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Complex Effects of Papain on Function and Inhibitor Sensitivity of the Red Cell Anion Exchanger AE1 Suggest the Presence of Different Transport Subsites

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Abstract. Band 3 (AE1), the anion exchanger of the human erythrocyte membrane, mediates not only fluxes of small hydrophilic anions (e.g., chloride, oxalate), but also the flip-flop of long-chain amphiphilic anions (e.g., dodecylsulfate). Treatment of erythrocytes with papain, long known to inhibit the transport of the former type of anions, accelerates the transport of the latter type. In an attempt to elucidate the basis of these opposite responses to papain, several small amphiphilic arylalkyl sulfonates and -sulfates were tested for the response of their transport, via AE1, to papain. Although all these probes are most likely transported by a flux and not by flip-flop, their transport was inhibited by papain only in some cases, but accelerated in others. Different responses to papain therefore most likely do not reflect differences between transport by flux or by flip.

The transports of different species of anions also differed considerably in the changes of their sensitivity, to noncovalent and some covalent inhibitors, brought about by papain treatment. While oxalate transport remained as sensitive as in native cells, transports of small amphiphilic anions lost their sensitivity to a major extent, regardless of the inhibition or acceleration of their transport by papain.

The results are discussed in the light of present concepts of the structural organisation of AE1, and interpreted in terms of a model of different transport subsites for different species of anions in this transporter.

Key words: Erythrocyte membrane — Band 3 (AE1) — Anion transport — Flip-flop — Papain

Dedicated to Professor Hermann Passow on the occasion of his 75th birthday.

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Introduction

Transport of anions across biomembranes usually requires membrane proteins. Hydrophilic, inorganic and small organic, anions are transported between the extracellular and the intracellular water phase by electrically silent anion exchangers [12, 35, 55] or cation/anion cotransporters [44, 65]. Alternatively, membrane channels provide for conductive transmembrane diffusion of such anions [17, 19].

In recent years, the transport of amphiphilic anions has also come into the focus of interest [34, 54, 61, 66]. Due to a hydrophobic, aliphatic or aromatic, moiety of the molecule in addition to the hydrophilic anionic "headgroup" — e.g., a sulfate, sulfonate or phosphate group — such anions will insert into the lipid domain of artificial and biological membranes with high affinity. Due to their low water solubility and the resulting high content in the bilayer, the transmembrane transport of such anions is most adequately described in terms of their translocation from one leaflet of the bilayer to the opposite one, i.e., in terms of a flip-flop. While flip-flop of amphiphiles was originally observed as a nonmediated process in lipid membranes, it has now become clear that specialized membrane proteins can catalyze passive and even active uphill flip (inward) and flop (outward) processes of biologically relevant substrates [2, 64, 70].

We have provided evidence in recent years that AE1 (band 3), the anion transporter of the red blood cell, does not only operate as a high capacity exchanger [55] and a low capacity conductive channel [19], but can also act as a flippase mediating the translocation of membrane-bound long-chain sulfate, sulfonate and phosphate compounds including derivatives of phosphatidic acid and its mono-esters [34, 54, 57, 61, 66].

In the "flip mode" of operation of AE1, the long-chain amphiphilic substrates are assumed to approach their transport site from the lipid bilayer and not from the

aqueous phase as in the case of the "flux mode". It has therefore been an intriguing question whether and which transport characteristics of transport via band 3 might differ in the flip mode from those in the flux modes of operation. Besides some quantitative differences, concerning inhibitor sensitivity, the band 3-mediated translocation of long-chain amphiphilic anions indeed exhibits a qualitative peculiarity in that this process is enhanced by a pretreatment of the cells with papain or by butanol present during transport measurement [34, 54, 61, 66]. Both treatments are well known to inhibit the transport of "normal" hydrophilic anions such as chloride, sulfate or oxalate [18, 26, 27, 50]. These findings suggested that the opposite responses of anion transport to both, papain and butanol, might arise from different modes of transport (i.e., flip-flop or flux).

In an attempt to define the structural details of amphiphilic anions that are responsible for these anomalous properties of their translocation via band 3 and to substantiate or refute the above mentioned hypothesis, we have now studied the effects of papain and butanol on the transport of a number of amphiphilic anions with structural features placing them between the hydrophilic, inorganic and small organic, anions on the one side, and the typical, lipid-like, long-chain amphiphilic anions on the other side. This intermediate class was represented in our study mainly by anions containing $-SO_3^-$ or $-OSO_3^-$ groups connected to naphthalene and benzene directly or via a short alkyl spacer (*see* Fig. 1). As will be shown, within this class of probes, termed "small amphiphilic anions" in the following, we observe the transition, with respect to their response to papain (and butanol), between hydrophilic anions represented by oxalate, and the long-chain amphiphilic anions represented by SDS and NDSU. Moreover, it turned out that even simple aliphatic dicarboxylate anions of different chain length vary quantitatively in their response to papain.

Materials and Methods

Materials

Human erythrocyte concentrates were obtained from the local blood bank and used within 14 days. Erythrocytes were isolated by centrifugation and washed three times with isotonic saline. Incubation media contained (mM): KCl (90), NaCl (45), sucrose (44) and NaH_2PO_4/Na_2HPO_4 (12.5; pH 7.4) (medium A).

10-α-Naphtyl-1-decanol was prepared from 1,10-decanediol by a five-step synthesis according to ref. [33]. The probes NMSU, NESU, PESU and NDSU were prepared from the corresponding alcohols (α-naphthylmethanol, 2-α-naphthyl-1-ethanol, 2-phenyl-1-ethanol, 10-α-naphtyl-1-decanol) by reaction with sulfurtrioxide dimethylformamide complex [58]. The identity of all probes was confirmed and their purity estimated by NMR, mass and IR spectroscopy.

Bovine serum albumin (fraction V) was obtained from Päsel-Lorei (Hanau); DNDS from Pfaltz and Bauer (Waterbury, CT); DIDS from Calbiochem; PLP from Serva (Heidelberg). Chymotrypsin, DEPC, DTNB, erythrosin B, MESNA, niflumic acid, 1-NS, papain, TNS and WRK were purchased from Sigma (Deisenhofen); *n*-butanol, sulfurtrioxide dimethylformamide complex, 1-naphthalenemethanol, 2-α-naphthyl-1-ethanol, 2-phenyl-1-ethanol, phenylmethane-sulfonylchloride and HBS from Aldrich; ABS, ANS and 2-NS from Fluka (Buchs); PS from Molecular Probes (Eugene, Oregon); NSU from ICN Biomedicals Inc. (Aurora, Ohio); (1-^{14}C)-SDS from ARC (St. Louis MO.); (^{14}C)-oxalic acid, (2-^{14}C)-malonic acid and (2,3-^{14}C) succinic acid from NEN.

Modification of Erythrocytes

Erythrocytes were pretreated with DIDS (50 μM) by a 30 min incubation at 37°C in medium A (pH 7.4, 10% hct), and subsequently washed twice with medium A.

Exofacial proteolytic cleavage of band 3 was carried out by treatment of erythrocytes in medium A (50% hct) with cysteine-activated papain (0.1–3 mg/ml cells, 60 min, 37°C, pH 7.4) or chymotrypsin (2 mg/ml cells, 60 min, 37°C, pH 7.4) followed by two washings with medium A containing 0.2 g dl^{-1} albumin and two washings with medium A.

Cells were modified with the carboxyl-reactive Woodward reagent K (WRK) essentially following the procedure elaborated by Jennings and Anderson [28]. Briefly, cells were suspended (hct 20%, 0°C) in medium A at pH 6.8–8. Solid WRK was added at a final concentration of 2 mM. After 10 min incubation, the cells were sedimented and washed twice with medium A. Alternatively, the 10 min with WRK were followed by 2 sequential additions of $NaBH_4$ (4 mM) with 5 min interval. After further incubation for 5 min, the cells were sedimented and washed.

Cells were modified with the histidine-reactive agent diethylpyrocarbonate (DEPC) following the procedure described by Izuhara et al. [25] for resealed ghosts. Whole erythrocytes were suspended in medium A (hct 10%, 0°C) at pH 8. DEPC was added to final concentrations between 5 and 20 mM as given in the Results. After 30 min incubation, the cells were sedimented by centrifugation, washed twice with medium A and retitrated to pH 7.4.

Measurement of Uptake and Release of Amphiphilic Anions

Measurement of Initial Membrane Binding and Subsequent Uptake of Small Amphiphilic Anions

Binding and uptake of the probes were quantified by measuring the decrease of probe concentrations in the extracellular medium. Packed erythrocytes were suspended (25% hct) in medium A at the desired temperature. Inward transport was initiated by adding the probe to the cell suspension from a concentrated stock solution (final concentrations, in the total suspension, of naphthyl probes: 40 μM, of MESNA: 400 μM, of phenyl probes: 4 mM). After suitable time intervals, samples were taken and centrifuged (6,600 × *g*, 30 sec) to stop the uptake. To obtain the fluorescence of the extracellular medium at "zero-time" (c_0), probe was added to medium A at a concentration 1.33 times its initial concentration in the total suspension, in order to account for the 25% cell mass in the suspension. Further treatment was the same as described below.

Measurement of Release of Small Amphiphilic Anions from Preloaded Cells

The release of probe anions from preloaded cells was quantified by measuring the increase of their concentration in the extracellular medium. For preloading, packed erythrocytes were suspended in

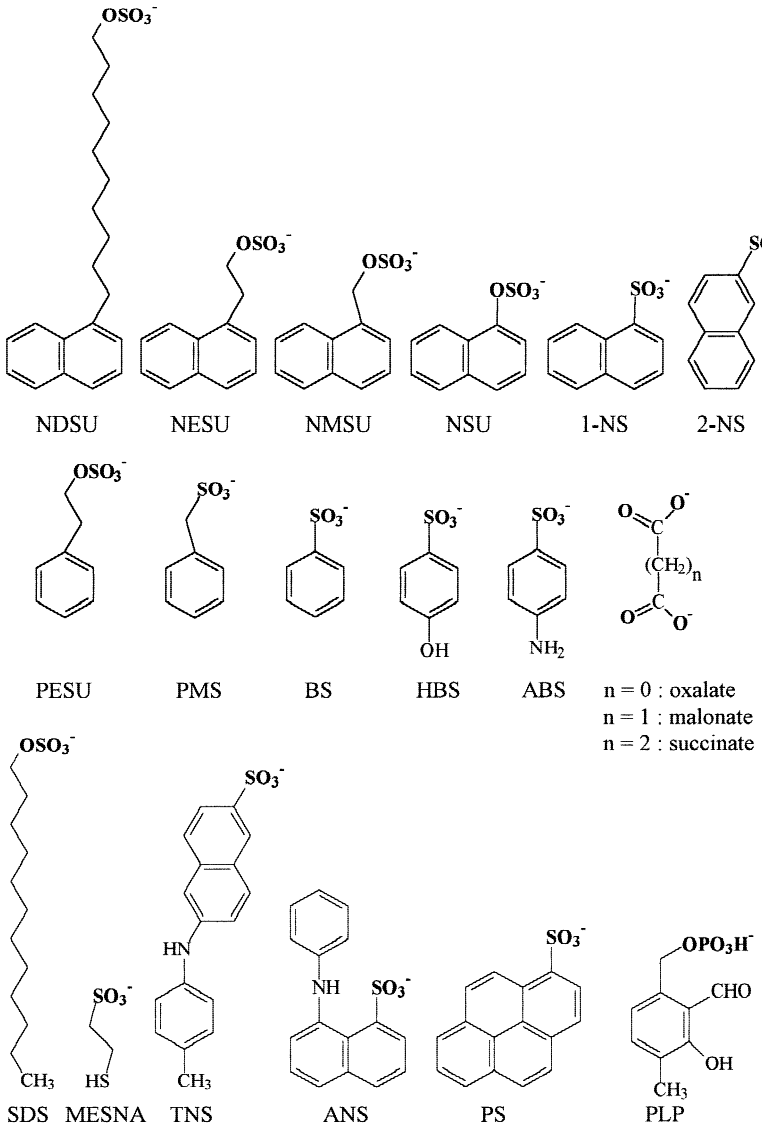

Fig. 1. Structure and abbreviated names of the hydrophilic and amphiphilic anions used in this study

medium A (20% hct, 37°C) containing the probe (final concentrations of fluorescent probes: 120 μM, of absorbing probes: 10 mM, of MESNA: 4 mM). These suspensions were incubated for 30–60 min at 37°C. Subsequently, the cells were washed twice with medium A at 0°C. Outward transport was initiated by resuspending the probe-containing cells in medium A (hct 25%), at appropriate temperatures. After various time intervals, 500 μl samples of the suspension were removed and the supernatants separated from the cells by centrifugation (6,600 × *g*, 30 sec).

Preparation of Samples for Fluorescence or UV Spectroscopy

General Procedure

Aliquots of the supernatants and the "zero-time" medium were diluted with adequate volumes of medium A for spectroscopic analysis. If necessary, hemoglobin released due to hemolysis was precipitated by adding 50 μl perchloric acid (60%) per 1 ml medium. Protein-free suspensions were analyzed by fluorescence spectroscopy (wavelengths given in Table 1), using a Shimadzu spectrofluorometer (RF 5001 PC) or UV-spectroscopy using a Pharmacia Biotech Ultrospec 2000.

Special Procedures

To quantify TNS and ANS, supernatants were diluted with medium A containing the cationic amphiphile HTAB (10 mM) to increase the fluorescence yield.

For the determination of ABS the absorption of 20-fold diluted supernatants was measured at 415 nm and 249 nm. The absorption at 415 nm, where ABS does not absorb, served to correct for the contamination by hemoglobin, which also absorbs at 249 nm, on the basis of calibration curves prepared by lysing packed red cells with water at defined dilution. MESNA was quantified by SH group determination. Supernatants were diluted 10-fold with medium A and the absorption at 412 nm was measured before and after addition of 0.1 ml volume of DTNB (1 mM in 1 g dl^{-1} sodium citrate).

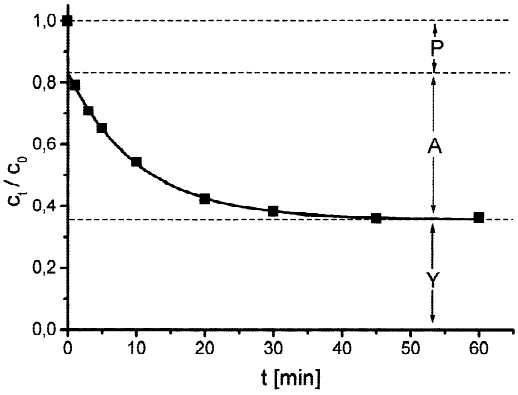

Fig. 2. Schematic time curve of the change of the extracellular concentration of a small amphiphilic anion added to a suspension of erythrocytes (hct. 25%). Concentrations normalized to the initial concentration. For the explanation of the three phases P, A and Y *see* text

Analysis of Data Points

In the influx experiments, the concentration of the small amphiphilic anions in the medium (c_t) changed in a biphasic time course upon addition of cells, as shown schematically in Fig. 2. The first, essentially instantaneous, decrease (P) reflects the binding of the probe to the outer leaflet of the membrane, the subsequent slower exponential decline (A) the movement of the probe to the inner leaflet and the cell interior, ending with a steady state distribution at the level Y of probe concentration in the medium.

From the difference between the initial relative fluorescence or absorption of the medium and the fluorescence or absorption at the point of intersection of the exponentially fitted curve A with the ordinate, partition coefficients for the probes between the outer membrane layer and the external medium were calculated.

This coefficient (K_P) was defined as

K_P = mol probe per ml membrane outer leaflet/mol probe per ml medium

and calculated as

$$K_P = (P/Y + A) \cdot [(100 - hct)/hct] \cdot 200$$

The factor 200 stems from the assumption that 1 ml cells contains a volume of 5 μl lipid in its outer membrane leaflet, which serve as solvent for the probe bound initially.

To derive rates of uptake from the data points for the slower phase of decline, normalized concentrations c_t/c_0 were fitted for $t > 0$ to an exponential function of the type

$$f(t) = Y + A \cdot e^{-k \cdot t}$$

where k = rate constant of the transport process. Rates of release were calculated analogously. The steady state distribution ratio $(c_i/c_e)_\infty$ of the probes between the intra- and the extracellular space was calculated by the equation.

$$(c_i/c_e)_\infty = [1/Y - (1 + P + P \cdot r)] \cdot [(100 - hct)/hct] \cdot 1/0.65$$

where Y and P have the meaning defined in Fig. 2 and r is the distribution ratio for the probe between the outer and the innner leaflet of the lipid bilayer. r is about 0.25 for anionic amphiphiles at low probe levels [49, 54, 57]. To be related to the intracellular aqueous space of 65% of the total cell volume, the numbers have to be divided by 0.65.

Table 1. Excitation, emission and absorption wavelengths (nm) used for the direct quantification of the amphiphilic anions

Probe	Excitation λ_{ex}	Emission λ_{em}	Absorption λ_{abs}
1-NS	282	333	
2-NS	275	338	
NSU	280	334	
NMSU	280	337	
NESU	280	337	
NDSU	281	337	
ANS	377	479	
TNS	315	440	
PS	345	374	
PLP	292	394	
BS			262
PMS			258
PESU			257
ABS			249
HBS			271

Measurement of the Transbilayer Translocation of the Long-Chain Amphiphilic Anions NDSU and SDS

The transbilayer reorientation (flip) of the fluorescent probe NDSU was measured using the procedure described for an analogous alkyl phosphate in [34]. Probe concentrations were quantified by fluorescence spectroscopy at the wavelengths given in Table 1.

The flip of ^{14}C-labeled SDS was measured following the same principle, but with appropriate modifications. Briefly, ^{14}C-SDS was inserted into the outer membrane leaflet of erythrocytes by incubation of the cells (hct 30%, 0°C, 2 min) in medium A containing 10 μM unlabeled and 30 nC_i/ml ^{14}C labeled SDS. After two washings in 10 volumes of medium A, 600 μl loaded cells were suspended and incubated (hct 10%) in medium A at 10°C. 300 μl aliquots were sampled at zero time and at appropriate time intervals and diluted with 900 μl ice-cold medium A containing 0.8 g dl^{-1} BSA to extract SDS present in the outer leaflet. After 2 min, the cells were spun down (20 sec, 10,000 × g), the supernatant was removed and the cells washed once with 1 ml medium A at 0°C.

Following lysis of the cells with 5 vols. water, protein was precipitated by addition of 1 ml isopropanol. After centrifugation, the radioactivity in the supernatant, corresponding to SDS translocated to the inner membrane leaflet, was measured by liquid scintillation counting and related to the total radioactivity in the same amount of hemolyzed cells not treated with BSA. Data were used to calculate the rate coefficients for the unidirectional flip and the steady-state distribution of the probe between the inner and outer membrane leaflet as described in [34].

Measurement of Self Exchange of Small Aliphatic Dicarboxylates

Self-exchange of oxalate, malonate and succinate was measured by following the tracer efflux at concentration equilibrium and appropriate

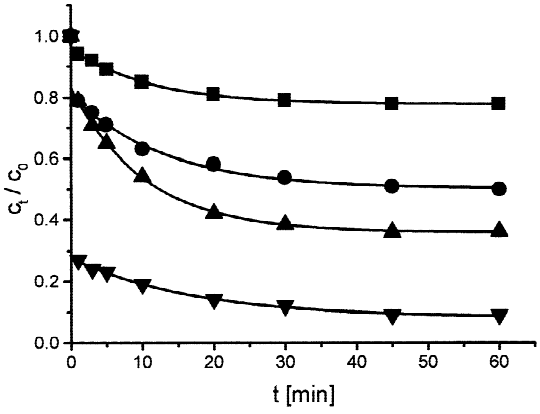

Fig. 3. Time course and extent of the initial binding and subsequent uptake of various amphiphilic sulfonates into erythrocytes as reflected by the decrease of the normalized concentration in the extracellular medium. ■ BS 4 mM, 20°C; ● 1-NS 40 μM, 10°C; ▲ NESU 40 μM, 20°C; ▼ PS 20 μM, 15°C. Note that all kinetics start at 1.0 at 0 min.

temperatures (hct 5%) from cells preloaded with the ^{14}C-labeled probes (2 mM). Rate coefficients for the flux were calculated from the increase of extracellular radioactivity by standard procedures [15].

Abbreviations

1-NS, 1-Naphthalenesulfonate; 2-NS, 2-Naphthalenesulfonate; ABS, 4-Aminobenzenesulfonate; ANS, 8-Anilino-1-naphthalenesulfonate; BADS, 4-Benzamido-4′-aminostilbene-2,2′-disulfonate; BS, Benzenesulfonate; DEPC, Diethylpyrocarbonate; DIDS, 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid; DNDS, 4,4′-Dinitrostilbene-2,2′-disulfonic acid; DTNB, 5,5′-Dithio-bis-(2-nitrobenzoic acid); HBS, 4-Hydroxybenzenesulfonate; HTAB, Hexadecyltrimethylammonium bromide; MESNA, 2-Mercapto-1-ethane-sulfonate (Na salt); NAP-taurine, N-(4-azido-2-nitrophenyl)-2-aminoethanesulfonate; NBD-taurine, N-(7-Nitrobenzofuran-4-yl)taurine; NESU, 2-(α-Naphthyl)-1-ethyl-sulfate; NDSU, 10-(α-Naphthyl)-1-decyl-sulfate; NMSU, α-Naphthyl-methyl-sulfate; NSU, α-Naphthyl-sulfate; PESU, 2-Phenyl-1-ethyl-sulfate; PLP, Pyridoxal-5-phosphate; PMS, Phenylmethanesulfonate; PS, 1-Pyrenesulfonate; SDS, 1-Dodecylsulfate; TNS, 6-(p-Toluidino)-2-naphthalenesulfonate; WRK, N-Ethyl-5-phenylisooxazolium-3′-sulfonate.

Results

Initial Binding and Subsequent Uptake of Amphiphilic Anions

As a basis for the investigation of the effects of papain and butanol on the transport of small amphiphilic anions via the anion exchanger, rates of uptake of these probes were determined by following the decrease of their concentration in the extracellular medium after addition of cells. To avoid major side effects of the probes, the lowest concentrations still allowing quantitative spectroscopic analysis were chosen. As already explained in Methods, a biphasic decrease of probe concentration is always observed, consisting of a very fast and a subsequent slower phase, which arise from the initial, instantaneous binding of the probe to the cell surface and its subsequent slower uptake into the cytoplasm, most likely in exchange for intracellular chloride.

Table 2. Initial binding of amphiphilic anions to the outer cell surface, expressed in various terms. K_P: Membrane/water partion coefficients, calculated as described in Methods

Probe added [μmol/ml suspension]	Probe initially bound [nmol/ml cells]	K_P	Copies bound/ band 3
1-NS [0.04]	17.8	79 ± 20 (16)	0.9
2-NS [0.04]	29.9	144 ± 36 (7)	1.5
NSU [0.04]	23.2	106 ± 27 (15)	1.2
NMSU [0.04]	29.9	144 ± 140 (14)	1.5
NESU [0.04]	22.7	103 ± 19 (14)	1.1
ANS [0.04]	54.3	315 ± 39 (4)	2.7
TNS [0.04]	91.8	791 ± 78 (8)	4.6
PS [0.02]	45.2	1642 ± 200 (8)	2.3
PLP [0.04]	36.1	175 ± 97 (4)	1.8
BS [4]	1319.0	57 ± 24 (8)	66
PMS [4]	371.0	15 ± 27 (7)	19
PESU [4]	1153.0	49 ± 20 (6)	58
ABS [4]	155.0	6 ± 11 (8)	8
HBS [4]	391.0	16 ± 11 (8)	20
MESNA [0.4]	22.0	9 ± 11 (5)	1.1
SDS* [0.04]	119.1	80 000 (2)	6.0

* [57]. Number of experiments in parentheses.

Figure 3 presents primary experimental data obtained by this technique for a number of representative anions. It becomes evident from this diagram that the fast and the slow phases of the uptake differ considerably in extent and rate for the different probes. Table 2 compiles information concerning the initial membrane binding of the probes included in our study. As expected, the small amphiphilic anions bind to the outer membrane surface only with a rather low affinity. This is also true for the phenyl containing anions, in spite of the high amounts of probe initially bound, which are a mere consequence of the much higher concentration in the medium (4 mM) than in the case of the naphthyl containing anions (40 μM). Partition coefficients derived from the concentrations bound per ml cells should be regarded with some caution and therefore be considered as apparent, since it is not possible, by the technique used, to distinguish superficial adsorption and binding to membrane proteins from the true insertion into the bilayer. The numbers obtained, which range between 10^2 and 10^3 for the naphthyl derivates and below 10^2 for the phenyl derivates, therefore represent upper limits. They have to be compared with those for long-chain amphiphilic an-

Table 3. Rate coefficients (k) of uptake (at 10°C), final distribution between intra- and extracellular space $(c_i/c_e)_\infty$ and maximal fractional inhibition by DIDS (k_I/k) for the amphiphilic anions

Probe	[mM]	k*[min^{-1}]	$(c_i/c_e)_\infty$	k_I/k
1-NS	[0.04]	0.173 ± 0.023 (8)	2.6	0.01
2-NS	[0.04]	0.224	3.3	0.01
NSU	[0.04]	0.067 ± 0.011 (5)	6.6	0.01
NMSU	[0.04]	0.016 ± 0.002 (4)	3.3	0.01
NESU	[0.04]	0.012 ± 0.001 (4)	8.2	0.02
NDSU**	[0.04]	0.026	0.2	0.20
ANS	[0.04]	0.039	18.2	0.17
TNS	[0.04]	0.009	19.5	0.01
PS	[0.02]	0.006	29.5	0.01
PLP	[0.04]	0.018	4.4	0.01***
BS	[4]	0.032	1.0	0.01
PMS	[4]	0.007	0.9	0.02
PESU	[4]	0.023	1.5	0.01
ABS	[4]	0.009	0.9	0.01
HBS	[4]	0.004	1.0	0.01
MESNA	[0.4]	0.035	0.8	0.01
SDS**	[0.04]	0.026	0.25	0.20

* k-values without standard deviations are means from 2–3 independent experiments.

** Measured at three higher temperatures and extrapolated to 10°C on the basis of the Arrhenius equation. c_i/c_e refers to the distribution between the two leaflets of the lipid bilayer in these cases.

*** [5].

Numbers calculated from kinetics as shown in Fig. 1 and as described in the Methods. k values not corrected for the steady-state distribution of the anions.

ions such as SDS, for which we have obtained, by the same technique, partition coefficients of about 10^5. We have not tried to establish a relationship between the molecular structure and the measured partition coefficients, since in addition to the problem of superfacial adsorption, anionic probes are likely to bind to membrane proteins. The bound amounts of the naphthalene-derived sulfonates used in our study correspond to not more than 1–2 × 10^6 probe molecules bound to a cell with more than 10^6 copies of membrane proteins.

The second, slower phase of the decrease of extracellular probe concentration, resulting from the translocation of the probe to the inner membrane leaflet and into the intracellular space, was quantified by calculating first order rate coefficients. Steady-state distributions $(c_i/c_e)_\infty$ of the probes were determined as described in Methods, correcting for the probe bound to the outer and inner leaflet of the lipid bilayer. As evident from Table 3, $(c_i/c_e)_\infty$ values differ considerably among the various anions, but quite consistently exceed the value of about 0.7 expected for monovalent anions comparable to Cl^- [10]. This intracellular excess of probe almost certainly results from binding of the anions to hemoglobin and other cytoplasmic proteins.

Due to this complication, the rate coefficients calculated from the uptake kinetics were not corrected for compartment sizes. A further complication arises from the fact that many of the small amphiphilic anions, like their long-chain homologues, are inhibitors of anion exchange via band 3. This inhibitory influence, which will be dealt with in more detail below, may also affect their own transport rates to an unknown extent, since the type of inhibition is presently not clear and may well be non-competitive (i.e., allosteric).

For all these reasons, it is probably not surprising that no simple relationship between the structure of the small amphiphilic anions and their transport rates becomes evident from Table 3, although all these anions are moving exclusively via AE1, as shown by our finding (Table 3) that the stilbene disulfonate DIDS completely suppresses their uptake. Translocation of these anions by other transporters of the red cell membrane, such as the monocarboxylate transporter or the ATP-dependent flippase, is very unlikely since such transporters are only little, if at all, sensitive to stilbene disulfonates [13, 3]. As expectable, the long-chain amphiphilic anions NDSU and SDS, moving by flip-flop [57] have a minor stilbene-disulfonate insensitive component (*cf.* Table 3). The effects under study in this work, however, involve the Band 3-mediated component [57]. It can be stated safely that all these monovalent sulfonates and sulfate monoesters are transported orders of magnitude slower than Cl^- and related halide anions [9]. It seems also justified to assume that, with the possible exception of PS, all small amphiphilic anions included in our work move via AE 1 in the flux mode of operation, since their low membrane/water partition coefficients (*see* Table 2) favor an approach to the transport site from the aqueous phase. Transport rates of small polar dicarboxylates (C_2 and C_3) measured under comparable conditions (2 mM in 150 mM Cl^-, 10°C) are of an order of magnitude comparable to that of the small amphiphilic anions (oxalate 0.0500 min^{-1}, malonate 0.0013 min^{-1}), while rates for the slightly larger succinate (0.0001 min^{-1}) or even sulfate [45] are considerably lower.

Interestingly, the translocation, via band 3, of the two long-chain alkyl sulfates NDSU and SDS, which mainly involves an AE1-mediated flip process across the bilayer [57], does essentially not differ in its rate from the transport of smaller analogs moving via AE1 by flux.

Self Inhibitory Effects of Amphiphilic Sulfonates

To obtain some basic information on the interaction of small amphiphilic sulfonates with their transporter, we studied the concentration dependence of the uptake. Rate coefficients decreased with increasing extracellular substrate concentration and the corresponding fluxes exhibited saturation. Double reciprocal plots provided lin-

ear regressions, from which half saturation constants (K_m values) in the submillimolar range were derived (1-NS: 259 μM, NSU: 151 μM, NESU: 84 μM).

One might ascribe these kinetics to the saturation of the transport site. It can, however, not be excluded that "self inhibition" via a "modifier site" was involved. The decrease of the fluxes at high substrate concentrations, which usually serves as an indicator for an inhibitory modifier site [9, 55], may not have been observable since the range of concentrations of the sulfonates that could be used was limited by low solubility or lytic effects. The presence of an exofacial inhibitory modifier site has been established for NBD- and NAP-taurine, amphiphilic sulfonates structurally comparable to the sulfonates studied here [16, 36, 37, 38]. NAP-taurine, which has been studied most throroughly, is transported via band 3 with an apparent K_m at its transport site of at least 2–4 mM at 140 mM chloride [37]. In contrast, an exofacial inhibitory modifier site binds NAP-taurine, at the same chloride concentration, with a tenfold lower IC_{50} [37]. The uptake of amphiphilic sulfonates can therefore obviously exhibit apparent saturation by binding of the substrate to an allosteric modifier site on band 3 at concentrations below those required for saturation of the transport site [*see also* 36, 37].

If this is also the case for the amphiphilic sulfonates used in our study, they should inhibit the band 3-mediated transport of other anions via two binding sites. Such inhibition should go along with supralinearity in a Dixon plot (1/flux *vs.* inhibitor concentration) which has indeed been reported for NAP-taurine and related sulfonates [37, 38]. Although inhibition of the transport of hydrophilic anions could indeed be demonstrated for our small amphiphilic sulfonates (*see* below), supralinearity in the Dixon plots was not observed up to 70% inhibition, i.e., $2 \times IC_{50}$ (*data not shown*). This might indicate, on the one hand, that our amphiphilic sulfonates do not bind to a modifier site but only to the transport site. On the other hand, their affinity for the transport site might be so low that saturation and competitive inhibition at this site do not occur before inhibition — and apparent saturation of transport — via the putative "modifier site" become operative. Both, apparent saturation of their own uptake and inhibition of other anions would then result from an interaction with the "modifier site".

Inhibitory Influence of the Amphiphilic Sulfonates on the Self-exchange of Hydrophilic Anions Via Band 3

As already mentioned above, the amphiphilic sulfonates inhibit the band 3-mediated transport of hydrophilic anions. We have studied this inhibition using oxalate as a hydrophilic test anion. Oxalate is transported via band 3 like sulfate, though much more rapidly, and shares with sulfate and chloride the patterns of sensitivity to many modifying interventions [31]. The IC_{50} values of amphiphilic sulfonates for inhibition of oxalate self-exchange vary by a factor of about 20 between 1-NS (IC_{50} = 1.3 mM), NSU (IC_{50} = 110 μM) and NESU (IC_{50} = 60 μM). The last two numbers closely correspond to the apparent K_m values for the uptake of these anions (*see above*), while in the case of 1-NS the IC_{50} is 5 times higher than the apparent K_m. For the three tested amphiphilic sulfonates, as for NAP-taurine [20], inhibition proved to be mutually exclusive with DNDS, but also with some other inhibitors, e.g., niflumate (*data not shown*), while phloretin acted partly additive with the sulfonates (*data not shown*), as it does with NAP taurine [20].

Since, on the other hand, DNDS is mutually exclusive with many other, formally competitive or noncompetitive, inhibitors, and is probably an allosterically competitive inhibitor of anion transport [55, 59], it seems safe to postulate that the amphiphilic sulfonates belong to the group of anions and agents affecting anion transport via band 3 by allosterically coupled, mutually interacting binding sites. In line with this concept, we could also demonstrate mutual exclusion for the inhibitory effects of pairs of the small amphiphilic sulfonates on dicarboxylate transport (*data not shown*).

Influence of Papain on the Transport of Amphiphilic and of Hydrophilic Anions

Papain and chymotrypsin treatment of intact human erythocytes lead to an exofacial cleavage of band 3. Papain cuts the protein into four fragments [26, 32]. Two larger fragments of about 60 and 28 kDa and one smaller fragment of 7 kDa remain associated with each other within the bilayer [40]. A fourth small exofacial peptide of 14 amino acids (1 kDa) is released from the membrane. Chymotrypsin cleaves band 3 into only two fragments of 60 and 35 kDa, which remain associated with each other in the bilayer [26].

Exofacial cleavage of band 3 by papain has long been known to inhibit (up to about 85%) the self-exchange transport of hydrophilic anions such as chloride [27], sulfate [26] or phosphate [50], while cleavage by chymotrypsin has no such effect [26]. The same is true for oxalate transport (Table 4). In contrast, the band 3 mediated flip-flop of a number of *long-chain* amphiphilic anions was recently shown to be accelerated by papain treatment, while also being insensitive to chymotrypsin [34, 54, 61, 66]. The question was, therefore, how the transport of the *small* amphiphilic anions would respond to papain (and chymotrypsin) treatment.

As expected, chymotrypsin had essentially no effect (*data not shown*). Papain, however, inhibits or stimulates transport, depending on the type of anion. The uptake of the small amphiphilic anions with larger hydro-

Table 4. Decreasing inhibitory influence of papain on the self exchange of aliphatic dicarboxylates, via band 3, with increasing chain length. Cells were treated with 1 mg (Pap 1) or 3 mg (Pap 3) papain/ml cells (60 min, 37°C, pH 7.4).

Probe	k/k_0	
	Pap 1	Pap 3
Oxalate (20°C)	0.40 ± 0.07	0.21 ± 0.04
Malonate (35°C)	0.55	0.37 ± 0.04
Succinate (42°C)	0.63	0.57 ± 0.07

k_0: Rate constant for untreated cells. *k:* Rate constant for papain treated cells. Mean values from 4–7 experiments where standard deviations are given.

phobic constituents is accelerated (Table 5), though not to the extent observed in the case of long-chain amphiphilic anions such as NDSU or SDS. In contrast, the uptake of the amphiphilic anions with smaller hydrophobic constituents is inhibited by papain, but to a somewhat lesser extent than the transport of Cl^- [32] or oxalate. The small amphiphilic anions thus range intermediately between the hydrophilic and the long-chain amphiphilic anions even to the extent that the transport of some of them (ABS, HBS, PLP) remains essentially unaffected by papain treatment. Considering that, as stated above, all our small amphiphilic anions move via AE1 by flux, the opposite, inhibitory or accelerating, effects of papain on their transport cannot be related to differences in the mode of transport, i.e., flip or flux, as proposed in our earlier work [54].

To obtain more detailed information on the significance of certain structural properties of the amphiphilic sulfonates for their response to papain, probes with the same aromatic moiety but differing in the length of the alkyl spacer between the aromatic and the anionic group were compared. This included naphthyl probes (1-NS, NSU, NMSU, NESU, NDSU) and phenyl probes (BS, PMS, PES). As evident from Table 5, a clear correlation exists between the length of the aromatic spacer and the effect of papain cleavage on the transport rates. With increasing length of the spacer the transport of the probes becomes more and more accelerated, or less inhibited.

A comparable dependence, of the response to papain, on the structure of the transported anion could also be demonstrated, in parallel experiments, for homologous aliphatic dicarboxylates. As shown in Table 4, inhibition of their self-exchange by papain treatment (at intermediate and maximally effective enzyme concentrations) decreased with increasing distance between the two carboxyl groups. For technical reasons, these experiments had to be carried out at different temperatures for the various anions. We could demonstrate, however, that the effect of papain was independent of the temperature at which the fluxes are measured (*data not shown*).

Table 5. Influence of papain pretreatment (1 mg/ml cells, 60 min, 37°C, pH 7.4) on the transport, via band 3, of small and long-chain amphiphilic anions

Probe	k/k_0
1-NS	0.5 (4)
2NS	0.7 (3)
NSU	3.2 (2)
NMSU	4.8 (4)
NESU	11.3 (2)
NDSU*	50.0 (2)
ANS	3.9 (2)
TNS	3.6 (2)
PS	3.3 (2)
PLP	1.2 (2)
BS	0.6 (2)
PMS	1.4 (3)
PESU	2.5 (2)
ABS	1.2 (2)
HBS	0.9 (2)
MESNA	0.4 (2)
SDS*	70.0 (2)

* Cells were treated with 0.1 and 0.15 mg papain/ml erythrocytes and the observed accelerations extrapolated to 1 mg / ml cells. k_0: Rate constant for untreated cells. *k:* Rate constant for papain treated cells. Number of experiments in parentheses.

In early studies of Jennings and Adams [27] on the effect of papain on the obligatory exchange transport of inorganic anions via band 3, some evidence was provided indicating that different steps of the transport cycle are affected differently. It was proposed that papain strongly inhibits the outward translocation rate constant of the exchange process while somewhat accelerating the inward rate constant. A resulting shift of the distribution of the anion (chloride) binding sites of AE1 between the inward and the outward oriented conformation was proposed to affect the apparent (effective) but not the true (intrinsic) substrate affinity of the transporter.

A comparable difference between influx and efflux was not observed in our previous studies on the acceleration, by papain, of the flip-flop of long-chain amphiphilic anions via band 3. Inward flip and outward flop of these probes were equally accelerated [53]. The same now proved to be true for the transport of the small amphiphilic anions. Regardless of the direction of papain's effect, uptake and release of the probes are affected by papain in the same direction, as shown for two examples in Fig. 4. Although our data were obtained under experimental conditions and with probes different from those used by Jennings and Adams [27], the transmembrane distribution of the binding sites on band 3 must have been the same in influx and efflux experiments, since in our experiments influx and efflux were mea-

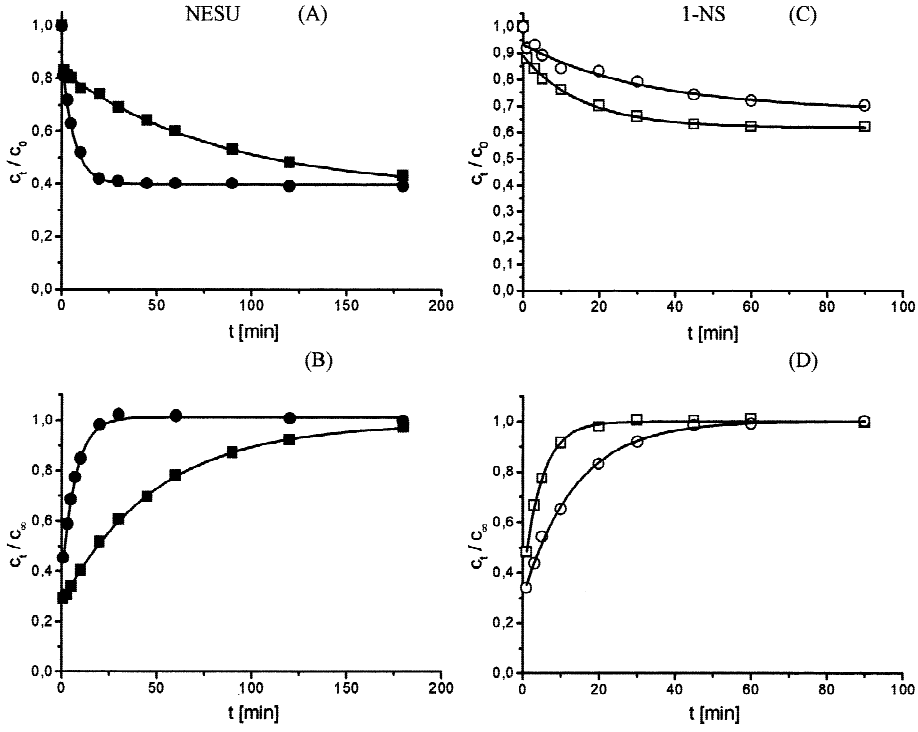

Fig. 4. Influx (*A,C*) and efflux (*B,D*) of amphiphilic sulfonates are equally influenced by papain treatment. ■, □ controls; ●, ○ cells pretreated with papain (1 mg/ml, 60 min, 37°C, pH 7.4).

sured in standard media containing chloride as the major anion on both sides of the membrane and at low levels of the test anions (*see* Table 2). The effect described by Jennings and Adams can therefore not underlie our observation of different responses of transport in dependence on the substrate anion studied following papain treatment.

One possible explanation for the oppositely directed, inhibitory or accelerating, effects of papain might be that different sites or steps of cleavage of band 3 are involved. To address this question, the dependence of both, inhibition and acceleration, on the applied concentration of papain was studied. Figure 5 demonstrates that inhibition and acceleration saturate, with the same concentration of about 0.5 mg papain/ml required for the half maximal effect. Figure 5 also demonstrates that the transport inhibition saturates at $k/k_o \sim 0.2$ in the case of our hydrophilic and small amphiphilic anions after saturating treatment with papain. We conclude from this observation that, as in the case of chloride [27], papain-modified band 3 still transports at about 20% of the control rate.

This correspondence supports the concept of a common event underlying inhibition and acceleration, most likely related to the formation of the 28 kD fragment by cleavage of human band 3 at Gln 630 [32, 50].

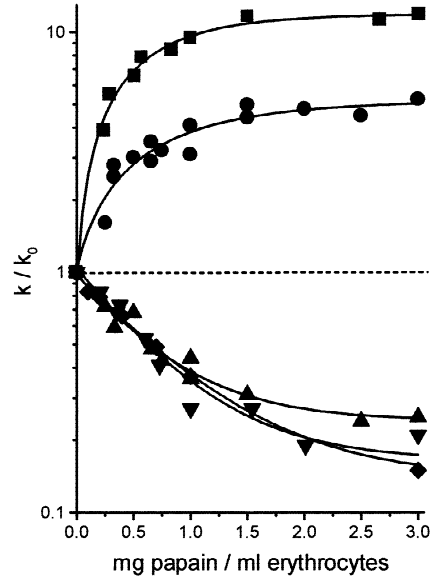

Fig. 5. Dependence, on the concentration of papain, of its inhibitory and stimulating effects on the normalized transport rate (k/k_0) of different anions. Cells pretreated with papain for 60 min, 37°C, pH 7.4. ■ NESU 10°C; ● NSU 6°C; ▲ 1-NS 10°C; ▼ MESNA 30°C; ♦ oxalate 15°C. Note the logarithmic scale of the ordinate!

Table 6. Influence of inhibitors on the band 3-mediated transport of small aliphatic dicarboxylates before (0) and after treatment of the cells with 1 mg/ml (P1) or 3 mg/ml (P3) papain for 1 hr at 37°C

Inhibitor [μM]	Oxalate (20°C)			Malonate (35°C)			Succinate (42°C)		
	$(k_I/k)_0$	$(k_I/k)_{P1}$	$(k_I/k)_{P3}$	$(k_I/k)_0$	$(k_I/k)_{P1}$	$(k_I/k)_{P3}$	$(k_I/k)_0$	$(k_I/k)_{P1}$	$(k_I/k)_{P3}$
Tetrathionate [2000]	0.27	0.25	0.33	0.42	0.50	0.58	0.57	0.70	0.85
Niflumate [20]	0.12	0.14	0.22	0.22	0.35	0.50	0.30	0.51	0.68
Erythrosin [15]	0.16	0.12	0.23	0.16	0.28	0.45	0.31	0.59	0.75
DNDS [7.5]	0.31	0.38	0.47	0.40	*n.t.*	0.59	0.50	*n.t.*	0.77

k: Rate constant for controls. k_I*:* Rate constant in presence of inhibitor. *n.t.:* not tested.

Inhibitor Sensitivity of Anion Transport Following Papain Treatment as a Function of Substrate Structure

Noncovalent Inhibitors

One of the characteristic features of anion transport via band 3 is its sensitivity to numerous noncovalently bound inhibitors, having in common not much more than their capacity to bind to biological membranes [11, 12, 14, 55]. It has been observed [27, 50], that after papain treatment the sensitivity of the transport of inorganic anions (chloride, sulfate and phosphate) to such inhibitors was diminished or even abolished. Long-chain amphiphilic anions translocated by flip-flop via band 3 behaved similarly [34, 61], in spite of a response to papain opposite to that of the inorganic anions. On the other hand, oxalate self-exchange via band 3, which exhibits many features characteristic for inorganic anion transport [31] remains fully sensitive to its inhibitors (including the small amphiphilic sulfonates (*data not shown*)) after a treatment of the cells with papain (Table 6), although papain inhibits the transport of oxalate, like that of the inorganic anions, up to about 80%.

In view of these discrepancies, we also studied the inhibitor sensitivities of the uptake of three small amphiphilic anions responding oppositely to papain treatment: NSU (stimulated by papain), 1-NS, and MESNA (both inhibited by papain) (Table 7). The four inhibitors used as examples in these experiments are considered to exert their effects via different mechanisms and binding sites: allosterically competitive (DNDS [59]), noncompetitive (erythrosin [39, 47], niflumate [7]) or mixed (tetrathionate [15, 55]).

In native cells ($(k_I/k)_0$ in Table 7), these four inhibitors had about the same effects on the transport of the amphiphilic anions as on the transport of oxalate (*cf.* Table 6), which responds like the inorganic anions. After treatment with papain at 1 and 3 mg/ml cells, however, all four inhibitors lost their effects on the transport of NSU as well as of 1-NS and MESNA to a major extent. Niflumate even accelerated the transport of these anions in papainized cells. Only weak inhibitory effects could be elicited at very high inhibitor concentrations. 100 μM DNDS (about 25 times the normal IC_{50}) produced 50% inhibition of the uptake of 1-NS, 500 μM induced 60% inhibition. These numbers do certainly not fit into a simple kinetic scheme. In the case of NSU , 500 μM even produced only 40% inhibition (*data not shown*).

Not unexpectedly, the self-inhibitory effects of the small amphiphilic sulfonates described above were also decreased in papain-treated cells. The half saturation constants increased more than 10-fold (*data not shown*).

To further increase the complexity of the situation, it also turned out (Table 6), that within the class of aliphatic dicarboxylates the sensitivity to inhibitors decreased following papain treatment, like the response to papain per se (Table 4) when the distance between the carboxylate groups was increased from oxalate to succinate. Quite evidently, the sensitivity of anion transport to noncovalently bound inhibitors varies after papain treatment, like the effect of papain itself, with the anion species under study. In contrast, DIDS, as a covalent inhibitor, was still fully inhibitory after papain in all cases we have studied. This finding clearly shows that the tested anions still migrate exclusively via band 3 in the papain-treated cells.

Anion Replacement Experiments

The heterogeneity in the response to modifying effects before and after papain treatment became also evident when chloride as the major anion in the suspension was replaced by other anions. It has long been known that in native erythrocytes replacement of chloride by nitrate inhibits, while replacement by sulfate (or methanesulfonate) enhances the self-exchange of inorganic anions and of oxalate [11, 68]. The same is true for the small amphiphilic anions (*data not shown*). Not unexpectedly, the effect of anion replacement proved to be unaffected by papain treatment in the case of oxalate. In contrast, both the inhibitory effect of nitrate as well as the enhancing effect of methanesulfonate were attenuated after papain pretreatment in the case of the small amphiphilic sulfonates (*data not shown*).

Table 7. Influence of inhibitors on the band 3-mediated transport of small amphiphilic anions before (0) and after treatment of the cells with 1 mg/ml (P1) or 3 mg/ml (P3) papain for 1 hr at 37°C

Inhibitor	NSU (6°C)			1-NS (10°C)			MESNA (30°C)		
[μM]	$(k_I/k)_0$	$(k_I/k)_{P1}$	$(k_I/k)_{P3}$	$(k_I/k)_0$	$(k_I/k)_{P1}$	$(k_I/k)_{P3}$	$(k_I/k)_0$	$(k_I/k)_{P1}$	$(k_I/k)_{P3}$
Tetrathionate [2000]	0.17	0.76	0.97	0.24	0.88	0.86	*n.t.*	*n.t.*	*n.t.*
Niflumate [20]	0.22	1.05	1.18	0.26	1.40	2.33	0.38	0.58	1.43
Erythrosin [15]	0.29	0.90	0.97	0.27	0.85	0.91	0.28	0.52	1.04
DNDS [7.5]	0.39	0.91	0.93	0.33	0.66	0.74	0.41	0.78	0.94

k: Rate constant for controls. k_I*:* Rate constant in presence of inhibitor. *n.t.:* not tested.

Covalent Modifiers

As reported above, the transport of all our test anions in papain-treated cells was fully sensitive to covalently bound DIDS. Sensitivity to covalently bound inhibitors is, however, not generally maintained after papain treatment. This became evident when we analyzed the effect of Woodward's reagent K (WRK) and of diethylpyrocarbonate (DEPC). WRK, a carboxyl-reactive isoxazolium sulfonate, binds, under appropriate conditions, to two exofacially accessible glutamates on band 3 [28]. One, on the 28 kD C-terminal domain, has been identified as Glu 681 and is thought to be involved in H^+-cotransport with divalent anions [30], but may have even more general significance for the function of AE1 [6, 60]. The localization of the other binding site of WRK, on the N-terminal 60 kD domain, has not yet been identified [28].

In native cells covalent binding of WRK inhibits the transport, via band 3, of hydrophilic, mono- and divalent anions [28] including oxalate (Table 8). Subsequent reductive cleavage of the adduct by borohydride, which converts the free γ-carboxyl group of Glu 681 into an alcohol [30] stimulates the transport of hydrophilic divalent anions, including oxalate (Table 8), while inhibiting the transport of hydrophilic monovalent anions [29]. Long-chain [34, 53, 61] and small monovalent amphiphilic anions also follow this pattern, i.e., 1-NS and NESU are inhibited by WRK and WRK/BH_4^- (Table 8).

In papain-treated cells, the response to WRK and WRK/BH_4^- again varied with the species of the transported anion. As shown in Table 8, fractional inhibition (by WRK) and acceleration (by WRK/BH_4^-) of oxalate transport were only slightly diminished after papain, indicating essential additivity of the two modifications. In contrast, both, the WRK-induced inhibition of 1-NS and NESU, as well as the WRK/BH_4^- induced inhibition of NESU, were markedly diminished after papain treatment, which by itself inhibits 1-NS but stimulates NESU uptake. A similar suppression of an inhibitory effect of WRK/BH_4^-, after papain has been reported recently for a long-chain amphiphilic sulfonate [53].

Table 8. Influence of covalent modifiers on the band 3-mediated transport of small hydrophilic and amphiphilic anions before (0) and after (P) treatment of the cells with 3 mg/ml papain for 1 hr at 37°C

Inhibitor	Oxalate		1-NS		NESU	
[mM]	$(k_I/k)_0$	$(k_I/k)_P$	$(k_I/k)_0$	$(k_I/k)_P$	$(k_I/k)_0$	$(k_I/k)_P$
WRK [2]	0.19	0.33	0.28	0.86	0.28	0.85
WRK/BH_4^- [2/4]	3.77	2.49	*n.t.*	*n.t.*	0.25	1.12
DEPC [5]	0.28	0.89	*n.t.*	*n.t.*	*n.t.*	*n.t.*
DEPC [10]	0.28	0.41	0.49	3.93	1.03	1.16
DEPC [20]	*n.t.*	*n.t.*	0.45	9.60	0.82	1.50

k: Rate constant for controls. k_I*:* Rate constant in presence of inhibitor. *n.t.:* not tested.

The reverse sequence of treatment (first WRK, then papain) provided the same results in the case of 1-NS. After a treatment with WRK, which inhibited 1-NS uptake by about 75%, papain treatment did not produce further inhibition, although papain still modifies band 3 under these conditions, as indicated by its persistent inhibitory effect on oxalate transport under the same experimental conditions (*data not shown*). A slowing-down, by WRK, of the proteolytic effect of papain has previously been reported [28], indicating some sort of coupling between the WRK binding site and the cleavage site of papain at Gln 630. In contrast, papain maintained its stimulating effect on the residual uptake of NESU after a treatment of the cells with WRK (*data not shown*), in line with similar observations for a long-chain amphiphilic anion [61].

Modification of AE1 by DEPC, either in erythrocytes [25] or after its expression in *Xenopus* oocytes [51, 52], inhibits anion (Cl^-, phosphate) transport by reacting with one or more histidine residues, in particular most likely His 734, a residue thought to be oriented to the inner face of the membrane [25] and to play a role in a network of H-bonds taking part in the process of anion transport [51, 52].

As evident from Table 8, oxalate transport was also markedly inhibited by DEPC. Pretreatment of cells with 3 mg/ml papain, which inhibits oxalate transport, re-

Table 9. Effects of butanol on the transport of different types of anions via band 3

Probe	k/k_0
Sulfate*	0.2
Oxalate	0.2
1-NS	1.5 (2)
2-NS	1.5 (2)
NSU	1.4 (2)
NMSU	1.5 (2)
NESU	2.6 (2)
NDSU	4.3 (2)
PS	2.5 (2)
BS	0.9 (2)
PMS	1.3 (3)
PESU	1.0 (2)
HBS	0.9 (2)
MESNA	0.8 (2)
SDS	1.8 (2)

* [18]. k_0: Rate constant for untreated cells. *k:* Rate constant for cells in the presence of 100 mM butanol. Number of experiments in parentheses.

duced this inhibition. This indicates either a lowered binding affinity of the inhibitor or an altered effect of the bound inhibitor on the conformation of the transport domain. Small amphiphilic sulfonates reacted differently. The uptake of 1-NS was inhibited by DEPC, as expected. After pretreatment with papain, however, which by itself acts inhibitory (*see above*), the residual transport of 1-NS was considerably accelerated by DEPC. The uptake of NESU was at best slightly inhibited by DEPC. After papain, which by itself stimulates (*see above*), DEPC produced some minor enhancement. In this respect, the behaviour of NESU again coincides, according to preliminary results (*data not shown*), with the behaviour of the flip of long-chain amphiphilic anions.

Butanol Treatment

Like papain treatment, the presence of butanol affects anion transport via band 3 in a direction depending on the nature of the anion probe. While the transport of hydrophilic, inorganic and small organic, anions has long been known to become inhibited [14, 18], the flip-flop of long-chain amphiphilic anions has recently been shown to be enhanced [34, 54, 57, 66]. This discrepancy corresponds to that observed with papain. We therefore also tested the effect of butanol on the transport of the small amphiphilic anions. As evident from Table 9, the band 3-mediated transport of these anions is indeed not inhibited strongly like the transport of oxalate or inorganic anions. We observed either a very slight inhibition (BS, HBS, MESNA) or a minor enhancement, qualitatively corresponding to the response of the transport of these anions to papain.

Discussion

The results presented above further specify and extend our earlier observation [34, 54, 61, 66] that papain treatment of human erythrocytes does not only inhibit the movements of anions via the anion exchanger (AE1), but can, in contrast, also accelerate this anion transport. The present data substantiate our previous demonstration [53], that these opposite effects of papain are independent of the direction of transport via AE1, and clearly indicate that not only the direction of the effect of papain, inhibition or acceleration, but also the extent of both effects depends on the type of anion studied. Moreover, our results suggest that, in contrast to our initial assumption [54], the opposite effects of papain are not related to the difference in the possible pathways of access of anions to their transport site, i.e., from the aqueous phase in the case of normal "fluxes", or from the lipid bilayer in the case of transport by "flip-flop".

In parallel with its dual effect on band 3-mediated anion transport per se, papain treatment also affects the sensitivity of this process to noncovalent inhibitors to an extent again depending on the anion species tested, but not on the direction of papain's effect. In the case of the small amphiphilic anions that we have studied, a major loss of transport sensitivity to inhibitors is observed after papain (Table 7), in line with earlier data for the DNDS-induced inhibition of sulfate influx [27] and for the inhibition, by various noncovalent inhibitors, of the flip of long-chain amphiphilic anions via band 3 [34, 61], but in contrast to oxalate transport. This process largely maintains its inhibitor sensitivity after papain, while the fluxes of its slightly larger homologues, malonate and succinate, become less sensitive with increasing intensity of papain treatment (Table 6).

The response of anion transport to changes of the anion milieu shows a comparable pattern. While the transport of small amphiphilic sulfonates becomes less sensitive to such changes in papain-treated cells, oxalate transport maintains the response of the untreated controls (Table 8). Similar, but even more complex differences characterize the effects of sequential application of papain and the covalent modifiers WRK and DEPC (Table 8).

Attempts to interpret our results in structural terms have to take into account the primary sequence and folding models of the bilayer-associated domain of AE1. Original models [48, 69] have proposed 14 transmembrane spans. A somewhat more complex organization of the membrane domain is indicated by recent studies, which suggest that the number of transmembrane spans may be lower than 14, or may comprise transmembrane

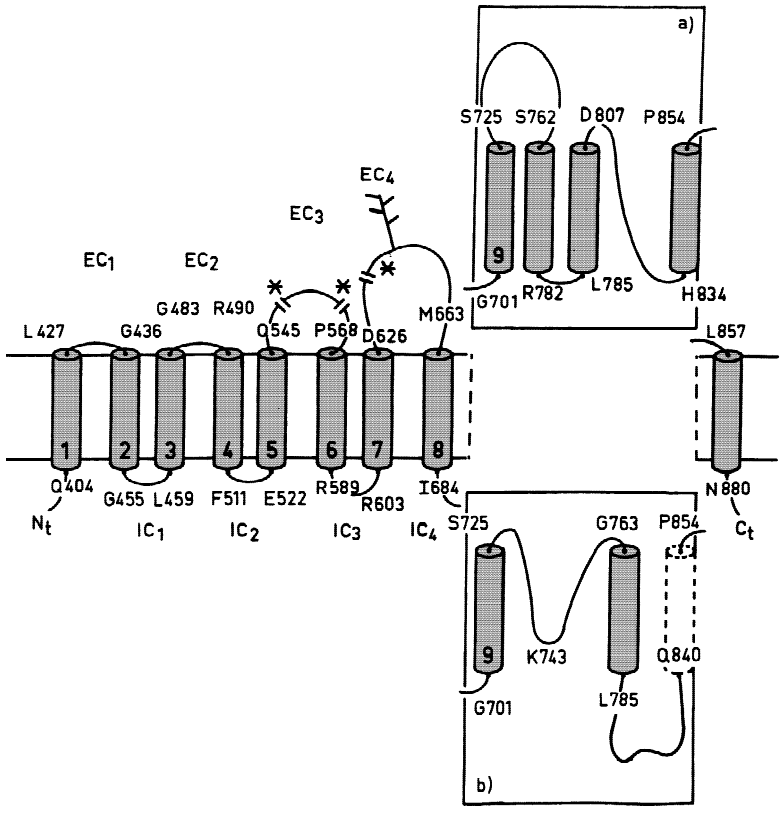

Fig. 6. Topology model of the membrane domain of the human erythrocyte anion transporter AE 1 (band 3) adapted from recent proposals in refs [21, 23, 56, 62]. Inserts (*a*) and (*b*) illustrate alternative concepts of the folding of the protein between Ser 725 and Pro 854 as proposed in [21, 62] (*a*) and [23, 56] (*b*). Positions given for bilayer entry and exit should be regarded as approximations. Sites of cleavage by papain are marked by asterisks.

stretches of the polypeptide chain not organized as transmembrane helices [21, 22, 23, 24, 56, 62, 63].

For the purpose of the following discussion the scheme given in Fig. 6 shows some essentials of the present concepts. The positions given on the polypeptide chain for its entry into and exit from the bilayer are approximations based on recent topological schemes [21, 56, 62, 63]. The stretch between residues Ser 725–Pro 854 is of particular interest. It seems to have, on the one hand, an unusual topology, and is considered, on the other hand, as a promising candidate for the yet unidentified substrate binding and translocation domain. The N-terminal residues of this stretch may be part of an exofacial vestibule leading to the substrate binding site [21], while the C-terminal end, which probably forms an intracellular loop, has been discussed as part of the transport-relevant binding site, in particular for small monovalent anions [1, 51, 52]. The intracellular loop IC 3 connecting TM 6 and TM 7 (Arg 589–Phe 608) may additionally be required for the exchange of divalent anions, e.g., sulfate [4] or oxalate, which are cotransported with H^+ in a process also involving Glu 681 in TM 8 [30]. The exofacial loop EC 4 (Asp 626–Met 663) connecting TM 7 with TM 8, which carries the N-glycosylation site (Asn 642), may also be a constituent of the anion transport machinery, possibly as a further component of the vestibule [62, 63]. Many mutations in this loop go along with a loss of transport activity [63].

Papain cleaves the membrane domain in two exofacial loops, EC 3 and EC 4, producing three fragments comprising transmembrane spans TM 1–5, TM 6–7, and TM 8–14 (in terms of the original 14 span model [69]), with the removal of a short peptide (Lys 551–Gln 564) from EC 3, which connects TM 5 with TM 6 [32]. Formation of these fragments by proteolytic cleavage in situ clearly affects the transport function of the protein, in somewhat surprising contrast to the recent observation by Groves et al. [24], that coexpression of these fragments, when generated by mutagenesis, gives rise to essentially normal, stilbene-disulfonate-sensitive transport of chloride in *Xenopus* oocytes. Groves et al. [24] have also provided indications that TM 6 and TM 7 are not essential for the transport of chloride, while no other transmembrane span can be omitted without loss of transport function. This finding, combined with the previous demonstration [32, 50] that the papain-induced cleavage of band 3 at Gln 630 in the putative exofacial loop EC 4 correlates with the papain-induced alteration of anion transport, supports hypotheses assigning the effect of papain to elements C-terminal from Gln 630. It may be speculated that the increased motional freedom of the new N-terminus of the cleaved loop EC 4 alters the conformation of the adjacent transport-mediating spans and loops in a way leading to the diverse alterations described in this work.

The question might of course be asked, whether

mechanisms other than mere backbone scission cause the transport effects of papain treatment. Two possible alternatives can be considered. First, the removal of the short peptide from residue Lys 551 to Gln 564 in EC 3 between TM 5 and TM 6, and second, the formation of net charges at the newly arising N- and C-termini of the membrane-bound proteolytic fragments. This latter event is not very likely to be a major factor since the corresponding fragments coexpressed in oocytes contain such additional charged termini without loss of transport function [24]. Testing the former possibility will require coexpression studies using either fragment TM 6–TM 14 truncated at its N-terminus or papain treatment of oocytes coexpressing fragments TM 1–TM 5 and TM 6–TM 14 analogous to the chymotrypsin treatment used to establish the surface localization of expressed fragments of AE1 [67].

Interestingly, papain also interferes with the effect of two covalent modifiers of anion transport, WRK, which reacts with Glu 681 in TM 8 near its cytoplasmic exit from the bilayer [30], and DEPC, which modifies histidines, in particular His 734, probably located not far from the exofacial exit of TM 9, but accessible from the cytoplasmic interface [25]. Both covalent modifications affect the transport of hydrophilic anions in a characteristic pattern [29, 30, 51, 52], also observed for the small amphiphilic anions (*see* Table 8). The flip of long-chain amphiphilic anions, while responding to WRK [34, 53, 61], is insensitive to treatment with DEPC (*data not shown*). The attenuation or even reversal of the effects of both covalent modifiers after treatment with papain (Table 8) suggests an allosteric coupling between the exofacial papain cleavage site and the probably endofacial binding sites of the two covalent modifiers.

The above discussion of the influence of papain on anion transport in terms of far-reaching conformational alterations of the protein can not yet provide a reasonable explanation for the opposite, inhibitory and enhancing, effects of a single proteolytic cleavage. This aspect can, however, be considered in terms of conceptual models of the functioning of the anion exchanger. According to Krupka [42], substrate specificity and transport velocity arise from the stability ("tightness") of the carrier-anion complex in its "activated state". This stability is related to the structure, conformation and internal mobility of the transport domain. Krupka also proposes different subsites for substrate binding in this transport domain, in order to account for the acceptance, as substrates, of structurally very diverse anions. Similar concepts have been put forward by Sekler et al. [60] on the basis of flux measurements in mutated AE1. These subsites must, however, be closely adjacent to each other or similar in structure, since they are equally sensitive to inhibitors of anion exchange via AE1 [43].

The presence of different "subsites", each defined as the particular assembly of amino acid side chains of AE1 involved in the formation of the activated state for a particular anion, may well account for the oppositely directed effects of papain treatment. We propose that propagated conformational changes in the transport domain of AE1, which follow the proteolytic cleavage, affect the subsites for different anions differently. The subsites involved in the transport of small hydrophilic (monovalent and divalent) and small amphiphilic anions are apparently rearranged by papain in a way which *impedes* the formation of the activated state, while the subsites responsible for the transport of larger and long-chain amphiphilic anions are rearranged in a way which *favors* the formation of an activated state. In cases where transport is insensitive to papain one would have to assume that the papain-induced change of conformation has no influence on the formation of the activated state.

The basic assumption, in this proposal, of a complex papain-induced change of conformation in band 3 is also supported by the differing changes in sensitivity (Table 6 and 7) of the various classes of anions to noncovalent inhibitors supposed, as outlined above, to act by allosteric mechanisms. The decrease in self-inhibitory potency of the amphiphilic sulfonates after papain treatment reported above appears of particular interest in this context. This decrease may not only enhance the papain-induced stimulation of the transport of some of these anions, but also diminish the inhibition of others. The available techniques to measure the transport rates of these anions preclude at present a more detailed investigation of this hypothesis.

It remains to be seen, how the varying extent to which the inhibitor sensitivity of the transport of different anions is diminished after papain treatment (Table 6 and 7) can be incorporated into the hypothesis outlined above. Since at least many of the noncovalent inhibitors probably act by allosteric mechanisms, one may assume that the allosteric coupling between the inhibitor binding sites and the putative subsites for the different classes of anions is affected to a different degree by the changes of conformation caused by papain.

Our finding that the inhibitor sensitivity of at least one anion, oxalate, is barely affected by papain treatment, while the sensitivity of other anions responds to a varying degree, also supports the view that the altered inhibitor sensitivities do not arise from an altered affinity of the inhibitor binding sites for their ligands, but from an altered (mainly less efficient) allosteric coupling between the inhibitor binding sites and the different anion transport subsites. How the papain-induced proteolytic cleavage of an exofacial loop of the protein affects an allosteric coupling along and between transmembrane spans remains to be clarified.

In some contrast to our interpretation, Lieberman and Reithmeier [46] have reported that the affinity of

band 3 for BADS, a noncovalent, formally competitive, stilbene disulfonate inhibitor, is greatly reduced after papain treatment. A similar effect has been reported for flufenamate, a noncompetitive inhibitor of anion exchange [8]. On the other hand, the closely related inhibitors DNDS and niflumate behave according to our concept, i.e., both inhibit oxalate transport with unaltered efficiency in papain-treated cells (Table 6) in spite of losing efficiency against the transport of chloride [27] and other anions (*see above*). This inconsistency remains to be resolved.

A final observation that deserves comment concerns the variable effects of butanol on the transport of the various types of anions via band 3, which qualitatively resemble those of papain. Combined with earlier evidence for alkanol-induced conformation changes in band 3 [18, 41], our findings suggest that butanol, and probably other alkanols, affect the subsites for different classes of anions in a way comparable to that responsible for the alteration by papain.

In conclusion, we have reported new data on anion transport via band 3 (AE1) supporting the concept of different binding subsites for different classes of anions and demonstrating the remarkable extent and diversity of the allosteric reactions, within the large but firmly coherent membrane domain of this protein, to the seemingly minor modification of interrupting, by papain, the continuity of the primary sequence at an exofacial site. We can also conclude, that the oppositely directed effects of papain treatment, i.e., inhibition *vs.* stimulation of transport, are not coupled to the putatively different pathways of substrate access to the transport sites on AE1 either from the aqueous phase, inducing "flux", or from the lipid bilayer, inducing "flip-flop". The different responses to papain are most likely related to structural features of the substrate anions leading to their interaction with differing binding subsites on AE1.

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