processes provide for the transverse mobility of phospholipids [14, 67], cholesterol [62], long-chain alkane derivatives like fatty acids [38], and of similar amphiphilic compounds located exclusively or predominantly in the hydrophobic membrane phase due to their low water solubility. Transport by flip-flop can have the properties of a simple diffusive process governed by the principles of the physicochemistry of lipids [31, 44]. More interesting, flip-flop can occur as a mediated process catalyzed by membrane proteins [15]. A growing number of ''flippases'' has been characterized since the original discovery of phospholipid translocases [15, 16, 30, 61].

Flippases can mediate passive gradient-driven and active ATP-driven movements of amphiphiles [14, 16, 62, 67]. In spite of an increasing body of information on this new type of transporters, many details concerning the molecular events during the flippase-mediated translocation of amphiphiles are still unknown, although general interest in this type of transport has been stimulated in recent years by the emerging relationships between flippases and transporters of the ABC-cassette family [27, 53] and the family of multispecific organic anion transporters (MOAT [39, 57]).

In recent studies, we have demonstrated that the long-known and well characterized anion transporter of the erythrocyte membrane, band 3 protein, can operate as a flippase [52, 60, 64] in addition to its normal function as an obligatory passive anion exchanger [33, 54] and a conductive ''channel'' [22, 33, 54] for a variety of anions. This flippase mode of operation of band 3 has so far been studied with fluorescent long-chain anions of the alkylsulfonate type [52] and phospholipid probes

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bearing one or two net-negative charges on their polar head group [60, 64]. All these anions proved to be translocated not only via band 3, but also via additional parallel pathways, such as the lipid bilayer and/or the ATP-dependent, vanadate-sensitive ''floppase'' [7, 60] of the erythrocyte membrane.

In the following, data will be presented on the flip of a long-chain amphiphilic anion of the alkyl phosphate type, 10-(α-naphthyl)-1-decyl-phosphate (NDP), translocated exclusively via band 3 and therefore suitable for studying the flippase mode of operation of band 3 without having to consider parallel pathways. Unexpectedly, this anion turned out to be cleaved endofacially by a cytoplasmic phosphatase. Attempts to overcome this untoward complication will also be reported.

Materials and Methods

Materials

Human erythrocyte concentrates were obtained from the local blood bank and used within 10 days. Erythrocytes were isolated by centrifugation and washed three times with isotonic saline. Incubation media contained (mM): KCl (90), NaCl (45), sucrose (44) and either NaH_2PO_4/Na_2HPO_4 (12.5; pH 7.4) (=KNPS) or HEPES (10.0; pH 7.0 or 8.0) (=KNHS).

Resealed ghosts were prepared according to ref. [41]. The fluorescent probe 10-(α-naphthyl)-1-decyl-phosphate (NDP) was prepared from 1,10-decandiol, α-naphthyllithium bromide and tetrabenzylpyrophosphate by a seven step synthesis in an overall yield of 22% [40]. Identity and purity of the product were confirmed by NMR, mass and IR spectroscopy.

Bovine serum albumin (fraction V, fatty-acid-free) was obtained from Päsel-Lorei (Hanau), 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) from Pfaltz and Bauer (Waterbury, CT), 4,4′diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) from Calbiochem (Bad Soden), eosin-5-maleimide from Molecular Probes (Eugene, OR), Woodward's reagent K (WRK) from Fluka AG (Neu Ulm), 2,4,6-trinitrobenzenesulfonate from ICN (Meckenheim), phloretin from Carl Roth (Karlsruhe), pyridoxal-5-phosphate (PLP) from Serva (Heidelberg) and dephostatin from Alexis (Grünberg). Dipyridamole, merocyanine 540, cantharidine, p-nitrophenylphosphate (pNPP), phosphotyrosine, phosphoserine and niflumic acid were purchased from Sigma (Deisenhofen), papain from Boehringer (Mannheim).

Modification of Erythrocytes

Erythrocytes were pretreated with DIDS or eosin-5-maleimide (50 μM, 30 min, 37°C, KNPS, pH 7.4, 10% hct.), TNBS (5 mM, 30 min, 37°C, KNPS, pH 8.0, 10% hct.), or WRK 2 mM followed by $NaBH_4$, 4 mM (both 10 min, 0°C, KNHS, pH 7.0, 10% hct.) and washed two times with KNPS or in the case of eosin-5-maleimide two times with KNPS containing 0.2 g dl^{-1} albumin, followed by two washes with KNPS.

Exofacial proteolytic cleavage of band 3 was carried out by treatment of erythrocytes in KNPS (50% hct.) with cysteine-activated papain (30 U/ml cells, 60 min, 37°C, pH 7.4) followed by two washes with 0.2 g dl^{-1} albumin in KNPS and two washes with KNPS.

Cells were pretreated (5 min) with PLP in KNPS (10 mM, 30% hct., pH 7.4, 37°C). After 5 min the reaction was terminated by addition of lysine (250 mM), cells were washed two times with KNHS (pH 8.0, 0°C) and treated with $NaBH_4$ (60 min, 0°C) followed by washing three times with KNPS.

Measurement of Inward Translocation (flip) of NDP

Packed erythrocytes (1 ml) were suspended in 2.5 ml KNPS (0°C) containing the fluorescent probe NDP (50 μl of a 4.4 mM ethanolic stock solution, final probe concentration 60 μM, equivalent to 200 nmoles/ml cells or a mole fraction of about 2% related to the lipid content of the erythrocyte membrane [13]), incubated for 10 min at 0°C to insert NDP into the outer membrane leaflet and washed two times with medium at 0°C. Transbilayer reorientation, which is neglible at 0°C, was initiated by resuspending the washed, probe-containing cells in the appropriate medium, usually KNPS (hct. 10%), at higher temperatures. To measure the time-dependent flip, 300 μl samples of the suspension were diluted after various time periods with 900 μl KNPS (0°C). After centrifugation (6,600 × *g*, 20 sec) cells were extracted twice with bovine serum albumin (1 g dl^{-1} in KNPS, 0°C, incubation 3 min) at 0°C to remove the probe from the outer leaflet and washed with medium. Subsequently, the cells were hemolyzed with 150 μl water. 150 μl of the hemolysate were transfered into a glass tube (to avoid contamination by plasticizers). The fluorescent probe was extracted from the membranes in the lysate with 800 μl isopropanol and 200 μl $CHCl_3$. NDP was quantified by fluorescence spectroscopy (excitation at 281 nm, emission 336 nm) using a Shimadzu spectrofluorophotometer (RF5001 PC). The residual fluorescence after albumin extraction (NDP in inner membrane leaflet, X_i) was related to the total NDP fluorescence, X_{tot}, in the same amount of hemolyzed cells not treated with albumin. An exponential function, $q \cdot [1 - \exp(-k \cdot t/q)]$, was fitted to the values for X_i/X_{tot}, after various times *t*, where *q* represents the fraction of probe in the inner leaflet under stationary conditions and *k* is the rate constant for the unidirectional flip.

Measurement of Hydrolytic Cleavage of NDP by RP-HPLC

The time-dependent hydrolytic cleavage of NDP to the corresponding alcohol was followed in intact erythrocytes and hemolysates prepared by dilution of packed erythrocytes with distilled water. Following the exposure to NDP, cells or lysates were extracted with isopropanol/$CHCl_3$ as described above and the extracts subjected to RP-HPLC [40], (C_{18} reversed-phase column KS 100/4 Nucleosil 100-5 C_{18} (Macherey & Nagel), mobile phase: methanol/acetate buffer (0.06 M) 5:1, flow rate 1 ml/min). Effluents were analyzed online by quantitative spectrofluorometry (sample size 30–50 μl).

Abbreviations

DENSA	5-(N-decyl)aminonaphthaline-2-sulfonic acid
DIDS	4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid
DNDS	4,4′-Dinitrostilbene-2,2′-disulfonic acid
LPM	Lysophosphatidylmethanol
NBD	N-(7-Nitrobenz-2-oxa-1,3-diazole-4-yl)
NBD-P	1 oleolyl-2[N-NBD]amino hexanoyl-sn-glycero-phospho
NDP	10-(α-naphthyl)-1-decyl-phosphate
NEM	N-ethylmaleimide
PTP	Protein tyrosin phosphatase
WRK	Woodward-Reagent K (N-ethyl-5-phenylisoxazolium-3-sulfonate)
PA	Phosphatidic acid
PM	Phosphatidylmethanol
TON	Turnover number

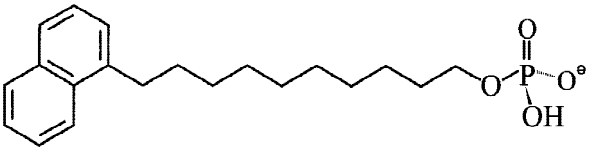

Fig. 1. Structure of the amphiphilic anionic probe NDP (10-(α-naphthyl)-1-decyl-phosphate).

Results

Flip and Intracellular Cleavage of NDP

Brief incubation of erythrocytes with the fluorescent long-chain amphiphilic anion NDP (Fig. 1) at 0°C results in a nearly complete insertion of the probe into the membrane. The membrane/water partition coefficient of the amphiphilic anion, derived from its redistribution from the membrane into the aqueous phase as described in ref. [52], is about $1.5 \cdot 10^5$ (pH 7.4). Due to this high membrane affinity, almost all of the inserted NDP will remain confined to the membrane upon resuspension of the ''loaded'' cells in fresh medium. NDP redistributed from the membrane into the aqueous phase (about 130 nM under our standard conditions) will be in true solution, since its critical micellar concentration is at least 100 μM, as can be extrapolated from data for simple alkylphosphates of comparable chain length [5].

Following the insertion of NDP into the outer membrane leaflet at 0°C, its time-dependent translocation to the inner leaflet was followed (21°C, pH 7.4) by quantifying the increase of the fraction of NDP not extractable by albumin (Fig. 2). By fitting the data to an exponential function (*see* Materials and Methods) a first-order rate coefficient (k) for the flip process of $(8.0 \pm 2.4) \cdot 10^{-3}$ min^{-1} ($n = 31$), equivalent to a half-time of 17 min, was obtained. The fraction of the probe present in the inner leaflet leveled off at about 20%.

Pretreatment of the cells with DIDS, a covalent inhibitor of band-3-mediated transport of hydrophilic anions [3], produces an almost complete suppression (≥95%) of the flip of NDP (Fig. 2), with a half-maximal inhibitory concentration (I_{50}) of about 1.5–2 μM (hct. 10%). This inhibition makes it likely that the transbilayer reorientation of NDP is mediated almost exclusively by the anion exchanger, band 3. NDP thus differs from the anionic amphiphiles DENSA [52] and lysophosphatidylmethanol (LPM) [64] as well as from a number of NBD-labeled anionic phospholipids [60] which were shown to move across the erythrocyte membrane via two pathways, one involving band 3 protein, and another one having the characteristics of a nonmediated process presumably involving the lipid domain. The concept of a translocation of NDP via band 3 is also supported by the observation (*data not shown*) that the

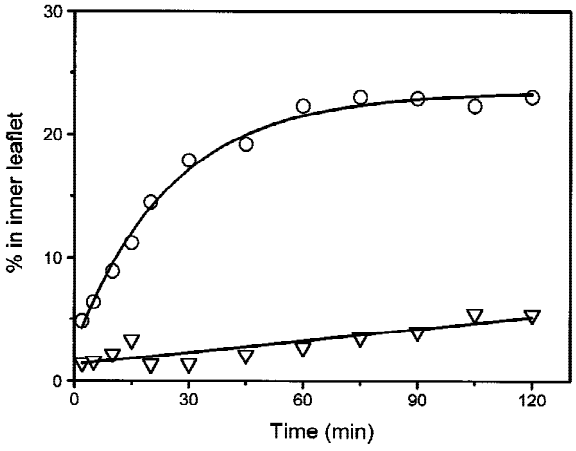

Fig. 2. Time-dependent translocation (flip) of NDP from the outer to the inner membrane leaflet of human erythrocytes at 21°C (○) and inhibition of the flip by DIDS (▽). Erythrocytes were pretreated with DIDS (50 μM, hct. 10%, 30°C, pH 7.4, 30 min). NDP (200 nmol/ml cells) was inserted into the outer membrane leaflet (0°C) and its translocation to the inner leaflet (21°C, pH 7.4, hct. 10%) quantified by fluorescence spectroscopy as described in Materials and Methods.

flip of NDP is enhanced by a factor of up to 10 when measured in chloride-free cell suspensions containing no hydrophilic anions known to be transported to a significant extent by band 3 except intracellular inorganic phosphate and the unavoidable OH^- (for details *see* Materials and Methods).

To characterize the flip of NDP in some detail, we studied its temperature dependence. These experiments revealed an unexpected peculiarity. At higher temperatures (≥30°C), the fraction of inserted fluorescent material not extractable by albumin first increased rapidly with time, as expected, but then decreased again (Fig. 3). Simultaneously, the total amount of NDP in the membrane, as quantified by RP-HPLC, decreased (Fig. 4) in parallel with the appearance of a new compound, which was identified as the corresponding alcohol, naphthyl decanol, arising from NDP by hydrolytic cleavage. In contrast to NDP, the alcohol is extractable by albumin from both membrane leaflets due to a very high transbilayer mobility. Dephosphorylation of NDP is thus responsible for the decrease of the non-extractable fluorescence during the progress of the flip process.

Characteristics of the Intracellular Dephosphorylation of NDP

When flip measurements of NDP were carried out in resealed ghosts containing only small residues of original cytoplasm [41], the decrease of NDP after its initial increase in the inner leaflet was much less pronounced than in native cells (Fig. 5). Moreover, a high level of NDP in the inner leaflet (50–60%) was reached in ghosts and

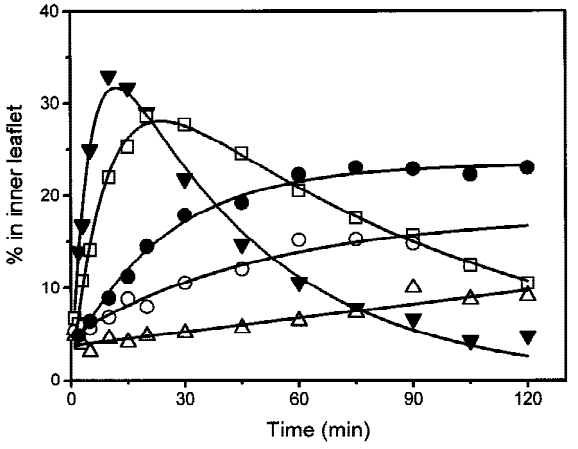

Fig. 3. Time-dependent translocation of NDP in human erythrocytes at different temperatures. NDP was inserted into the outer membrane leaflet (0°C) and the time-dependent change of the non-extractable fraction of NDP (pH 7.4, hct. 10%) followed at 37°C (▼), 30°C (□), 21°C (●), 15°C (○) and 5°C (△).

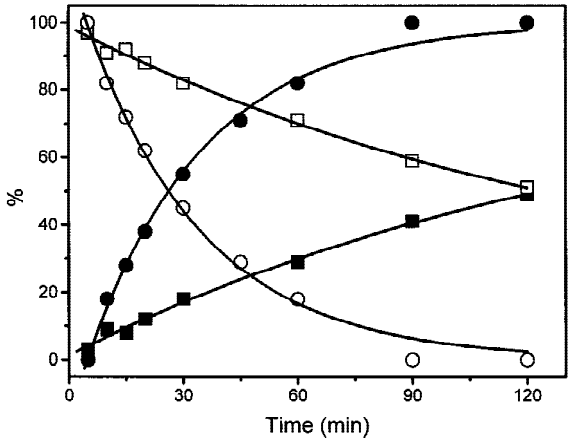

Fig. 4. Dephosphorylation of NDP under formation of the corresponding long-chain alcohol in erythrocytes at different temperatures. The time-dependent disappearance of NDP at 37°C (○) and 21°C (□) as well as the formation of the alcohol at 37°C (●) and 21°C (■) were followed by RP-HPLC using fluorometric detection.

essentially maintained. The dephosphorylation of NDP proceeded much slower in ghosts than in intact cells.

This finding suggested the catalytic function, in the cleavage of NDP, of a cytoplasmic component, such as a phosphatase. The presence, in erythrocytes, of cytosolic enzymes of this class, including serine/threonine and tyrosine phosphatases as well as ''acid phosphatases,'' is well established [1, 2, 6, 17, 55, 63, 66]. Indeed, membrane-free hemolysates cleaved NDP at substantial rates while isolated white ghosts exhibited no detectable activity. In hemolysates prepared by adding 2 vols of water to 1 vol packed erythrocytes the initial rate of cleavage, at an NDP concentration of 100 μM, was 4.3 μmol/l

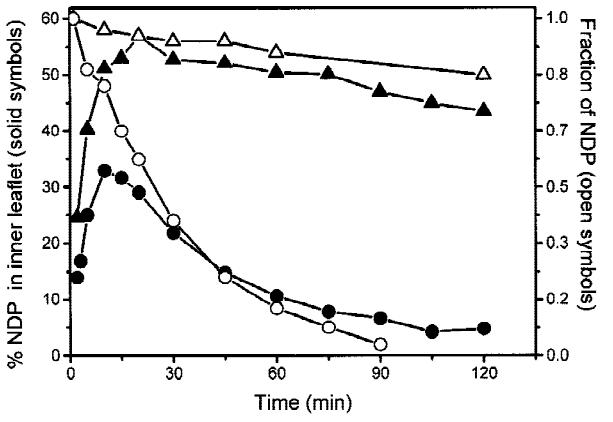

Fig. 5. Time-dependent flip (closed symbols) and dephosphorylation (open symbols) of NDP in resealed ghosts (△, ▲) and native erythrocytes (●, ○) at 37°C. Experimental details as described for intact cells.

lysate · min at 21°C. Removal of the ghost membranes did not alter the rate of cleavage. On the other hand, progressive dilution of these hemolysates did not go along with the expected proportional decrease of enzymatic activity: Upon increasing the dilution from 1:3 to 1;100, the rate of cleavage only decreased by a factor of 5. This indicates the presence of inhibitory effects of cytoplasmic constituents of the erythrocytes, which would be diminished by dilution.

While neither Mg^{++} nor ATP had any influence on the rate of cleavage, inorganic phosphate at the concentrations present intracellularly in our flip experiments (about 5–7 mM) had a significant (about 60%) inhibitory effect (*data not shown*). Conversely, inorganic phosphate depletion in whole cells and in 1:3 lysates by incubation with inosine enhanced the cleavage of NDP (*data not shown*).

Hydrolysis in 1:100 diluted hemolysates was also suppressed by a number of established [1, 55, 69] inhibitors of phosphatases. Substantial inhibition (⩾90% at concentrations ⩽1 mM) was obtained in 1:100 diluted hemolysates by Mn^{++}, Zn^{++}, arsenate, orthovanadate, phenylarsinoxide and NEM. For reasons yet unclear, all these compounds were somewhat less effective in more concentrated (1:3) lysates. Compounds acting as weaker inhibitors comprise dephostatin® [23] (35% at 100 μM), p-nitrophenylphosphate (66% at 1 mM), phosphotyrosine (50% at 1 mM), and phosphoserine (30% at 1 mM). KCl at 150 mM suppressed phosphatase activity by about 50%. In contrast, ATP (1 mM), Mg^{++} (5 mM), EDTA (1 mM), fluoride (5 mM), and cantharidin (100 μM) as well as tartrate (20 mM) proved to be ineffective.

NDPase-activity had a maximum at pH 6.5 which is compatible with the involvement of an ''acid phosphatase'' [2, 6, 63]. From the concentration dependence of the rate of cleavage in 1:100 hemolysates kinetic con-

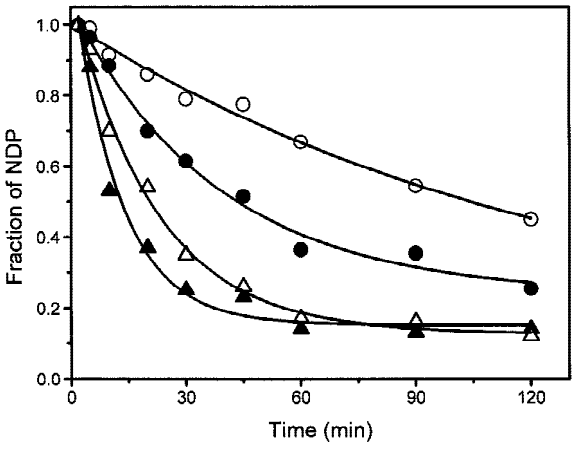

Fig. 6. Time-dependent hydrolytic cleavage of NDP in erythrocytes without interference of the transbilayer translocation process at 21°C (circles) and 37°C (triangles), in the absence (●, ▲) and presence (○, △) of vanadate (1 mM). The hydrolytic cleavage of NDP, translocated into the inner leaflet of the bilayer during an initial flip period and subsequent removal of NDP from the outer leaflet by albumin, was followed in the presence of DIDS (50 μM) by measuring the time-dependent decrease of albumin-inextractable fluorescence.

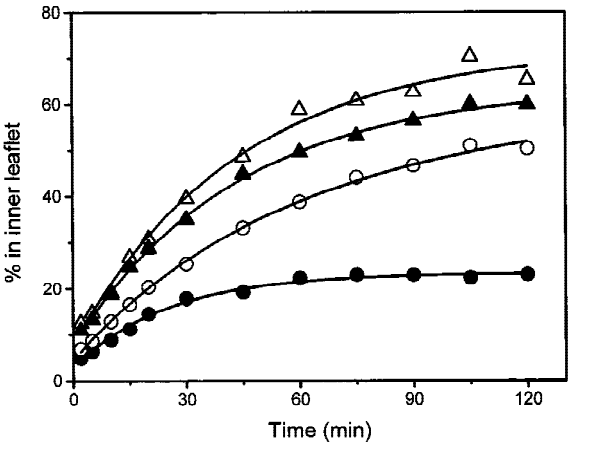

Fig. 7. Influence of vanadate (1 mM, open symbols) on the time course of the flip at 21°C and the stationary levels of transbilayer distribution of NDP, in intact cells (○, ●) and resealed ghosts (△, ▲). Controls: closed symbols.

stants were also derived by extrapolation (K_m = 225 μM, V_{max} = 3.9 μmol/l lysate · min^{-1}, pH 7.4, 22°C).

Considering this pattern of characteristics and inhibitors, in particular the inhibition by orthovanadate, Zn^{++}, NEM and phenylarsinoxide [55] and the lack of inhibition by fluoride, tartrate [17, 55] and cantharidin, an inhibitor of serine/threonine phosphatases [32], one may postulate that the NDPase activity is due to a cytoplasmic phosphotyrosine phosphatase (PTPase), although the agreement of our data with the available, somewhat contradictory data for erythrocyte PTPases [2, 6] is not complete. It has to be kept in mind, however, that the cleavage of long-chain alkyl esters by PTPases, which are claimed to be highly selective for aryl phosphates [68], has not been investigated previously.

To obtain information about the phosphatase-mediated cleavage of NDP in the intact cell, but without interference of the transbilayer translocation process, the time-dependent decrease of intramembrane NDP was measured following its primary accumulation in the inner membrane leaflet and removal of NDP from the outer leaflet in the presence of DIDS (*see* Materials and Methods) to prevent the band-3-mediated outward reorientation of NDP. The long-chain alcohol produced in the inner leaflet by phosphatase action could be extracted from both membrane leaflets by albumin due to its high transbilayer mobility. From the time-dependent exponential decrease of intramembrane NDP (Fig. 6), first order rate constants for the cleavage of the ester at 21°C (k = 0.012 min^{-1}, $t_{1/2}$ = 58 min), 30°C (k = 0,050 min^1, $t_{1/2}$ = 14 min) and 37°C (k = 0.070 min^{-1}, $t_{1/2}$ = 10 min) were derived. This temperature effect corresponds to an activation energy of about 80 kJ/mole. Vanadate inhibited the cleavage process by about 66%, which is somewhat lower than the inhibition found in hemolysates.

In view of its strong inhibitory effect, Mn^{++}, which can be introduced into native cells by the ionophore A23186, should allow the measurement of the flip of NDP almost without interference of its phosphatase-mediated cleavage. However, Mn^{2+} turned out to be a strong inhibitor of band 3-mediated anion transport processes, including the flip of NDP (*data not shown*). Therefore, vanadate, which has no such transport-inhibiting effects, had to be used to suppress the hydrolysis of NDP, although it does not completely inhibit NDPase activity in whole cells. Alternatively, rate coefficients were derived from initial flip rates, which are rather insensitive to subsequent processes.

Phosphatase inhibition by vanadate increased the slope of the flip kinetics in erythrocytes (Fig. 7). Since, however, the stationary level of NDP at saturation (q as defined in Materials and Methods) is higher in the presence of vanadate (0.56 ± 0.18 *vs.* 0.20 ± 0.03 in the controls) due to the slower cleavage of NDP, the rate *coefficient* (k/q) for the inward translocation of NDP derived from these kinetics, (8.6 ± 2.9) · 10^{-3} min^{-1} (at 21°C, n = 51), proved to be identical to that obtained in the absence of vanadate. First-order rate coefficients for the flip in resealed ghosts in the absence of vanadate [(k = 13 ± 3.4) · 10^{-3} min^{-1} (n = 12)] were somewhat higher than those in erythrocytes, but became only slightly enhanced in the presence of vanadate [(k = 15 ± 5.0) · 10^{-3} min^{-1} (n = 7)]. As expected, the nonextractable fraction of NDP at saturation was increased in re-

Table 1. Inhibition of the flip of NDP, at 22°C, by compounds known as inhibitors of the band 3-mediated anion exchange. Number of experiments in parentheses

Inhibitor	Concentration (mM)	% Inhibition
DIDS*	0.01	95 ± 3 (7)
Eosinmaleimide*	0.05	91 ± 6 (5)
2,4,6,-Trinitrobenzenesulfonate*	5	77 ± 11 (4)
Pyridoxal-5′-phosphate/$NaBH_4$*	20	84 (2)
DNDS	2	94 ± 2 (3)
Tetrathionate	20	85 (2)
Dipyridamole	0.02	85 ± 3 (3)
Phloretin	0.25	83 (2)
Niflumate	0.02	64 ± 11 (7)
Salicylate	20	45 ± 24 (4)
Erythrosin	0.025	68 ± 9 (3)
Merocyanine 540	0.02	34 ± 15 (6)
Pyridoxal-5′-phosphate	20	34 ± 11 (3)

* Cells pretreated with inhibitor, for details *see* Materials and Methods.

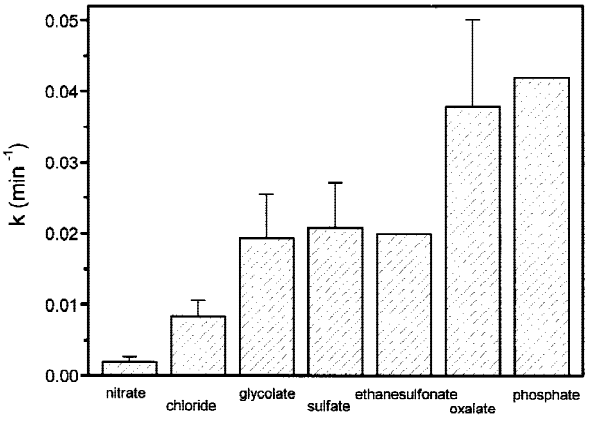

Fig. 8. Influence of the anion milieu on the flip rate of NDP. Intracellular Cl^- was replaced by other anions by 2 sequential 60 min incubations of the cells (37°C, hct. 10%) in media containing only these foreign anions. Subsequently, flip rates were measured in media containing the foreign anion (21°C). Mean values ± SD from 3–19 experiments.

sealed ghosts relative to native cells due to the absence of NDPase activity in the resealed ghosts.

Characterization of the Flip of NDP

It is presently not clear whether the flip mode of operation of band 3 is based on the obligatory exchange mechanism operative in case of hydrophilic anions [33, 54]. In view of the extremely low rates of the flip via band 3 (*see* Discussion) this can not be tested directly, e.g., by trans-acceleration experiments. Some indirect information on this point may be obtained, however, by investigating whether certain modifications which affect the exchanger function of band 3 have the same influence on the flip mode. Such studies are reported in the following.

(i) Inhibition of the flip of NDP was observed not only for DIDS, but also for a number of other compounds having in common the potency to inhibit band 3-mediated anion exchange (Table 1). These inhibitors were used at concentrations known to almost completely abolish the exchange of hydrophilic anions [3, 12, 13, 46]. Interestingly, some of these compounds, niflumate [8], erythrosin [43], salicylate [9, 50, 59], pyridoxal phosphate [4, 51] and merocyanine 540, proved to be only weak inhibitors of the flip of NDP. The observed weak effects were maximal ones in the sense that higher concentrations did not markedly increase flip inhibition. Nevertheless, band 3 is most likely the target of the inhibition. This assumption is also supported by the further observation that dipyridamole, a strong inhibitor of the transport of hydrophilic anions [47, 56] and of the flip of NDP, requires the presence of halides in both cases (*data not shown*).

(ii) Rates of self-exchange of anions via band 3 are dependent on the major anion species in the suspension. Replacement of Cl^-, in cells and medium, by nitrate or thiocyanate inhibits, while replacement by small monovalent organic and most divalent anions enhances the self-exchange of small hydrophilic anions such as chloride [10], phosphate [12], sulfate [58, 65] or oxalate. Kinetic analyses suggest that these effects largely result from competition between anions for the ''transport site'' [58], although noncompetitive (allosteric) components involving ''modifier sites'' on the transporter cannot be excluded [10]. The flip rate of NDP (measured after pretreatment of the cells with vanadate in order to suppress phosphatase activity) responded to changes of the anion milieu (Fig. 8) in a way comparable to hydrophilic anion exchange. These results are in line with preliminary data reported for the band 3-mediated flip of the amphiphilic sulfonate DENSA [52] and the anionic phospholipid NBD-PA (*data not shown*), while flip processes via the lipid domain are not sensitive to the anion milieu [60]. These findings suggest that the anionic headgroup of the amphiphiles interacts—during the flip process—with the sites involved in the obligatory exchange of hydrophilic anions.

(iii) The obligatory anion exchange is usually modeled by a ''ping-pong'' mechanism [54], in which the transport site is alternately accessible from either of the two sides of the membrane. This allows for a ''recruitment'' of all transport sites to the outer membrane surface when cells containing a transportable anion, e.g., chloride, are suspended in a medium containing only an essentially nontransportable anion, e.g., glutamate [37]. Under these conditions, the transport rate of a suitable test anion added to the extracellular glutamate medium will be markedly stimulated, compared to ''normal''

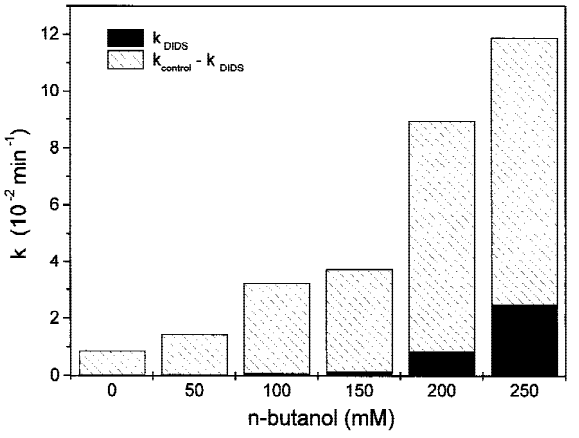

Fig. 9. Influence of butanol on the flip rate k of NDP, measured at 21°C in the absence or presence of DIDS (50 μM). Mean values from 2 experiments.

Table 2. Effectivity of flip inhibitors following stimulation of inward translocation of NDP by papain (5-fold) or 150-mM n-butanol (4-fold)

Inhibitor (mM)	Untreated**	Papain	n-Butanol
		% Inhibition	
DID (0.05)*	95	75 ± 11 (3)	93 (2)
Eosin maleimide (0.1)*	91	<10 (2)	92 (2)
Erythrosin (0.025)	68	<10 (2)	55 (2)
Dipyridamole (0.025)	85	36 (2)	76 (2)
DNDS (0.5)	94	32 (2)	90 (3)
Pyridoxal phosphate/$NaBH_4$ (10)*	84	10 (2)	56 (2)
Tetrathionate (20)	85	<10 (2)	69 (2)
Niflumate (0.025)	64	<10 (2)	41 (2)

* Cells pretreated with inhibitor, for details see Materials and Methods.
** From Table 1.
Number of experiments in parentheses.

conditions with Cl^- on both sides of the membrane, since the number of transfer sites exofacially available to this test anion is increased.

In experiments of this type, using NDP as a probe, flip rates increased from $(8.4 \pm 2.2) \cdot 10^{-3}$ min^{-1} ($n = 12$) in chloride medium to $(52 \pm 20) \cdot 10^{-3}$ min^{-1} ($n = 3$) in glutamate medium. While this increase may result from a ''recruitment'' and could be taken as evidence that the inward flip of NDP in fact involves exchange against cellular chloride, it has to be considered that the outward-directed transmembrane chloride gradient will also induce an inside-positive membrane potential due to the anion-selective membrane conductance of the erythrocyte membrane [54]. This membrane potential might be the cause of an acceleration of the inward flip of the anion NDP, if the flip were not based on an electrically silent exchange process but on the conductance via band 3, well established for hydrophilic anions [22]. In the present case, however, this alternative explanation is unlikely to apply since the enhancement in glutamate medium did not disappear when the membrane potential was collapsed by a K^+ conductance induced by valinomycin (*data not shown*).

(iv) Aliphatic alcohols inhibit the band 3-mediated exchange of hydrophilic anions [21]. In contrast, the flip of NDP via band 3 is enhanced by such alcohols, as shown for butanol in Fig. 9. The elevated flip is completely inhibited by DIDS and other inhibitors up to a butanol concentration of 150 mM (Table 2) and is thus clearly band 3-mediated. At higher butanol levels an additional DIDS-insensitive component appears, most likely representing flip via the lipid domain, which can obviously become permeable to NDP after suitable modification. In view of similar results for the flip via band 3 of other amphiphilic anions [52, 64], the peculiar stimulating effect of butanol can be regarded as a general paradoxical effect of alcohols on the flip mode of operation of band 3.

(v) Proteolytic digestion of erythrocytes by papain cleaves band 3 protein exofacially at three positions and produces two large and two small fragments [34, 36]. This treatment inhibits the self-exchange of hydrophilic anions [46]. In contrast, papain treatment as described in Materials and Methods accelerates the flip of NDP by a factor of 4.7 ± 1.1 ($n = 10$). A similar stimulation has previously been demonstrated for the flip of other types of anionic amphiphiles via band 3 [52, 64]. Pretreatment of cells with DIDS prior to papain prevents this flip acceleration (*data not shown*), indicating that the effect of papain is related to the modification of band 3, since DIDS also suppresses the papain-induced cleavage of band 3 [49]. Following modification of cells by papain the effectiveness of inhibitors of anion exchange on the flip of NDP proved to be diminished or even abolished (Table 2), as in the case of papain-induced (partial) inhibition of anion exchange [36].

(vi) Sequential treatment of erythrocytes with the carboxyl reagent WRK followed by $NaBH_4$, at pH 7, has been shown to convert the glutamate residue at position 681 of human band 3 protein to the corresponding alcohol and thereby to accelerate the self-exchange of the divalent anion sulfate while inhibiting that of the monovalent anion chloride [35]. In line with these findings, the band 3-mediated flip of two monoanionic long-chain amphiphiles, LPM and DENSA, is also inhibited by WRK/BH_4^- [52, 64]. In contrast, the flip of NDP proved to be insensitive to WRK/BH_4^- when measured at pH 7.4. Some inhibition became evident, however, at pH 6, while at pH 8.6 the flip was markedly enhanced (Table 3). This pattern may be a consequence of the changes of ionization, with pH, of NDP, which is mono-anionic at acid pH and thus prone to inhibition of its flip by WRK/

Table 3. Effect of the medium pH on the rate constant for the inward translocation of NDP

pH	WRK/BH_4^-	k/(10^{-3} min^{-1})	$k_{WRK}/k_{control}$	*n*
6	−	6.1	1	2
	+	4.2	0.69	2
7.4	−	8.6 ± 2.9	1	3
	+	8.0 ± 2.9	0.93	3
8.6	−	7.7 ± 1.2	1	3
	+	16 ± 4.7	2.08	3

21°C in control erythrocytes and in cells pretreated with WRK (2 mM at pH 7, 10 min, 0°C) followed by 4 mM $NaBH_4$ (10 min, 0°C).

BH_4^-, but di-anionic at alkaline pH and thus prone to flip stimulation following WRK/BH_4^- treatment. At neutral pH, where NDP will be present in both forms of ionization to a rather similar extent, the opposite effects of WRK/BH_4^- probably cancel each other.

The effectiveness of the weak inhibitors of the flip of NDP (*cf.* Table 2) was somewhat increased after WRK/$NaBH_4$ treatment, while that of dipyridamole, but not other strong inhibitors, was markedly decreased (*data not shown*). This feature has previously been reported for the flip of anionic NBD phospholipids via band 3 [60].

While phosphatase inhibitors were usually present in the above experiments aiming at the characterization of the flip of NDP, some basic characteristics of the flip could be derived from initial flip rates measured in the absence of such inhibitors. According to our results (*data not shown*), the amount of NDP translocated per minute increases linearly with the amount of probe initially inserted into the outer membrane leaflet in the range between 90 and 330 nmoles/ml cells, equivalent to molar ratios of NDP inserted per copy of band 3 between 4 and 16. Moreover, from an Arrhenius plot of the rate constants, derived from a linear fit of the initial part of the flip kinetics in intact cells and resealed ghosts at temperatures between 5° and 30°C, an activation energy of 116 kJ/mol was derived. This is comparable to the activation energies reported for the translocation of other amphiphilic [52, 64] and some hydrophilic anions [24, 29, 54] via band 3.

NDP as an Inhibitor of Anion Exchange

A flip of NDP via band 3 implies that the probe interacts with the transport protein. Since NDP is an amphiphilic anion, one would expect that it also inhibits the exchange of hydrophilic anions like many other alkyl- and aryl-derived anions [13]. This proved indeed to be true. Self-exchange of malonate—used as a probe for band 3 function (at 25°C)—is suppressed by NDP. 50% inhibition (at a hct. of 5%) were obtained at 25 μM (Fig. 10), equivalent to 500 nmoles/ml cells. A Hill plot of the dose-response curve indicates a stoichiometric 1:1 binding of NDP at concentrations below 30 μM. At higher levels, inhibition increases overproportionally and with a higher stoichiometry, as evident from the Dixon and the Hill plot. Our flip experiments were usually carried out at NDP levels of 200 nmoles/ml cells, equivalent to a concentration of 10 μM added to the aqueous phase at 5% hct. In preliminary experiments (*data not shown*) we could also demonstrate that the extent of NDP-induced inhibition of anion self-exchange (tested with ^{14}C-malonate (2 mM)) increases or decreases upon replacement of Cl^- as the major anion in the cell suspension by other anions in a way comparable to the changes of the flip rate of NDP *per se* (Fig. 8) when measured in these media. This parallelism in anion sensitivity may be taken as evidence that the flip of NDP and anion self-exchange are at least related processes.

Discussion

This study extends the analysis of the flip mode of operation of the anion exchanger of the erythrocyte membrane to a long-chain monoalkylphosphate. As could be shown, this type of an amphiphilic anion has the advantage over related alkylsulfonates and diacylglycerophosphates that it does not permeate to any significant extent via the lipid domain of the membrane. On the other hand, NDP has the disadvantage that it is cleaved enzymatically once having reached the inner leaflet of the membrane. In the following, aspects resulting from these two properties shall be discussed in addition to new insights into the flip via band 3 arising from this study.

Enzymatic Cleavage of NDP

The enzyme activity catalyzing the hydrolysis of NDP resides in the cytosol as indicated by its essential absence in ghost membranes. Phosphatase activities of the erythrocyte cytosol have previously been characterized using various substrates and inhibitors as well as pH-dependences [1, 2, 6, 17, 63, 66]. NDP is cleaved by a phosphatase activity with some characteristics of a protein tyrosine phosphatase (PTPase) (*see above*). This activity may be ascribed to one of the low-molecular-weight acid phosphatases structurally characterized by genetic and protein biochemical techniques only recently [17, 66] but known to be present and functionally investigated since decades [1, 63]. PTPases are usually taken to be much more specific for aromatic phosphate esters like p-nitrophenylphosphate, β-naphthylphosphate or phosphotyrosine than for alkyl and related phosphate esters [66, 68]. The alkyl esters tested so far, however, mainly had rather short polar alkyl residues like a glyc-

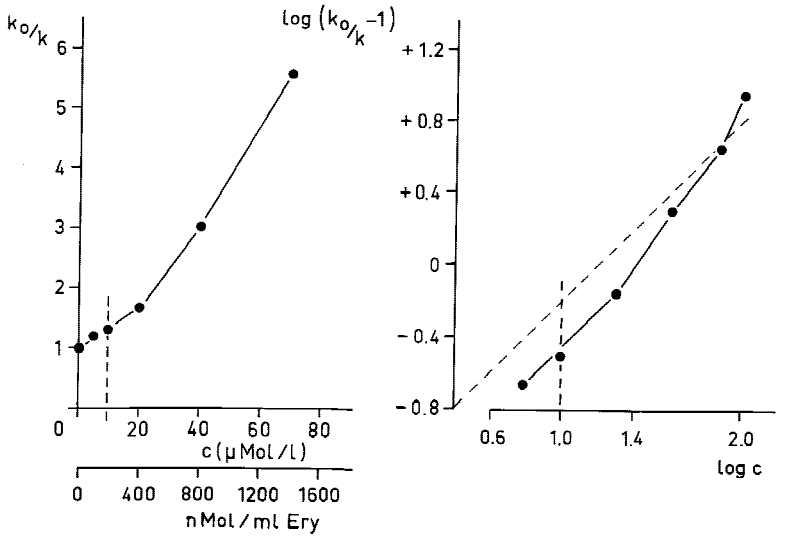

Fig. 10. Inhibition of the self-exchange of malonate (2 mM in KNPS) by NDP. Rates of self-exchange were measured, using ^{14}C-malonate, at 25°C and 5% hct. by conventional techniques. Rate coefficients in the presence of inhibitors, normalized to the control rates, were plotted according to Dixon (*A*) and Hill (*B*). The thick hatched vertical lines indicate the concentration usually applied in flip experiments. The thin hatched line in 10 *B* corresponds to a Hill coefficient of 1. Mean values from 3 experiments.

eryl group in α-glycerophosphate [68]. In NDP, the long apolar hydrocarbon chain next to the phosphate headgroup may well facilitate, like an aromatic group, the approach of the phosphate group to the active site of PTPase, known to be located in a deep cleft of the enzyme protein [11, 55]. The inhibitory effect of aromatic phosphate esters on the cleavage of NDP (*see* Results) also points to the involvement of a PTPase.

Due to its high membrane affinity and its molecular geometry, NDP is intercalated to a major extent between the phospholipids, perpendicular to the bilayer surface. For energetic reasons, its phosphate head group is probably located in the region of the acyl ester bonds of the phospholipids and not in the region of their phosphate groups. Cleavage of NDP by the cytosolic phosphatase may therefore have to occur in an environment less polar than water, somewhat distant from the bilayer interface and below the meshwork of the membrane skeleton. As an alternative, one might ask whether the very small fraction of the anionic probe present in the cytosolic phase, but in continuous equilibrium with the large membrane pool, could serve as substrate for the enzyme. This interpretation seems questionable, however, in view of our finding (*data not shown*) that two commercial phosphatases (acid phosphatase from potatoe [Type IV S Sigma P1146], and alkaline phosphatase from bovine intestine [Fluka 79385]), which both readily dephosphorylate NDP in saline solution, were ineffective when NDP was presented intercalated between the membrane phospholipids of white ghosts (*data not shown*).

The cytoplasmic NDPase thus seems to have specific structural properties not present in other phosphatases, which allow its access to a bilayer-intercalated alkyl phosphate. This specificity might be related, as probably in other PTPases [69], to a specific binding to the membrane, mediated by membrane skeletal proteins. If this is the case, NDP added to hemolysate should be protected from cytoplasmic NDPase in the presence of lipid membranes devoid of skeletal proteins. In agreement with this assumption, we could show in preliminary studies that addition of lecithin vesicles (1 mg/ml) to membrane-free hemolysates goes along with a strong suppression of the enzymatic cleavage of NDP, indicating that the intercalation of the amphiphile into the pure lipid layer renders it inaccessible to the phosphatase. In contrast, addition of unresealed white erythrocyte ghosts (up to 1 mg lipid/ml, corresponding to a hct. of 20%) to the same lysates did not have such a protective effect. In further agreement with our interpretation, addition, to a lysate, of DIDS-treated erythrocytes, in which NDP and enzyme have only access to the cytoskeleton-free outer leaflet of the membrane, markedly reduced the enzymatic cleavage of NDP, almost like the pure lecithin vesicles (*data not shown*). All these observations are reconcilable with the concept that the cytoplasmic NDPase requires membrane skeletal elements for its action on membrane-intercalated alkyl phosphate esters.

Flip of NDP Via Band 3

Figure 2 and Table 1 provide the evidence that NDP moves from the outer to the inner leaflet of the membrane almost exclusively via an inhibitor-sensitive pathway, most likely band 3. The subsequent discussion is based on this concept, already presented elsewhere [52, 64]. From flip rate constants obtained in resealed ghosts under conditions largely undisturbed by NDPase activity (0.081 min^{-1} at 37°C) and the amounts of probe initially introduced into the outer membrane layer (200 nmoles/ml cells under standard conditions, equivalent to $12 \cdot 10^6$ probe molecules per cell) one can calculate an ''influx'' of NDP from the outer to the inner leaflet of 16 nmoles/ml cells/min at 37°C. At 21°C, the rate is about 10-fold

Table 4. Comparison of rates of flip via band 3 (k_{B3}), of amphiphilic long-chain phosphates and sulfonates. Flip rates measured at 37°C

Anion	k_{B3} (10^{-3} min^{-1})	k_{B3} as % of k_{total}
NDP	81	100
DENSA	31	70
^{14}C-LPM	10	54
NBDP-Methanol	9	20
NBD-Phosphatidate	2	65
NBD-P-Glycol	0.8	30
NBD-P-Glycerol	0.3	40
NBD-P-Glycolate	0.06	80
NBD-P-Hydroxyethanesulfonate	0.05	90
NBD-P-Hydroxybutyrate	0.03	50

Data calculated, except those for NDP, from refs. [52, 60, 64].

smaller. These numbers can be compared directly with numbers for the other anionic flip probes studied so far, provided that fluxes are in the domain of linear concentration dependence, i.e., the rate constants are independent of the substrate concentrations. This is true for DENSA, LPM and probably further probes.

Table 4 compiles rate constants for the flip via band 3 of the probes studied so far. These constants are all lower than those for NDP and vary by more than three orders of magnitude. The simple sulfonate DENSA, moves almost as fast as NDP. LPM and the methyl ester of NBD-PA (i.e., NBDP-Methanol) are intermediate, while other esters of phosphatidate (NBDP-derivatives) having a considerably larger volume of their head group are, not unexpectedly, rather inadequate substrates for a flip via band 3. All these anions are mono-anionic at pH 7.4. The di-anionic NBDP-esters bearing one charge on the phosphate group and the other one on the substituting anionic group, penetrate via band 3 even more slowly. Table 4 also shows that NDP is the only amphiphile anion ''flipping'' exclusively via band 3.

To compare an intramembrane flip via band 3 with normal transmembrane exchange fluxes via this transporter, it would seem appropriate to determine turnover numbers (TON) for substrate-loaded band 3 by dividing a measured flip or flux by the number of copies of band 3 involved. In the case of chloride exchange flux at physiological concentrations, a TON of about $2.4 \cdot 10^6$ anions/band 3/min at 37°C can be derived from recent work of Knauf et al. [42] assuming approximate saturation of band 3 with its substrate. A TON for NDP was derived indirectly from an estimate of the number of copies of band 3 occupied by NDP at the intramembrane NDP concentrations present in our flip experiments. To this end, we took advantage of the inhibition, by NDP, of the self-exchange of hydrophilic anions via band 3. From the inhibitions and stoichiometries shown in Fig. 10, we derived a number of about 20% occupation of band 3 (total content $1.2 \cdot 10^6$ monomers per cell) for the membrane level of NDP of 200 nmoles/ml cells, i.e., $12 \cdot 10^6$ molecules per cell in 150 mM Cl^-. This means—on the basis of the most simple model—that of these probe molecules only $0.2 \cdot 1.2 \cdot 10^6 = 2.4 \cdot 10^5$, i.e., 2% of $12 \cdot 10^6$, are bound to the transporter, while almost 98% are either located in the bilayer or bound to other membrane proteins. Relating the measured flip rate of 16 nmoles/ml cells/min determined at this membrane level to the $2.4 \cdot 10^5$ band 3 monomers occupied by NDP we arrive at the very low TON of 5 NDP/band 3/min. The flip of an NDP anion via band 3 is thus a very rare event compared to the physiological anion exchange process. It has to be kept in mind, however, that TON's for other hydrophilic anions can also be much lower than those for Cl^- (or HCO_3^-), the major physiological substrates of band 3. Inorganic phosphate, for instance, is transported at a TON of the order of about $3 \cdot 10^3$ ions/band 3/min at 37°C (calculated from ref. 24), and phosphoenolpyruvate at only about 60 (extrapolated from data in [29]). Still lower TON's will probably apply to large hydrophilic organic sulfonates and phosphates.

An intriguing general problem in comparing kinetics of intramembrane translocation by flip with kinetics of transmembrane fluxes consists in defining the relevant, ''true'' substrate concentrations in the case of a flip process. While a level of 200 nmoles NDP/ml cells can be derived from the initial aqueous concentration of the substrate, its partition coefficient and the hematocrit, this is certainly not the substrate ''concentration'' seen by the transporter. It may seem reasonable to relate the intramembrane substrate level to the volume of the membrane lipid phase serving as a ''solvent'' for this substrate. On this basis we obtain a concentration of 200 nmoles NDP per 5 mg (≈5 μl) membrane lipid, equivalent to a 40 mM ''solution,'' if 1 mg membrane lipid is taken as 1 μl solvent and 1 ml erythrocytes as about 5 mg membrane lipid [13]. A membrane ''concentration'' twice as high could be assumed under our conditions if only the outer leaflet of the bilayer were taken to represent the *cis*-compartment of the translocation process. Whether estimates of this type are physically justified needs further consideration, since we are comparing an apparent concentration in a 2-dimensional solvent (the bilayer) with concentrations of hydrophilic anions in a true 3-dimensional fluid.

In any case, a lipid bilayer cannot be regarded as a well-stirred system. It might therefore also be asked whether the rate of association of NDP with its binding site on band 3 depends on the lateral diffusion coefficient of the probe which would thus limit the overall rate of the flip. A rough estimate, however, indicates that the membrane surface density of inserted NDP corresponding to a cell content of 200 nmoles per ml cells (15 pmol/cm^2

Table 5. Thermodynamic parameters of the transport, via band 3, of NDP and some hydrophilic anions

	k (37°C) Ions/B3/s	$\Delta H‡$ kJ/mole	$\Delta S‡$ J/mole/grad	$T\Delta S‡$ kJ/mole	$\Delta G‡$ kJ/mole
NDP	0.1	114	+103	32.0	82.2
Phosphoenolpyruvate[1]	1	136	+193	59.7	76.3
Orthophosphate[2]	50	134[4]	+219	67.8	66.2
Sulfate[3]	60	116[4]	+164	50.5	65.2

[1] Calculated from data in [29]
[2] From [24}
[3] From [26]
[4] Below transition temperature

membrane) and the lateral diffusion coefficient of the probe (at least $2 \cdot 10^{-8}$ $cm^2 \cdot sec^{-1}$) are sufficient to exclude the rate of approach of NDP to its transporter as the limiting step in the movement of NDP from one to the other leaflet of the bilayer. NDP is almost certainly in permanent equilibrium with its binding site on band 3.

To understand the low reorientation rate of NDP, two further approaches may be taken, both considering the formation of an activated (transition) state [26, 45] of the band 3-substrate complex as a crucial step in translocation. One approach is based on the thermodynamics of the activated state [25]. The enthalpy ($\Delta H‡$), entropy ($\Delta S‡$) and free energy ($\Delta G‡$) of its formation are determined according to Eyring [25] by the following equations

$$\Delta H‡ = E_{act} - RT$$

$$\Delta S‡ = R \cdot ln\,((Nh/RT) \cdot X)$$

$$X = k \cdot exp\,\Delta H‡/RT$$

$$\Delta G‡ = \Delta H‡ - T \cdot \Delta S‡$$

where E_{act} is the Arrhenius activation energy, R the gas constant, T the temperature (K), N Avogadro's number and h Planck's constant; k, a rate constant (sec^{-1}), is here equated with the TON (calculated as described above).

From the activation energy for the flip of NDP (116 kJ/mole) and the TON at 37°C (about 0.1 sec^{-1}) we arrive at the numbers given in Table 5. Data for related anions have been added for reasons of comparison. For the particular purpose of our problem, one would like to compare the phosphate ester NDP with orthophosphate with respect to the contributions of the enthalpic and the entropic term to the rates of turnover. According to Galanter and Labotka [24], the activation energy for the transport of orthophosphate changes at about 37°C. We have used the value for the range below 37°C, since we have no indication from our measurements for a similar change in the case of NDP below 37°C. Using this value of the activation energy of orthophosphate transport, we conclude from the data that the 500-fold lower TON of the amphiphilic phosphate ester NDP, if compared with orthophosphate, is mainly due to an unfavorably low positive entropy of activation.

For the hydrophilic phosphate ester, phosphoenolpyruvate, and inorganic sulfate (below the ''break'' [26]) the high activation enthalpy is compensated by a high activation entropy which provides for a high rate of turnover. This is not the case for the amphiphilic NDP (*see above*) and a similar amphiphilic sulfonate like DENSA (*data not shown*). If, as is commonly argued (*see* e.g., [26]), high entropies of activation indicate major changes in the conformation of the carrier protein in the course of the formation of the activated state, the lack of such a high entropy in the case of NDP may suggest that no large conformation changes of the carrier are involved when this anion is translocated.

This assumption would also account for the low transport rate of amphiphilic anions in terms of Krupka's model concept of band 3-mediated anion transport. His model [45] attributes substrate specificity and transport velocity to the stability (''tightness'') of the carrier-substrate complex in its activated state, and assigns this stability to structural features of the substrate and of the binding region of the carrier. Considering the structure of NDP, one is tempted to speculate that the extended and bulky hydrophobic naphthyldecyl residue impedes the formation of a tight transition complex.

Based on these cautious conclusions one might ask, to what extent those characteristics that distinguish the flip mode of operation of band 3 from its exchange mode might be a consequence of the very loose transition complex. One remarkable peculiarity of the flip of amphiphilic anions concerns its low sensitivity to a number of strong inhibitors of the self-exchange of hydrophilic anions. This low sensitivity not only applies to the flip of NDP but has also been observed for the flip of other amphiphilic anions [52, 64]. For an interpretation of these differences the classification of the inhibitors of

anion exchange in terms of enzyme kinetics (competitive *vs.* noncompetitive) or carrier kinetics (site or access blocker *vs.* translocation blocker [18, 19, 20]) might be helpful.

Due to experimental and interpretational problems only a few of the many known reversible inhibitors have been characterized unequivocally in terms of the kinetic type of their inhibitory action (*see* [13] and [54] for reviews). Nevertheless it is possible to state that competitive (pyridoxalphosphate [51]) as well as noncompetitive (erythrosin [43], niflumate [8]) inhibitors of anion exchange are among the agents having significantly lesser effects on flip than on anion exchange. This classification can therefore not provide a basis for explaining the low effectivity of certain inhibitors.

Likewise, the ''low-effectivity'' inhibitors of the flip via band 3 are found in both classes of inhibitory agents distinguished with respect to their influence on the binding of substrate (35-Cl) to the transfer site, as detected by NMR spectroscopy [18, 19, 20]. Pyridoxalphosphate [48], salicylate [50] and tetrathionate [48] have been shown to be ''site (or access to site) blockers'' impeding Cl binding to the transfer site by either direct, ''steric'' competition or an allosteric mechanism [18, 19]. Niflumate and erythrosin (by analogy to eosin maleimide) belong to the class of ''translocation blockers'' [20] not interfering with substrate binding but preventing the configurational changes involved in anion exchange.

The weak effects of certain inhibitors of the exchange mode on the flip mode of operation of band 3 can thus not be assigned to established mechanistic features of their effects. It may be speculated, however, that these weak flip inhibitors probably interact with regions of band 3 protein whose native, unperturbed state is not as necessary for the flip process of amphiphilic anions with long hydrophobic tails, as it is for the exchange of small hydrophilic anions. This proposal can be reconciled with the concept of a loose, i.e., ''inefficient,'' transition complex between flipping anions and band 3 if it is hypothesized that the domains of band 3 not crucially involved in the formation of a transition complex with amphiphilic flip probes are those providing for the formation of a tight, highly efficient transition complex with small hydrophilic anions and for the strong inhibition of the transport of this type of anions by the weak inhibitors of the flip process. Since NDP itself is an inhibitor of the exchange of hydrophilic anions (*see* Fig. 10) it may also be proposed that the low sensitivity of the flip of NDP to certain inhibitors is due to a perturbation, by NDP itself, of the domains binding these particular inhibitors. In other words, NDP and possibly the other amphiphilic anions moving by flip would be something like ''intrinsic inhibitors'' of their own transport due to effects on the conformation of their binding regions on the transporter. Conformational changes of band 3 protein induced by interaction with amphiphilic anions are clearly indicated by calorimetric data [28].

The importance of a transition complex in determining the flip rate of NDP can also be reconciled with the stimulating effect of limited proteolysis by papain and of aliphatic alcohols on the flip of long-chain amphiphiles (*see above*). This effect contrasts with the inhibitory effect of these modifications on the exchange of small hydrophilic anions [21, 34, 49]. It may be proposed that proteolysis of band 3 or alcohols enhance the flexibility of domains of band 3 which form the putative transition complex with long-chain amphiphilic anion and thereby increase its stability. This hypothesis is also supported by our observation (*data not shown*) that the flip stimulation by papain does not go along with a decrease of the activation enthalpy. Stimulation must therefore be of entropic origin, probably reflecting a gain in configurational flexibility.

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