Biochimica et Biophysica Acta, 687 (1982) 147–155
Elsevier Biomedical Press

BBA 71114

INHIBITION OF ANION TRANSPORT IN THE RED BLOOD CELL BY ANIONIC AMPHIPHILIC COMPOUNDS

I. DETERMINATION OF THE FLUFENAMATE-BINDING SITE BY PROTEOLYTIC DISSECTION OF THE BAND 3 PROTEIN

J.-L. COUSIN and R. MOTAIS

Groupe de biologie marine du Département de biologie du Commissariat à l'Energie Atomique, Station Marine, B.P. No. 68, 06230 Villefranche-sur-Mer (France)

(Received December 8th, 1981)

Key words: Anion transport; Cl^- transport; Band 3 protein; Flufenamic acid; Proteolysis; Binding site; (Human erythrocyte)

Flufenamate, a non-steroidal anti-inflammatory drug, is a powerful inhibitor of anion transport in the human erythrocyte ($I_{50} = 6 \cdot 10^{-7}$ M). The concentration dependence of the binding to ghosts reveals two saturable components. [^{14}C]Flufenamate binds with high affinity ($K_{d_1} = 1.2 \cdot 10^{-7}$ M) to $8.5 \cdot 10^5$ sites per cell (the same value as the number of band 3 protein per cell); it also binds, with lower affinity ($K_{d_2} = 10^{-4}$ M) to a second set of sites ($4.6 \cdot 10^7$ per cell). Pretreatment of cells with 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), a specific inhibitor of anion transport, prevents [^{14}C]flufenamate binding only to high affinity sites. These results suggest that high affinity sites are located on the band 3 protein involved in anion transport. Extracellular chymotrypsin and pronase at low concentration cleave the 95 kDa band 3 into 60 kDa and 35 kDa fragments without affecting either anion transport or [^{14}C]flufenamate binding. Splitting by trypsin at the inner membrane surface of the 60 kDa chymotryptic fragment into 17 kDa transmembrane fragment and 40 kDa water-soluble fragment does not affect [^{14}C]flufenamate binding. In contrast degradation at the outer membrane surface of the 35 kDa fragment by high concentration of pronase or papain decreases both anion transport capacity and number of high affinity binding sites for [^{14}C]flufenamate. Thus it appears that 35 kDa peptide is necessary for both anion transport and binding of the inhibitors and that the binding site is located in the membrane-associated domain of the band 3 protein.

Introduction

The anion transport across the erythrocyte membrane has been shown to be inhibited by various chemically unrelated compounds [1–5]. However it can be observed that they are all amphiphilic compounds. Structure-activity studies performed with several sets of congeners showed that inhibitory potency is mainly correlated with hydrophobic character of the molecules and electron withdrawing ability of the substituents [6–11]. At the same time it appeared that the band 3 protein which is involved in anion transport [12–14] could be the site of action of certain of these molecules [15]. In a more detailed study performed with a non-steroidal anti-inflammatory drug, niflumate, which is a powerful reversible inhibitor, we gave convincing evidence that this drug binds

Abbreviations: SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; H_2DIDS, dihydro analog of DIDS; EDTA, ethylenediaminetetraacetic acid. Throughout this paper albumin refers to bovine serum albumin. kDa, kilodalton.

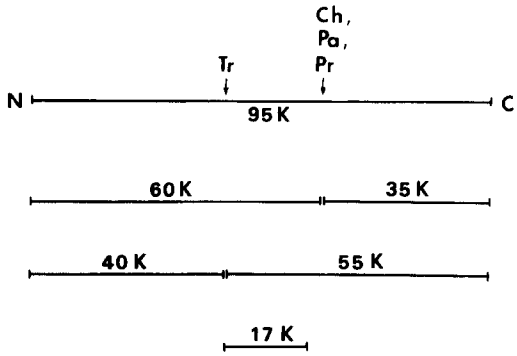

Fig. 1. Summary of major proteolytic cleavages in the band 3 protein. N, NH_2-terminal; C, COOH-terminal; Ch, Pa, Pr, respectively chymotrypsin, papain, pronase cleavage site outside cell; Tr, trypsin cleavage site inside cell; K, kDa adapted from Ref. 45.

to the band 3 protein [16]. The present work deals with an attempt to obtain information about the localization of the binding site of amphiphilic compounds on band 3 by proteolytic digestion of the protein. Indeed this transmembrane protein can be cleaved by proteolysis of erythrocytes or ghosts at well defined loci (Fig. 1; Refs. 17–23). Externally applied chymotrypsin and pronase (at low concentration) cleave the band 3 protein into 60 kDa and 35 kDa integral fragments without affecting anion transport [14,20]. Trypsin treatment, at the cytoplasmic face of the membrane obtained from chymotrypsinized cells produces 17 kDa and 35 kDa membrane-associated fragments and removes a 40 kDa water-soluble fragment at the cytoplasmic face of the protein [21,24,25]. Removal of this fragment has little effect on anion transport [26–28]. This suggests that anion transport is carried out by the membrane-associated domain of the band 3 protein. Since 17 kDa peptide contains most of the binding sites for 1-isothiocyanobenzene sulfonate [22] and H_2DIDS [27] which compete with Cl^- for the transport site [29], it appears that this peptide is essential for anion transport. But 35 kDa peptide also participates in the control of transport since digestion of this peptide by papain is accompanied by inhibition of anion transport [30].

In this context we have evaluated in parallel the effects of proteolytic treatments of the band 3 protein on anion transport and reversible binding of flufenamate. This study was performed with flufenamate instead of its very close analog niflumate used in precedent paper because we had at our disposal the radioactive form of flufenamate. The results show that both drugs have the same binding pattern to erythrocyte membrane: high affinity binding sites protectable by SITS (presumably band 3) and low affinity binding sites. The binding is not alterated by proteolytic treatments which do not inhibit chloride self-exchange. Conversely, digestions which inhibit anion permeability affect the number of high affinity sites in the same ratio.

In the accompanying paper [50] we report a structure activity study with flufenamate related compounds.

It has been possible to define the role of each part of the molecule in the binding to the site and to obtain information about the chemical properties of the inhibitor recognition site.

Materials and Methods

Red blood cells. Human red cells collected into citrate dextrose solution was obtained from the blood bank and stored at 4°C. Cells were prepared by washing three times in phosphate-buffered saline (150 mM NaCl/5 mM sodium phosphate, pH 7.4). SITS treatment was carried out as described previously [16]. Enzymatic digestion of the outer membrane surface was carried out in phosphate-buffered saline at 37°C for 1 h at 50% hematocrit 1 mg/ml chymotrypsin (α-chymotrypsin from bovine pancreas; Sigma) 0.3 mg/ml papain (from *Papaya carica*; Boehringer) or at 37°C for 20 min at 30% hematocrit 0.2 or 5 mg/ml pronase (Calbiochem B grade). Papain was dialysed 24 h against phosphate-buffered saline and was activated by incubating stock solution of 300 μg/ml in 5 mM dithiothreitol/1 mM EDTA just prior to use. Digestions were terminated with diisopropyl phosphofluoridate (for chymotrypsin; 0.25 mM final) or iodoacetic acid (for papain; 5 mM final). Cells were washed twice at room temperature in 10 vol. bovine serum albumin (three times after pronase treatment) and twice in 10 vol. phosphate-buffered saline.

Erythrocyte ghosts. Hemoglobin free erythrocyte ghosts were prepared according to Fairbanks et al. [31] but using 10 mM sodium phosphate instead of

5 mM. For trypsin treatment of the unsealed ghosts, one volume of packed ghosts was resuspended with an equal volume of phosphate buffered saline containing 100 μg/ml trypsin (from bovine pancreas; Sigma) for 30 min at 37°C. Digestion was terminated with soy bean trypsin inhibitor (Worthington biochemical Co; 200 μg/ml final). The ghosts were centrifuged and washed in 150 mM NaCl and then 'stripped' in 20 vol. 0.1 M NaOH at 0°C [32], centrifuged and washed once in 20 vol. water at 0°C.

Analytical techniques. Electrophoresis was performed on gels containing 5% acrylamide and 0.2% dodecyl sulfate following the procedure of Fairbanks et al. [31] modified by Steck and Yu [32].

Protein was determined according to the method of Lowry et al. [33]. Total phosphorus (P_i) was determined by the method of Razzel [34].

^{36}Cl flux measurements. Chloride equilibrium exchange was measured at 0°C in phosphate-buffered saline (pH 7.4) at 0.5% hematocrit using the previously described automatic technique [35].

[^{14}C]Flufenamate binding to unsealed ghosts. Ghost suspension with a known equivalent cell concentration was prepared by diluting ghosts pellet (of which we measured P_i content) in ice cold 155 mM sodium phosphate (pH 7.4). One volume of this suspension was added to an equal volume of ice-cold sodium phosphate containing various concentrations of [^{14}C]flufenamate and stored 20 min at 0°C. Total [^{14}C]flufenamate concentration was determined on an aliquot of the suspension which was then centrifuged 20 min at 0°C (Sorvall RC_2B, rotor SS 34, 20 000 rev./min) and [^{14}C]flufenamate concentration measured in the supernatant (free drug concentration). Bound drug was calculated from total and free concentrations and expressed as the number of bound molecules per equivalent cell. The data were plotted according to the method of Scatchard [36].

Since in proteolysed membranes protein determination is unsuited to estimate, as usual, the number of equivalent cells, we determined total phosphorus in the membrane assuming that it was not affected by proteolysis. We used $2.60 \cdot 10^9$ cells/μmol P_i as a constant factor relating total phosphorus and number of equivalent cells. This value was determined as follows: we measured in parallel total phosphorus and membrane protein contents in membranes from control cells and found 0.750 μmol P_i/mg of membrane protein. This can be converted into total phosphorus per cell using $1.95 \cdot 10^9$ cells/mg of membrane protein [37] giving $2.60 \cdot 10^9$ cells/μmol P_i.

[^{14}C]Flufenamate was purchased from the Centre d'Etudes Nucléaires, Saclay, France.

Results

[^{14}C]Flufenamate binding to the erythrocyte membrane

The binding of [^{14}C]flufenamate to the human erythrocyte membrane was performed over a large concentration range. The data were plotted according to the method of Scatchard [36]. Fig. 2 shows two components. Assuming that these represent two classes of independent sites, the number of flufenamate binding sites (n) and the dissocia-

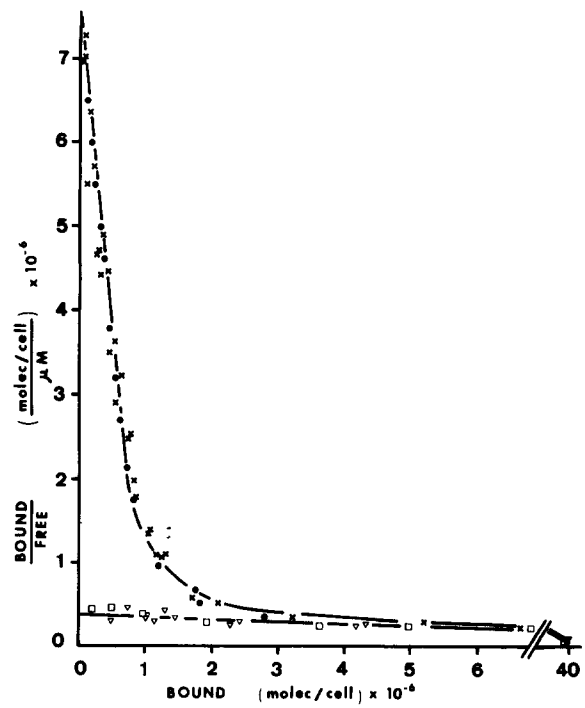

Fig. 2. Scatchard plot of [^{14}C]flufenamate binding to hemoglobin-free unsealed ghosts (●, × two different experiments) and ghosts from cells treated by SITS (▽, □ two different experiments). Ordinate: bound flufenamate (10^5 molecules per equivalent cell) divided by concentration of free drug (μM). Abscissa: bound flufenamate (10^5 molecules per equivalent cell).

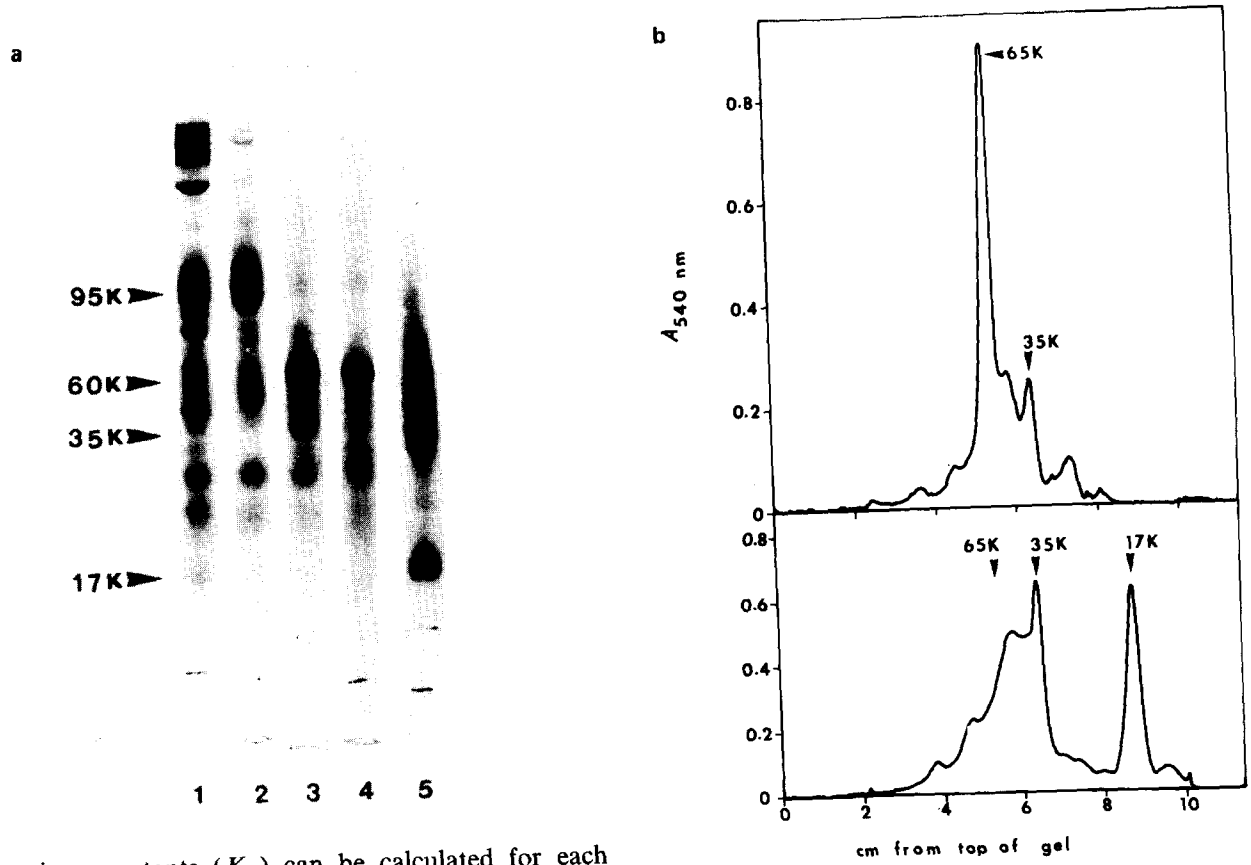

Fig. 3. Effects of proteolytic enzymes on the band 3 protein. (A) Electrophoretograms of membranes proteins stained with Coomassie blue: Except for control sample (gel 1) all the ghosts samples were 'stripped' by alkaline treatments. Gel 1, ghosts obtained from untreated cells. Gel 2, ghosts obtained from untreated cells and than 'stripped'. Gel 3, ghosts obtained from chymotrypsin-treated cells. Gel 4, ghosts obtained from cells sequentially treated with chymotrypsin and papain. Gel 5, ghosts obtained from chymotrypsinized cells subsequently trypsinized. K, kDa. (B) Scans (A at 540 nm) of gels 3 (upper scan) and 5 (lower scan).

tion constants (K_d) can be calculated for each population [38,39].

Strikingly, the number of high affinity sites ($n_1 = 0.85 \cdot 10^6$) is close to the number of band 3 copies per cell i.e. 10^6 [40] and the apparent dissociation constant ($K_{d_1} = 1.2 \cdot 10^{-7}$ M) is close to the I_{50} for the inhibition of anion self-exchange ($I_{50} = 5.3 \cdot 10^{-7}$ M). We also performed binding experiments with membranes of cells pretreated by SITS as described in Materials and Methods. SITS is one of the stilbene disulfonate derivatives which are known to be specific inhibitors of anion transport and bind specifically to band 3 protein. Under our conditions SITS was covalently bound to such an extent that chloride self-exchange was fully inhibited. After this treatment the first population disappears whereas the second class is not affected (open signs Fig. 2). These results suggest that the population 1 represents the binding sites of flufenamate to the band 3 protein involved in the control of anion transport. This agrees with our previous results with niflumate, a structural analog of flufenamate [16]. The flow affinity sites are much more numerous: $46 \cdot 10^6$ per cell with an apparent dissociation constant of 10^{-4} M. This suggests that membrane lipids participate in this class of binding sites.

The action of chymotrypsin on anion transport and the protein in band 3

In Figs. 3 and 4, except sample 1 (control), all the membrane samples were treated with 0.1 M NaOH before electrophoresis. This treatment ('stripping') elutes most of the weakly associated membrane proteins [32] making the integral membrane proteins or fragments more apparent.

Sample 3 in Fig. 3 shows that external chymotrypsin cleaves the 95 kDa band 3 into 60 kDa and 35 kDa fragments as previously reported by several laboratories [14,19–21]. These bands are still present on the gel after stripping indicating that they are integral membrane peptides [32]. It has been suggested by Grinstein et al. [27] that the 35 kDa fragment is susceptible to degradation by external chymotrypsin. We performed proteolysis in various conditions including those used by the authors, but we never observed further degradation of the 35 kDa fragment; This agrees with the results of Jennings and Passow [30]. Since the band 3 can be regenerated by H_2DIDS cross-linking of the 60 kDa and 35 kDa fragments even after extensive external chymotryptic proteolysis, these authors concluded that both fragments are present in the membrane in equimolar amounts.

We also tested the effects of chymotryptic cleavage on the chloride self-exchange across the membrane, the inhibitory activity of flufenamate and the binding of this drug to the high affinity sites considered as located on the band 3 protein (see above). The results summarized in Table I show: (1) Chymotrypsin cleavage of the band 3 does not lead to an inhibition of anion transport as also found by other workers [14,20]. (2) Flufenamate still continues to act as an inhibitor in the chymotrypsinized erythrocytes with the same inhibitory potency (I_{50} unchanged). (3) The number of flufenamate high affinity sites per cell n_1 and the apparent dissociation constant K_{d_1} are not affected by chymotrypsin treatment of eryhtrocytes. These observations suggest that the native conformation of the protein is not drastically altered by chymotryptic cleavage.

Double digestion of the band 3 protein by chymotrypsin and trypsin

Unsealed ghosts derived from chymotrypsin-digested erythrocyte were exposed to trypsin and then stripped with NaOH. The electrophoretogram (Fig. 3 sample 5) shows two major bands with apparent molecular weights of 35000 and 17000. The 35 kDa fragment is identical in appearance with that produced by chymotrypsin alone, indicating that it is resistant to trypsin. The 17 kDa band represents what is left of the 60 kDa chymotryptic fragment after removal of a water-soluble 40 kDa fragment from the cytoplasmic surface [21]. The 35 kDa band overlaps a smear extending from 30 to 70 kDa. This smear contains a large fraction 4.5

TABLE I

EFFECTS OF ENZYMATIC TREATMENTS ON CHLORIDE SELF-EXCHANGE IN HUMAN RED CELLS, INHIBITION BY FLUFENAMATE AND FLUFENAMATE BINDING TO UNSEALED GHOSTS

Cells were exposed to enzymes as described in Materials and Methods. In each case, a portion of cells was used for measurement of $^{36}Cl^-$ self-exchange and the molar concentration of flufenamate producing a 50% reduction of ^{36}Cl self-exchange was determined (I_{50}). Another portion of the cells was used for the preparation of ghosts. In one case ghosts were treated with trypsin; [^{14}C]flufenamate-binding experiments were then performed with a portion of ghosts. In each case dissociation constant and number of binding sites per cell were determined as in Fig. 1. The other portion of ghosts was 'stripped' with 0.1 M NaOH. After solubilization in sodium dodecyl sulfate, electrophoresis was performed in 5% polyacrylamide gel (0.2% SDS)

Proteolytic treatment	Choride self-exchange		Flufenamate binding	
	% control	Flufenamate I_{50} (M)	No. of sites (% of control)	Dissociation constant K_{d_1} (M)
Control	100	$5.8 \cdot 10^{-7}$	100	$1.2 \cdot 10^{-7}$
Chymotrypsin	95	$6.1 \cdot 10^{-7}$	86	$1.2 \cdot 10^{-7}$
Chymotrypsin + papain	39	$4.4 \cdot 10^{-7}$	42	$0.9 \cdot 10^{-7}$
Chymotrypsin + trypsin			100	$1.2 \cdot 10^{-7}$
Pronase (0.2 mg/ml)	100	$6.3 \cdot 10^{-7}$	95	$1.4 \cdot 10^{-7}$
Pronase (5 mg/ml)	47	$7.9 \cdot 10^{-7}$	42	$1.4 \cdot 10^{-7}$

region of the band remaining after alkaline treatment and overlaps the non-digested fraction of the 60 kDa fragment (Note that about twice as much protein was put on the gel 5 as on the others). In this case it is difficult to appreciate the completeness of the tryptic digestion, the different fractions can be distinguished more easily on densitometric scans. Comparison of quantitative integrated peaks of gels 3 and 5 reveals that more than 80% of the 60 kDa fragment was digested by trypsin.

We also performed external chymotrypsin proteolysis followed by proteolysis of unsealed ghosts by chymotrypsin instead of trypsin. In both cases we observed the same electrophoretograms.

These treatments did not affect either the number of binding sites or the affinity of flufenamate (Table I). This indicates that the cytoplasmic 40 kDa fragment does not play an essential role in the binding of flufenamate. Likewise removal of this fragment does not affect anion transport [27]. Thus the binding site, like the transport site is located in the membrane-associated domain of the band 3 protein.

Double digestion of the band 3 protein by chymotrypsin and papain

Intact cells were sequentially exposed to chymotrypsin and papain. An electrophoretogram of stripped membranes shows only one major band of approx. 60 kDa (sample 4 in Fig. 3). This fragment is apparently identical to the 60 kDa fragment obtained with chymotrypsin alone (see Jennings and Passow [30]). After papain, the 35 kDa chymotryptic band is no longer visible on the gel indicating a degradation of this fragment from the outer membrane surface by papain. A small amount of this fragment could be present on the gel but not seen because this glycosylated fragment is poorly stained with Coomassie blue. Similarly, we observed only the 60 kDa fragment with external papain alone.

It is unclear why degradation is more extensive than the splitting of the 35 kDa fragment into a 30 kDa fragment reported by Jennings and Passow [30].

Table I shows that exposure of the chymotrypsinized cell to external papain decreases the rate of chloride efflux to 39% of its value in control cells. The number of flufenamate binding

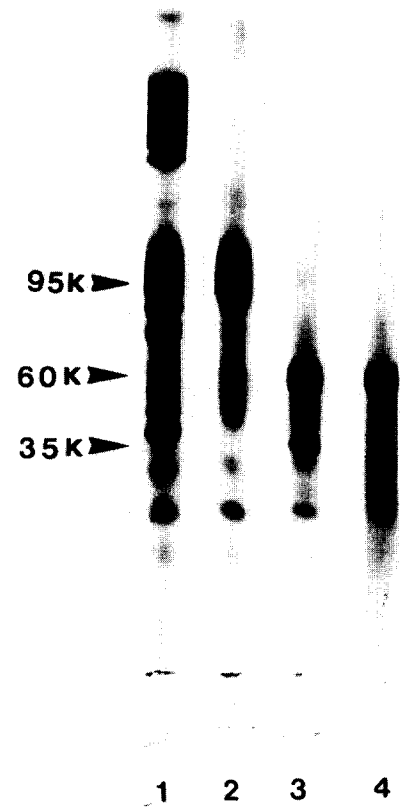

Fig. 4. Effect of pronase on the band 3 protein. Excepi for control sample (gel 1) all the ghosts samples were 'stripped' by alkaline treatment. Gel 1, ghosts obtained from untreated cells. Gel 2, ghosts obtained from untreated cells and then 'stripped'. Gel 3, ghosts obtained from cells treated with a low pronase concentration (0.2 mg/ml). Gel 4, ghosts obtained from cells treated with a high pronase concentration (5 mg/ml). K, kDa.

sites per cell in component 1 is reduced to 42% of the original value. The resting sites in component 1 accomodate flufenamate with an unaffected affinity (K_{d_1} unchanged) and the drug inhibits the residual chloride efflux from the cells with the same potency (I_{50} unchanged). This suggests that the inhibition of anion transport and the reduction of the number of inhibitor binding sites produced by papain may be related to the digestion of the 35 kDa fragment.

Digestion of the band 3 by pronase

In our experimental conditions (37°C; pH 7.4; 30% hematocrit for 20 min) 0.2 mg/ml pronase, at the outer surface of the membrane, splits the band 3

protein into 60 kDa and 35 kDa peptides (Fig. 4). This enzyme concentration does not affect chloride transport, the number of flufenamate binding sites in component 1, the affinity of the inhibitor for the binding sites and its inhibitory activity (Table I). This action of low pronase concentration is similar to that of chymotrypsin. In contrast to chymotrypsin, but like papain after chymotrypsin, at high pronase concentration (5 mg/ml), the 35 kDa fragment is further degradated, anion transport is reduced to 40% of the control value and the number of flufenamate binding sites decreases to 42% of the original value. This high pronase concentration does not affect the susceptibility of the residual flux to flufenamate nor its affinity for the resting sites in component 1 of the Scatchard plot. Thus 35 kDa peptide appears essential to the control of anion transport.

Discussion

The amphiphilic compound flufenamate (a non-steroidal anti-inflammatory drug) is a strong reversible inhibitor of anion transport (50% inhibition of chloride self-exchange is achieved with $5 \cdot 10^{-7}$ M of the drug). The Scatchard plot shows that the erythrocyte membrane exhibits two classes of [^{14}C]flufenamate binding sites. Results presented above suggest that the inhibitory activity of the drug is related to its binding to high affinity sites (component 1) and that these sites are located on the band 3 protein involved in anion transport. This conclusion is also supported by the observation that inhibition of anion transport by proteolytic fragmentation of band 3 is accompanied by a reduction of flufenamate binding sites in component 1 of the Scatchard plot.

The number of binding sites in component 2 of the Scatchard plot suggests that participation of the membrane lipids in this class of binding sites. This is also supported by the observation that this class corresponds to a concentration range in which the drug induces strong morphological change and protects erythrocytes against hypotonic hemolysis (Cousin and Baroin, unpublished data; Ref. 41) suggesting an adsorption of the molecules on the lipid phase of the membrane [42,43].

One implication of our binding parameters is that the binding of flufenamate to band 3 has only a single component. Now the band 3 protein is present in the membrane as a non-covalent dimer [46–48] and there exist data suggesting that binding of DBDS (4,4′-dibenzamido-stilbene-2,2′-disulfonate an analog of DIDS) to one subunit reduces affinity for binding to the other [49]. It is possible that such a negative cooperativity in binding of flufenamate exists but it is difficult to demonstrate because of the presence of the second class of sites containing numerous sites.

Externally-applied pronase at low concentration and chymotrypsin cleave the 95 kDa band 3 protein into peptides of 60 kDa and 35 kDa [14,17,19–21] without affecting either anion transport, the inhibitory activity of flufenamate, its binding pattern to the high affinity binding sites (presumably band 3) or the inhibitory activity of DIDS [27]. This suggests that the digestion does not drastically affect the native conformation of the protein with respect to anion transport and binding of the inhibitors and agrees with the observations that this treatment does not affect the covalent binding of H_2DIDS and the subsequent inhibition of anion transport [27,30].

Under these conditions there is no detectable change in 60 kDa peptide but the 35 kDa peptide is digested. These observations suggest that the 35 kDa fragment is involved in the control of anion transport and binding of flufenamate. Although, in our experiments, papain cleaved the 35 kDa chymotryptic fragment into much smaller fragments than the 30 kDa peptide found by Jenning and Passow [30], our results are nevertheless compatible with their proposition. These authors showed that the bifunctional reagent H_2DIDS, that binds at the transport site [29] can cross-link the 60 kDa and 35 kDa fragments but that no cross-linking can be achieved when the 35 kDa fragment is digested with papain. They conclude 'that a portion of this peptide participates, together with a portion of the 60 kDa fragment, in the control of anion transport'. However this does not agree with part of the work of Grinstein et al. [27] who reported a degradation, by external chymotrypsin, of the 35 kDa fragment without inhibition of anion transport. They conclude that this fragment does not contribute to the control of anion transport. We however, as did Jennings and Passow [30], always observed the 35 kDa fragment

on the gels after external chymotryptic proteolysis even under drastic conditions. Trypsinization of unsealed ghosts derived from chymotrypsinized cells cleaves the 60 kDa peptide into 17 kDa integral fragment and 40 kDa water-soluble fragment but is without effect on the 35 kDa chymotryptic fragment [21]. Removal of the 40 kDa fragment does not affect anion transport in inside-out vesicles [26,27] and flufenamate binding (Table I) suggesting that this fragment is not very important either for the structure or for the mechanism of the anion transport system. The observation that binding of covalent reagent to this fragment does not affect transport [44] supports this view.

The fact that the 17 kDa fragment contains most of the H_2DIDS binding sites [27] and that H_2DIDS competes with Cl^- for the transport site [29] suggests that this fragment participates in the structure of the transport site.

In conclusion, the transport site is located on the membrane-associated domain of the band 3 protein and its functional structure needs both the 17 kDa and the 35 kDa fragments (but not the 40 kDa water-soluble fragment). This work shows that the same conclusion can be drawn for the flufenamate binding site.

Three possibilities remain however: (1) the flufenamate-binding site is located on the 35 kDa peptide, (2) this fragment participates with the 17 kDa peptide in the binding site, (3) the 35 kDa fragments is necessary to maintain the structural organization of the site located on the 17 kDa fragments. Furthermore, we showed that niflumate and SITS are mutually exclusive inhibitors. Thus, there exists a close correlation between the modification of the transport site by proteolysis or by irreversible inhibitors and the modification of the binding site of flufenamate and niflumate. The sites appear distinct since niflumate does not compete with Cl^- [16] but they must be in allosteric interaction.

Some of the treatments performed in the above experiments are known to produce a mixture of right-side and inside-out vesicles. If flufenamate binds only to one face of the membrane, the binding determination could be affected by the sidedness of the different membrane preparations. Another possibility is that the membrane permeability of flufenamate is very high making the binding sites accessible whatever the sidedness of flufenamate action and of different membrane preparations.

We therefore attempted to measure the time course of the efflux of flufenamate from [^{14}C]flufenamate-loaded cells (10^{-3} M of drug; hematocrit 50%; 0°C, pH 7.4 for 5 h) into a flufenamate-free medium. The concentration of radioactive isotope in the external medium reached a plateau in less than 2 s after mixing and was stable for more than 30 min. In fact isotope equilibration represents both the release of molecules bound to the membrane and exit of flufenamate through the membrane. Nevertheless it is clear that the membrane permeability of this drug is considerable, which can be explained by the very high lipophilic character of the molecule (see Ref. 50).

This observation shows that the sidedness of flufenamate cannot be determined by this method but indicates that in our binding experiments flufenamate had access to the binding sites whatever the sidedness of the membrane preparation and the sidedness of the drug action.

Acknowledgements

The technical assistance of Ms. Corinne Raschi is gratefully acknowledged. This work was supported by the Délégation Générale à la Recherche Scientifique et Technique (No. 75-7-0052) and by the Centre National de la Recherche Scientifique (ERA No. 495).

References

1 Deuticke, B. and Gerlach, E. (1967) Klin. Wochenschr. 45, 977–983
2 Passow, H. and Schnell, K.F. (1969) Experientia 25, 460–468
3 Wieth, J.O. (1970) J. Physiol. (Lond.) 207, 581–609
4 Gunn, R.B. and Tosteson, D.C. (1971) J. Gen. Physiol. 57, 593–609
5 Schnell, K.F. (1972) Biochim. Biophys. Acta 282, 265–276
6 Motais, R. and Cousin, J.-L. (1976) Am. J. Physiol. 231, 1485–1489
7 Motais, R. and Cousin, J.-L. (1977) Proc. Int. Union Physiol. Sci. 13, 530
8 Motais, R. and Cousin, J.-L. (1977) in International Conference on Biological Membranes: Drugs, Hormones and Membranes (Bolis, L., Hoffman, J.F. and Straub, R.W., eds.), pp. 219–225, Raven Press, New York

9 Motais, R., Sola, F. and Cousin, J.-L. (1978) Biochim. Biophys. Acta 510, 201–207
10 Cousin, J.-L. and Motais, R. (1978) Biochim. Biophys. Acta 507, 531–538
11 Barzilay, M., Ship, S. and Cabantchik, Z.I. (1979) Membrane Biochem. 2, 227–254
12 Ho, M.K. and Guidotti, G. (1975) J. Biol. Chem. 250, 675–683
13 Cabantchik, Z.I. and Rothstein, A. (1974) J. Membrane Biol. 15, 207–226
14 Passow, H., Fasold, H., Lepke, S., Pring, M. and Schumann, B. (1977) in Membrane Toxicity (Miller, M.W. and Shamoo, A.E., eds.), pp. 353–379, Plenum Press, New York
15 Zaki, L., Ruffing, W., Gärtner, E.M., Fasold, H., Motais, R. and Passow, H. (1977) 11th FEBS Meeting, Copenhagen A4, 17671
16 Cousin, J.-L. and Motais, R. (1979) J. Membrane Biol. 46, 125–153
17 Bender, W.W., Garan, H. and Berg, H.C. (1971) J. Mol. Biol. 58, 783–797
18 Bretscher, M.S. (1971) J. Mol. Biol. 59, 351–357
19 Triplett, R.B. and Carraway, K.L. (1972) Biochemistry 11, 2897–2903
20 Cabantchick, Z.I. and Rothstein, A. (1974) J. Membrane Biol. 15, 227–248
21 Steck, T.L., Ramos, B. and Strapazon, E. (1976) Biochemistry 15, 1154–1161
22 Drickamer, L.K. (1976) J. Biol. Chem. 251, 5115–5123
23 Drickamer, L.K. (1977) J. Biol. Chem. 252, 6909–6917
24 Fukuda, M., Eshdat, Y., Tarone, G. and Marchesi, V.T. (1978) J. Biol. Chem. 253, 2419–2428
25 Steck, T.L., Koziarz, J.J., Singh, M.K., Reddy, G. and Köhler, H. (1978) Biochemistry 17, 1216–1222
26 Rice, W.R. and Steck, T.L. (1977) Biochim. Biophys. Acta 468, 305–317
27 Grinstein, S., Ship, S. and Rothstein, A. (1978) Biochim. Biophys. Acta 507, 294–304
28 Lepke, S. and Passow, H. (1976) Biochim. Biophys. Acta 455, 353–370
29 Shami, Y., Rothstein, A. and Knauf, P.A. (1978) Biochim. Biophys. Acta 508, 357–363
30 Jennings, M.L. and Passow, H. (1979) Biochim. Biophys. Acta 554, 498–519
31 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
32 Steck, T.L. and Yu, J. (1973) J. Supramol. Struct. 1, 220–232
33 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 263–275
34 Razzel, W.E. (1967) Methods Enzymol 12A, 118–125
35 Cousin, J.-L. and Motais, R. (1976) J. Physiol. (Lond.) 256, 61–80
36 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672
37 Lepke, S., Fasold, H., Pring, M. and Passow, H. (1976) J. Membrane Biol. 29, 147–177
38 Fletcher, J.E., Spector, A.A. and Ashbrook, J.D. (1970) Biochemistry 9, 4580–4587
39 Hunston, D.L. (1975) Anal. Biochem. 63, 99–109
40 Steck, T.L. (1974) J. Cell Biol. 62, 1–19
41 Inglot, A.D. and Wolna, E. (1968) Biochem. Pharmacol. 17, 269–279
42 Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. USA 71, 4475–4461
43 Seeman, P. (1972) Pharmacol. Rev. 24, 583–655
44 Dissing, S., Jesaitis, A.J. and Fortes, P.A.G. (1979) Biochim. Biophys. Acta 553, 66–83
45 Reithmeier, R.A. (1979) J. Biol. Chem. 254, 3054–3060
46 Clarke, S. (1975) J. Biol. Chem. 250, 5459–5469
47 Yu, J. and Steck, T.L. (1975) J. Biol. Chem. 250, 9170–9175
48 Nigg, E. and Cherry, R.J. (1979) Nature (London) 277, 493–494
49 Dix, J.A., Verkman, A.S., Solomon, A.K. and Cantley, L.C. (1979) Nature (London) 282, 520–522
50 Cousin, J.-L. and Motais, R. (1982) Biochim. Biophys. Acta 687, 156–164