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Transport Domain of the Erythrocyte Anion Exchange Protein

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Summary. The anion transport domain of the anion exchange protein (AEP) of human erythrocyte membranes (band 3, 95 kD mol wt) was probed with the substrate and affinity label pyridoxal-5'-phosphate (PLP). Acting from outside, this probe labels two chymotryptic fragments of 65 and 35 kD of AEP but only the 35-kD fragment is protected from labeling by reversibly acting disulfonic stilbenes (DS). It is shown here by functional studies and by immunoblotting with anti-PLP antibodies that transmembrane gradients of anions determine the availability of a 35-kD fragment *lys* residue to surface labeling by PLP, in analogy with their effects on labeling of 65-kD fragment by DS. On this basis, it is suggested that both fragments contribute to the formation of the transport domain. However, unlike DS, PLP blocks transport when reacted from within resealed membranes, indicating that the 35-kD fragment might contain components of the mobile unit of the AEP. Using impermeant fluorescence quenchers of PLP of both complexation type (anti-PLP antibodies) or collisional type (acrylamide) as topological probes for PLP-labeled sites, it is deduced that the 65-kD PLP-labeled and the 35-kD PLP-labeled *lys* groups are inaccessible to macromolecules from either surface, but the 65-kD PLP-*lys* is accessible to low molecular weight molecules from without while the 35-kD PLP-labeled *lys* shows accessibility primarily from within the cell surface. The studies indicate that the accommodation of a wide class of anions by AEP might be associated with the flexibility of the transport domain of the protein and its capacity to undergo transport-related conformational changes.

Key Words erythrocyte · membrane proteins · transport · fluorescence · antibodies

Introduction

The assignment of the anion transport domain to particular transmembrane fragments of the erythrocyte band 3 protein (95 kD), has largely depended on the combined use of specific labeling agents and selective proteolytic treatments (for reviews *see* refs. 3, 6, 24, 35). The functional involvement of the 65-kD chymotryptic fragment (or its 17-kD tryptic subfragment) was originally suggested on the basis of studies conducted with the competitive, affinity label DIDS (4,4'-diisothiocyano-2,2'-stilbene disulfonate) or related congeners [7, 31]. However, more recent studies, have indicated that the 35-kD fragment might also be essential for the function [18, 20, 22, 33, 34, 40]. This raises the possibility that the transport domain might be associated, directly or allosterically, with various transmembrane fragments of the band 3 polypeptide.

To test this hypothesis we have used two types of probes which we had previously introduced in the field of anion transport. One is pyridoxal-5'-phosphate (PLP), an amino specific reagent which can demonstrably serve as a substrate [4] and, in combination with $NaBH_4$, as an affinity label [4, 5] for *lys* groups in 65 and 35 kD fragments of band 3. Inhibition of transport is associated primarily with labeling of *lys* 869 of the murine erythroid AEP or of *lys* 852 in the human erythroid AEP [23], residing in the 35-kD fragment [23, 33, 34, 40]. The second probe used, DNDS (4,4'-dinitro-stilbene-2,2'-disulfonate), is a noncovalent dinitro-congener of DIDS which interacts reversibly and competitively with the anion transport site [1, 10, 12], probably by binding to the 65-kD fragment. However, despite the fact that stilbene disulfonate binding to band 3 is associated with the 65-kD fragment, it blocks PLP binding to the 35-kD fragment of band 3 [33, 34, 40], thus suggesting a binding domain which is common to both types of probes and which is comprised of portions of at least two transmembrane segments [3, 18, 20, 34, 35]. Similar properties of mutual inhibitory effects between DNDS and amino reactive reagents [18], carboxyl-reactive reagents [21] and other reagents have also been described [14, 16, 27].

We demonstrate here by structural and functional studies, that by manipulating the transmembrane gradient of anions it is possible to "recruit" the PLP labeling site (i.e., the putative transport site) on the 35-kD fragment to either membrane surface, similar to what has been observed for the disulfonic stilbene binding site on the 65-kD fragment [1, 10, 12]. Based on the ping-pong model of anion transport [13, 16] and on the mutual interactions

between PLP and stilbene disulfonate binding sites [33, 34, 40], our studies clearly prove that both of the chymotryptic transmembrane fragments provide components of the anion translocating domain and implicate *lys* residues on the 35-kD fragment in the formation of the anion recognition domain. It is also demonstrated here that PLP can block the function by binding to cytoplasmic-facing components of band 3. Using collisional quenchers and complexation quenchers, we show data which are consistent with the idea that, when labeling is carried out from without, the PLP-*lys* (35-kD fragment) conjugate is found in a membrane pocket which are inaccessible to macromolecules from either membrane surface but which is accessible to small molecules only from the inner membrane surface.

Materials and Methods

Materials

2,2′-dinitro-4,4′-disulfonic stilbene was from ICN, K&K Chemicals, fluorescein-β-galactoside was from Research Organics, rabbit IgG ABC detection kit was from Vector. All immunochemicals were from Amersham and Pyridoxal-5′-phosphate, Na-borohydride and all other agents were from best available grades obtained from Sigma Chemical.

Red cells from blood (O^+) donated by healthy subjects were prepared within 3 weeks after withdrawal, by repeated washes with phosphate buffere saline (PBS: 150 mM NaCl, 10 mM Na-phosphate, pH 7.4, unless stated otherwise). All solutions were checked for osmotic strength in a Wescor osmometer apparatus.

Changes in the internal composition of cell Cl for SO_4 was accomplished by incubating the cells (10% hematocrit) at 37°C for 4 consecutive (2 hr) periods with 110 mM $NaSO_4$, 10 mM HEPES, pH 7.4, separated by centrifugation and changes of medium. Changes in cell Cl concentration were obtained by the nystatin technique as described elsewhere [26].

Labeling of Intact Cells

Labeling of intact cells with PLP-$NaBH_4$ was carried out by reacting a 20% cell suspension in the indicated isosmotic medium with 4–25 mM PLP (Sigma) in the presence or absence of 0.5 mM DNDS (ICN K&K), first for 10 min at 4°C and subsequently for 5 or 60 min at 37°C, as indicated. The reaction was terminated by addition of 50 mM lysine (pH 8.0, cooling to 4°C and washing twice with ice-cold medium. The PLP-*lys* Schiff's bases were irreversibly fixed by reacting the labeled cells with 10 mM $NaBH_4$ for 0.5–1 hr at ice-cold temperature [34].

PLP Labeling of Resealed Ghosts (RG)

Pink ghosts, containing 1/100th the original Hb concentration, were labeled either during or after the resealing process and in the absence or presence of 0.5 mM DNDS. Briefly, 1 ml packed cells (50% hematocrit) were incubated for 5 min at 4°C with 50 ml of medium containing 4 mM $MgSO_4$ and 1.7 mM acetic acid, centrifuged for 5 min at 12,000 rpm (Sorvall SS-4) and resuspended in 310 mosM medium containing 110 mM Na_2SO_4, 5 mM HEPES, 5 mM CAPS, 5 mM NaH_2PO_4, pH 8.0, with or without 0.5 mM DNDS and with or without 25 mM PLP. After an initial 10-min incubation at 0°C, the suspensions were transferred to 37°C for another 60-min incubation period. Reaction of external PLP was stopped by washing the ghosts with sulfate medium containing 50 mM lysine-HCl ± 0.5 mM DNDS at pH 8.0 and with medium alone, at 4°C. Where applicable, cells or ghosts were subsequently reacted with PLP for 15 min as described above, either in the presence or absence of 0.5 mM DNDS in sulfate medium. Half of the resealed ghost systems were reacted subsequently with 10 mM $NaBH_4$ as shown above.

Anion Transport

Anion transport of untreated or chemically modified cells was measured by CMTF, the continuous monitoring of transport by fluorescence method [9] after loading the cells (10% hematocrit) for 4 hr at 37°C with NBD-taurine (1 mM) dissolved in pH 7.4 HEPES-buffered isosmotic medium containing the Na salt of that anion which is present in cells (Cl or SO_4). A 10-μl aliquot of NBD-taurine loaded cells were jetted into a cuvette containing 2 ml medium placed in a 37°C thermostatted holder (Spex Fluorolog 2 spectrometer). Efflux of NBD-taurine was analyzed from the fluorescence tracings according to

$$k(t) = (dF/dt)_t/[F(\infty) - F(t)] \quad (1)$$

where $k(t)$ is the instantaneous rate constant of efflux and F the fluorescence intensity at times t and ∞ (after addition of 2 μl of 10% Triton X-100). Usually, 1,000 data points collected over the first 10 min of fluxes were used for the above calculations. The k appearing in the figures is the value for the initial (linear) phase of the profile. With resealed ghosts, we have monitored transport by CMTF using anti-NBD antibodies as external quenchers of fluorescence as described previously [11].

Biochemical Studies

Proteolysis of red cells labeled with PLP-$NaBH_4$ was carried out at 10% hematocrit in PBS containing 1 mg/ml chymotrypsin for 1 hr at 37°C, followed by two washes with PBS containing 1% bovine serum albumin and two washes with PBS alone.

Ghosts

Ghosts were prepared from PLP-$NaBH_4$ labeled cells by hypotonic lysis and repeated washes in 5 mM Na-phosphate, pH 8 [8]. A fraction of these unsealed ghosts (UG) was kept overnight at 4°C, resuspended in PBS in an N_2 atmosphere, and subsequently stripped of extrinsic proteins by treatment with 2 mM EDTA, pH 12, for 30 min on ice [41]. After centrifugation (39,000 × g × 30 min), the pellet was subjected to a similar extraction and finally resuspended in 36 mM Na-phosphate, pH 7.4. Another fraction was used for preparing right-side-out vesicles (ROV) or inside-out vesicles (IOV) [41].

Determination of PLP

Determination of PLP-associated with ghosts (either before or after alkaline stripping) was done by fluorescence (325 nm excitation, 398 nm emission). Calibration of PLP was accomplished by using PLP-labeled molecules such as PLP-lysine, PLP-egg albumin or PLP-lactalbumin. The stoichiometry of PLP bound to the latter was established by measuring their absorption spectrum and using the ε_{325} of 10,150 M^{-1} cm^{-1} [36]. The PLP-labeled proteins were prepared by using a 10 mg/ml protein solution and a 75 : 1 molar excess of PLP, both in 0.1 M Na-borate, pH 8.5, incubation for 2 hr at 37°C, cooling to 4°C, reaction with twofold excess of $NaBH_4$ for 30 min, neutralization with HCl and extensive dialysis. The resulting molar ratios (PLP/protein) were 3 : 1 for lactalbumin and 2 : 1 for egg albumin. Protein was determined by the BCA method (Pierce Chemicals). PLP-lysine was synthesized by reacting 1 mM PLP with 20 mM lysine, pH 8.0, for 1 hr at 37°C followed by 1 hr incubation at 5°C with 100 mM $NaBH_4$. After neutralization with HCl, excess lysine was replaced with Na by passing the mixture through a Dowex cation exchanger.

Anti-PLP Antibodies

Anti-PLP antibodies were raised in rabbits using PLP conjugated to keyhole limpet hemocyanin, which was prepared by the same protocol as that shown above for PLP-egg albumin. The antigen was injected subcutaneously in rabbits at 2 mg doses in a 1 : 1 homogeneous dispersion with Freund's complete adjuvant and subsequently boosted, at 1-month intervals, with similar dispersions using Freund's incomplete adjuvant. Two weeks after injections, the animals were bled and the serum immunoglobulins were precipitated with 40% $(NH_4)_2SO_4$. Antibody titers were determined by ELISA using PLP-egg albumin as antigen, β-galactosidase conjugated antirabbit IgG and fluorescein-β-D-galactoside as fluorogenic substrate. The assays were carried out routinely in Nunc 96-well immunoplates, and the fluorescein was read in microtiter fluorescence plate reader (Perkin Elmer) at 490–>520 nm using 10-nm slits. Alternatively, the antibody titers were obtained by following quenching of the fluorescence of 1 μM PLP-egg albumin in PBS, pH 8 (325–>398 nm), as a function of added antisera.

Fluorescence Quenching

Fluorescence quenching of PLP conjugated to either soluble proteins, or to ROV's, IOV's, UG's or RG's was performed both with complexation quenchers (i.e., anti-PLP antibodies) and with collisional quenchers (i.e., acrylamide, NaI and CsCl). For UG, ROV and IOV isolated from PLP-$NaBH_4$-labeled cells (± DNDS), the quenchers were added as 520 mosM solutions containing 50 mM NaCl and 5 mM Na-HEPES, pH 7.4, and the quencher at the indicated concentration (0–0.4 M for acrylamide and 0–0.2 M for the salts), while the osmolarity was compensated with sorbitol (for acrylamide) or with NaCl (for NaI and CsCl). Quenching studies in resealed ghosts were conducted after resealing them in 520 mosM solutions containing the above concentrations of quenchers and 50 mM NaCl-sorbitol solutions compensated to isosmolarity (520 mosM in all cases) and to isoionic strength.

Table 1. Conformation of transport sites under various experimental conditions

Media composition		Site conformation
Cells	Medium	("Sidedness")
Cl_i	Cl_o	"symmetric"
Su_i	Su_o	"symmetric"
Cl_i	Su_o	"outward"
Su_i	Cl_o	"inward"

SDS PAGE

SDS-PAGE [30] was applied to ghosts after alkali treatment. It was routinely followed by protein transfer into nitrocellulose paper and immunoblotting with anti-PLP rabbit antisera and anti-rabbit-IgG ABC kit (Vector Labs) (chloronaphthol staining). Densitometry tracings were made on the photographed blots using a laser densitometer. The areas under the specific peaks were integrated from the digital readings of the densitometry tracings and normalized to the total protein applied to the gel.

Results and Discussion

Effect of Anion Composition and Gradients on PLP Binding to Band 3 Polypeptides and Inhibition of Anion Transport

The anion composition of cells relative to that of the medium has been shown to have a dramatic effect on the exofacial binding of disulfonic stilbenes to the transport sites of the band-3 protein [10, 12, 14, 27] and on the associated inhibition of anion transport. The interpretation of these results according to the *ping-pong* model of obligatory anion exchange [16] is that a single anion transport site alternates between the inner and outer membrane surface and that the site can be recruited to an inward-facing or outward-facing conformation by placing in the medium facing that surface (*cis* side) anions that permeate considerably slower than those placed at the *trans* side [19, 39]. Thus, when SO_4 is present on one side of the cell membrane and Cl on the other, the transport sites are expected to be recruited to the SO_4-facing surface, as SO_4 permeates at about 10^{-4} the rate of Cl [19, 39] (Table 1). It was shown before that H_2DIDS or DNDS binding monitor the exposure of sites at the outer membrane surface [10, 12, 24, 26]. DNDS is implied to react with the 65-kD chymotryptic fragment of band 3, on the basis of chemical analogy to the covalent reactive congeners DIDS and H_2DIDS. On the other hand, in symmetric media PLP reacts with both the 65-kD

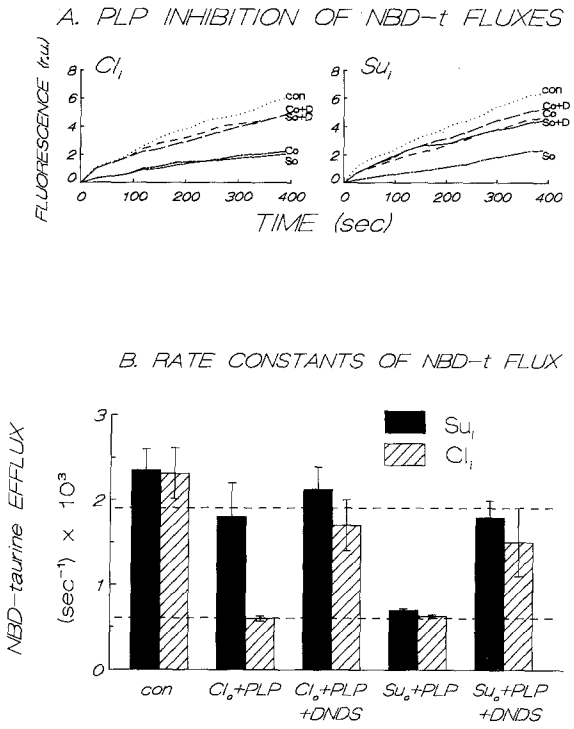

Fig. 1. The effect of transmembrane anion gradients on the susceptibility of the anion transporter to PLP. *Upper:* Red blood cells loaded with either Cl (Cl_i) or sulfate (Su_i) as the major transportable anion were reacted with PLP in Cl medium (C_o) or sulfate medium (S_o) either in the presence or absence of DNDS ($+D$). After fixation of the bound PLP with $NaBH_4$, the cells were extensively washed with the medium of the same composition as that used for the initial loading and incubated for 4 hr at 37°C in the same medium containing 1 mM NBD-taurine (*see* Methods for details). The fluorescent tracings of NBD-taurine efflux from the various treated cells were recorded using the above treated cells suspended in media symmetric in anion composition to those of cells (*con* denotes fluxes of NBD-taurine in Cl_i or Su_i cells which were reacted only with $NaBH_4$ in either Cl medium (Cl_o) or in sulfate medium (S_o), respectively. All tracings were normalized to the final fluorescence attained after lysis of the cells with detergent. *Lower:* Rate constants of the fluxes shown in the upper graph. The values of fluxes obtained with Cl-loaded cells (Cl_i) are depicted in *shaded* boxes and those obtained with sulfate-loaded cells are depicted in *black* boxes. The bars denote the standard deviation of the fluxes calculated over the first 200 points of the fluorescent tracings. $Cl_o + PLP$ and $Su_o + PLP$ represent reaction of cells with PLP-$NaBH_4$ in either Cl or sulfate medium, respectively. Analysis of the full traces by nonlinear regression based on a single exponential equation gave correlation coefficients of better than 0.97

and the 35-kD chymotryptic fragments [33, 34, 40]. We reacted Cl cells and SO_4 cells with PLP-$NaBH_4$ in both Cl and SO_4 media at pH 8.5 (so as to reduce SO_4 translocation), either in the presence or absence of DNDS. The cells were subsequently processed for measuring transport and for measuring PLP bound to band 3 or its 65 and 35 kD proteolytic fragments.

In Fig. 1 we depict the anion transport activity of cells that were chemically reacted in either symmetric or asymmetric conditions. The transport measurements were conducted in media symmetric with respect to anion composition, i.e., after all reagents were washed off and the cells were resuspended and equilibrated in media containing the same anion as that originally present in the cells. The transport measurements were based on the CMTF method, using the fluorescent substrate probe NBD-taurine. The calculated instantaneous rate constants of efflux $k(t)$ given in Fig. 1*B* provide a measure for the activity or number of functional transport sites available at the inner membrane surface at times t [9, 10]. Although the transport activities of Cl cells and SO_4 cells were similar in untreated cells (control) and were reduced by the same extent (80%) with PLP-$NaBH_4$ treatment in symmetric conditions, they were markedly different when chemical modification was carried out in conditions asymmetric with respect to anion composition. Thus, with Cl cells in SO_4 medium (i.e., outward conformation), a similar 80% inhibition was attained, most of which could be protected by the reversibly acting DNDS. However, with SO_4 cells in Cl medium (i.e., inward conformation), the inhibition attained was lower than 20% and most of it could not be protected by DNDS, unlike all previous cases. These results clearly indicate that a transmembrane anion gradient affects the susceptibility of the anion transporter both to PLP as well as to DNDS.

In order to assess the number of PLP binding sites and their locations in band 3 in cells, we prepared membranes from cells that were labeled with PLP as described above and subjected to chymotrypsinolysis. The isolated membranes were stripped of extrinsic proteins as described in Methods and used for determination of the number of PLP molecules bound per ghost protein. As shown in Fig. 2, in all conditions except when reaction with PLP-$NaBH_4$ was carried out in SO_4 cells in Cl medium (i.e., inward conformation), PLP binding was about 14 nmol/mg protein and in the presence of DNDS it was reduced to about 60% of the control level. However, when most transport sites were apparently recruited towards the inner surface, as in SO_4 cells reacted in Cl medium, binding of PLP was markedly reduced. The number of sites corresponding to maximal PLP binding found in the stripped membranes was 2.2×10^6 per cell or about 2.2–2.1 per band 3, based on our estimates of 1×10^{-9} mg protein per stripped ghost. However, since about 10–20% of the PLP label in intact cells is associated

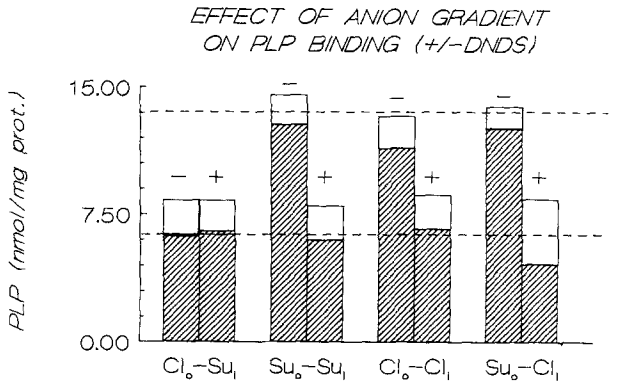

Fig. 2. The effect of transmembrane anion gradients on PLP-$NaBH_4$ binding to the anion transporter. Cells loaded with Cl (Cl_i) or sulfate (Su_i) were reacted with PLP-$NaBH_4$ (P) in the presence (+) or absence of DNDS (−) in either Cl medium (Cl_o) or sulfate medium (Su_o). A fraction of the cells were subsequently treated with chymotrypsin (hatched bars) or with buffered medium (open bars). Ghosts were prepared from all samples, stripped of extrinsic proteins with alkali-EDTA and analyzed for bound PLP by fluorescence and protein by the BCA method. Data are given in terms of nmol PLP bound per mg protein. The broken lines denote average levels of binding

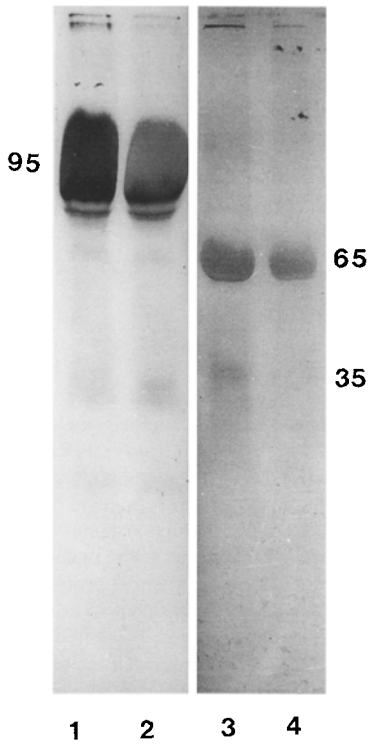

Fig. 3. The effect of transmembrane anion gradients on PLP labeling of red cells. Membranes were isolated from sulfate-loaded cells which were reacted with PLP-$NaBH_4$ in either sulfate medium (*1* and *3*) or Cl medium (*2* and *4*) followed by chymotrypsin (*3* and *4*) as described in Fig. 2. They were analyzed by SDS-PAGE (50 μg protein per track *1* and *2* and 35 μg per track *3* and *4*), electrotransferred onto nitrocellulose and immunoblotted with specific rabbit anti-PLP antisera as described in Methods

with glycophorin [4, 5], most of which could be removed by chymotrypsinolysis of cells (*unpublished observations*), we re-evaluated the PLP binding in membranes isolated from proteolysed cells. As seen in Fig. 2, the results of PLP binding were qualitatively the same as those obtained with unproteolysed cells. However, the protection of inhibition by PLP afforded by DNDS or by the apparent recruitment of sites towards the inner membrane surface induced by the transmembrane anion gradient, was about 50% of the value of the respective controls. Assuming the label is associated predominantly (90–95%) with band 3 or its fragments, the results would indicate that the protection afforded by either treatment is associated with about half the binding sites on band 3, i.e., with one of the two binding sites on band 3, as previously reported for PLP-$NaBH_4$ labeling in symmetric conditions [33, 34, 40]. Quantitatively, our results are more in agreement with those of Matsuyama et al. [33] than with those of Salhany et al. [40] who have apparently observed a considerably higher stoichiometry of PLP-binding to the 65-kD fragment, particularly when labeling was carried out with resealed ghosts [39]. However, with these preparations, an increased labeling might have resulted from the presence of a small fraction of contaminating unsealed ghosts, which not only have also their inner membrane aspects accessible to reaction by PLP, but also have considerably less hemoglobin than intact cells for sequestration of permeating PLP [4, 5].

The localization of the label in band-3 chymotryptic fragments, derived from cells labeled in symmetric and asymmetric conditions (± DNDS), was carried out on Western immunoblots after SDS-PAGE of the above membranes. Immunoblotting was performed on nitrocellulose paper using specific anti-PLP rabbit antibodies and biotin-avidin-HRP ABC kit (Vector) using chloronaphthol as the stainable substrate. Controls of bovine serum albumin-PLP, egg-albumin-PLP and lactalbumin-PLP were carried out in parallel and used for calibrating the system. In the present studies transfer of band 3 or its fragments was estimated to be higher than 80%, as judged by the Coomassie Blue staining of the gel after transfer (*not shown*). As seen in representative immunoblots presented in Fig. 3, the PLP label was predominantly associated with polypeptides in band 3 (95 kD) region when the reaction was carried out with intact cells in symmetric condi-

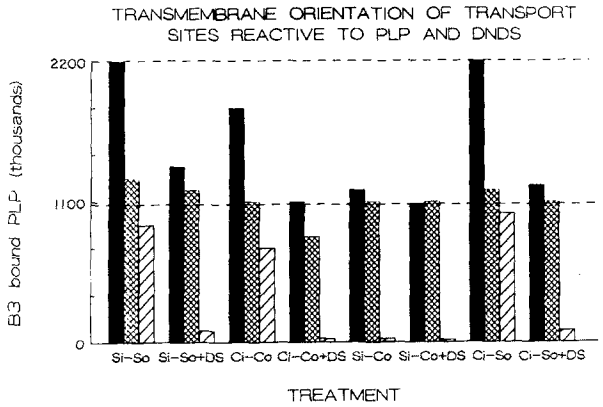

Fig. 4. The effect of transmembrane gradients on PLP labeling of red cells. Immunoblots of membranes isolated from cells treated as in Fig. 2 and as depicted in Fig. 3 were densitometry scanned and the peak areas in the 95-kD (black-filled boxes), 65-kD (cross-hatched boxes) and 35-kD (hatched boxes) bands were integrated with an Apple II computer interfaced to the densitometer. Data are given in terms of PLP bound per band-3 protein (or protein fragments), assuming the integrated and protein normalized 95-kD peak of membranes isolated from cells labeled with PLP-$NaBH_4$ corresponded to 2.2×10^6 sites per cell. C_i-C_o and S_i-C_o represent PLP-$NaBH_4$ reactions done respectively with either Cl-loaded cells or sulfate-loaded cells in Cl medium and Ci-S_o and S_i-S_o represent equivalent reactions done with either Cl-loaded cells or sulfate-loaded cells in sulfate medium. + *DS* represents systems labeled in the presence of DNDS

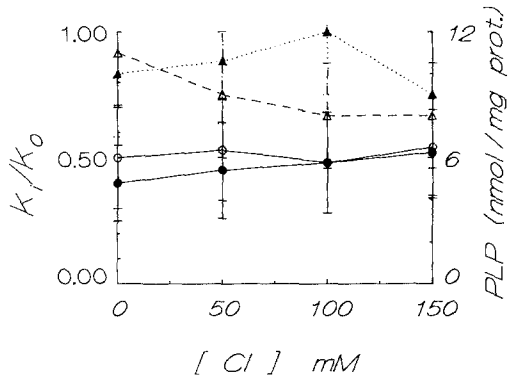

Fig. 5. Apparent competition of Cl and PLP for transport sites on the anion transport protein in symmetric and asymmetric media. Red cells loaded with Cl to different concentrations by the nystatin technique were loaded with NBD-taurine and analyzed for fluxes in symmetric media in the presence of 4 mM PLP (open circles) or treated subsequently with $NaBH_4$ in symmetric media, lysed and analyzed for bound PLP and protein (filled triangles). Red cells loaded with 150 mM Cl and with NBD-taurine were analyzed for fluxes in asymmetric media, containing different concentrations of Cl in the presence of PLP (filled circles) or treated also with $NaBH_4$ and analyzed for PLP bound per mg protein in isolated ghosts (open triangles). Data are given as fractional transport activity k_i/k_o, where k is the rate constant of the respective system obtained in the presence (k_i) or in the absence of PLP (k_o)

tions. After chymotrypsinolysis the PLP label was found to be associated with both the 65-kD and 35-kD fragments. However, when reaction was done with sulfate-loaded cells in Cl medium [17] (inward conformation), labeling of both the 95-kD and the 35-kD fragment resulting from proteolysis was markedly reduced. A quantitative measure of the degree of PLP labeling of membranes isolated from the various treated cells (Fig. 2) was obtained by densitometry of PLP immunoblots normalized per amount of original band-3 protein (Fig. 4). Major differences in the labeling intensities of band 3 are clearly apparent when comparing membranes isolated from cells treated in media of symmetric as opposed to asymmetric anion composition. Again, as in the above studies, SO_4 cells in Cl medium (inward conformation) were protected from labeling to the same extent as that afforded in the presence of DNDS in symmetric conditions. Most of the protection was apparent in the 35-kD chymotryptic fragment, while considerably less protection was detected on the 65-kD fragment (Fig. 4).

In order to assess whether the factor which determines the extent of labeling of the 35-kD fragment by PLP is in competition with the external anion or the availability of sites due to the transmembrane gradient of anions, we carried out both labeling and transport measurements (Figs. 4 and 5). The fact that PLP labeling in symmetric conditions was not significantly different when performed in either symmetric Cl media or SO_4 media, but varied greatly when the medium was of an asymmetric nature (Fig. 4), suggested that the gradient of anions significantly affected the availability of putative labeling sites and transport sites. However, we have found that varying the external Cl concentrations symmetrically at both sides of the membrane had a minimal effect on the extent of labeling and on the ensuing inhibition of transport (Fig. 5). On the other hand, both binding and inhibition of transport increased with increasing Cl_i/Cl_o (at constant physiological Cl_i). However, it should be stressed that the observed changes in PLP labeling and inhibition observed in asymmetric Cl_i–Cl_o conditions (Fig. 5) were considerably less pronounced than those obtained in Cl_o–SO_{4_i} conditions (Figs. 1 and 2). This is probably due to the fact that PLP has a much higher probability of reacting with external transport sites which have been retained in an outward conformation after having been translocated from the Cl to the sulfate medium, due to the considerably slower translocation rates of carrier sulfate complexes [17].

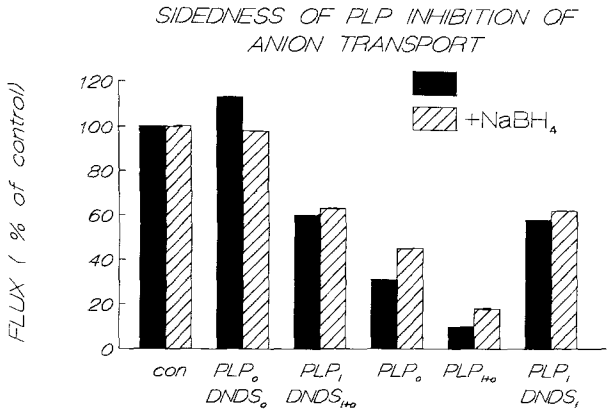

Fig. 6. Inhibition of anion transport by reaction with PLP and PLP-$NaBH_4$ from within and from without resealed ghosts. Ghosts which were partially depleted of Hb were resealed in the presence or absence of PLP ± DNDS and subsequently treated or not with PLP ± DNDS as labeled and reacted (hatched boxes) or not (black boxes) with $NaBH_4$, loaded with NBD-taurine and analyzed by the CMTF for inhibition of fluxes. Data are given in terms of fluxes relative to control, the corresponding untreated cells

Sidedness of PLP Inhibition

PLP can serve not only as a label for anion transport sites but also as a substrate of the system. Thus, although it is likely that inhibition of transport by PLP could be exerted by reaction with inward-facing conformation of the transport sites, labeling from within can also be of a less specific nature. This question was approached experimentally by resealing PLP in Hb-depleted cells (resealed ghosts) in the presence of the reversible inhibitor DNDS. The latter was found to confer protection from PLP only when present extracellularly, but not when sealed inside ghosts (Fig. 6). The reactions were carried out with surplus PLP (25 mM) to overcome possible depletion of intracellular reagent because of reaction with residual Hb and with cytoplasmic-facing membrane proteins. When applied to the inner surface and retained there by resealing, DNDS had no effect on anion transport, neither had it any effect when applied extracellularly after resealing and subsequent removal by washing. However, when PLP was reacted both inside and outside the cells, transport was inhibited to higher degree than when reacted outside alone (Fig. 6, PLP_{i+o} *vis a vis* PLP_o) or when reacted only inside (Fig. 6, PLP_{i+o} *vis a vis* PLP_i + $DNDS_{i+o}$). The inhibitory effects of PLP from within the cells and those from without the cells were nearly additive when compared to the effects of PLP applied to both sides of the cell membrane at the same time. We assume that PLP applied inside the ghosts reacted with transport sites only from within and not from without, inasmuch as DNDS conferred full protection to the external sites and presumably prevented any (band 3 mediated) egress of PLP. Similar to what has been already shown with NAP-taurine, whose reaction with cytoplasmic components of red cell membranes inhibited external H_2DIDS labeling of band 3 [15, 25], cytoplasmic PLP-$NaBH_4$ binding to cytoplasmic components reduced binding of ($^3H_2$DIDS) to red cells (*not shown*).

Topographical Nature of the PLP Binding Sites

External binding of PLP-$NaBH_4$ to red cell membrane components shows an approximately 2 : 1 stoichiometry with band 3 and, after chymotrypsinolysis, a 1 : 1 stoichiometry with both the 65-kD and 35 kD fragments. The *lys* group in the 35-kD fragment labeled by PLP was identified as *lys* 869, near the C-terminus of the murine erythroid protein [23] (or *lys* 852 of the human erythroid protein, ref. 42), while the *lys* on the 65-kD fragment remains to be more firmly defined. To determine the location of those *lys* groups relative to the plane of the membrane, we applied high molecular weight and low molecular weight quenchers of PLP-*lys* fluorescence from either one or both surfaces of the membrane. Anti-PLP antibodies are effective complexation quenchers of PLP fluorescence when applied to PLP-labeled proteins (Fig. 7*A*,*a–d*) or to PLP conjugated to lysine (*not shown*). More than 90% quenching can be observed on proteins, except that at higher concentrations the antibodies contribute some background fluorescence of their own. However, no detectable changes in fluorescence, i.e., quenching, could be observed when saturating amounts of anti-PLP antibodies are added to unsealed ghosts isolated form PLP-labeled cells in any of the following conditions: with (Fig. 7*B*, *e* and *d*) or without (Fig. 7*B*, *a–b*) DNDS, after (Fig. 7*B*, *b* and *e*) or before proteolysis (Fig. 7*B*, *a* and *d*), and also in the presence of the non-ionic detergent Triton X-100 (*not shown*). Only after SDS solubilization, SDS-PAGE, transfer and immunoblotting could antibody binding to PLP be observed, as shown above (Fig. 3). This apparent inaccessibility of macromolecules to PLP associated with anion transport sites is analogous to the lack of accessibility of those sites to disulfonic stilbenes coupled to dextrans, unless a spacer arm of several carbons separated the blocker from the macromolecule [2].

Among the low molecular weight collisional-type quenchers such as CsCl, NaI and acrylamide,

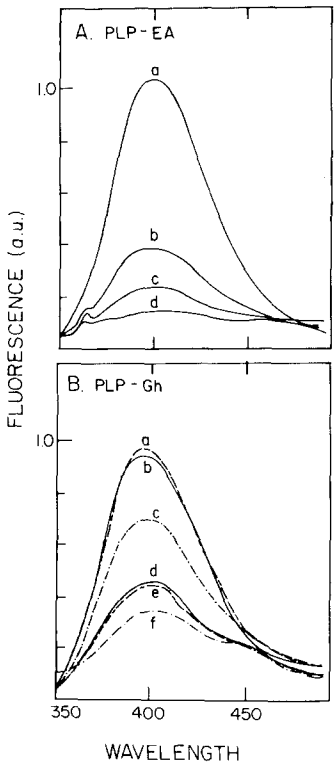

Fig. 7. Quenching of PLP conjugates with anti-PLP antibodies and collision quenchers. Emission spectra of PLP conjugates (excitation 325 nm): (*A*) PLP-egg albumin in PBS were titrated with increasing amounts of anti-PLP antibodies (*a*, 0 μl; *b*, 50 μl; *c*, 100 μl; and *d*, 150 μl of antibodies added to 2 ml PBS containing 100 nM of PLP conjugated to egg albumin, *PLP-EA*). (*B*) Unsealed ghosts derived from cells treated with PLP-$NaBH_4$ without (*a–c*) or with DNDS (*d–f*) cells containing the equivalent of 100 nM conjugated PLP (*PLP-Gh*) (*a–c*) or 40 nM PLP (*d–f*), were treated with anti-PLP (0 μl in *a* and *d*, 150 μl in *b* and *e*) or with 0.4 M acrylamide (*c*, *f*)

we found the latter to be the most efficient quencher of PLP fluorescence (Table 2). However, in all instances the degree of quenching of PLP in ghosts was considerably lower than that of soluble proteins, most probably indicating differences in accessibilities.

The sidedness of the quenching was studied with white resealed ghosts, right-side out (ROV) and inside-out (IOV) vesicles. The results were qualitatively similar in ghosts and vesicles, although they were more reproducible with the first ones (Fig. 8). White ghosts derived from PLP-$NaBH_4$-labeled cells (± DNDS) were lysed and used either for preparing ROV's and IOV's in low ionic strength buffer [41] or ghosts resealed in media containing various concentrations of the above-mentioned quenchers or sorbitol as control. The latter solute was also used for compensating the osmolarities (520 mosM) of both media inside and

Table 2. Stern Volmer constants K_{SV} of various quenchers of PLP fluorescence (M^{-1})

Preparation	Acrylamide	NaI	CsCl[a]
PLP-lysine	6.32	7.3	1.5
PLP-egg albumin	2.63	3.2	0.9
Unsealed ghosts	1.18	1.5	0.6
Unsealed ghosts (+DNDS)	0.65	2.2	0.4

The Stern Volmer plots [37] were carried out in 5 mM Na-phosphate solutions, pH 7.4, compensated with either sorbitol or Cl.

[a] The values of K_{SV} for CsCl were obtained in the 0–0.1 M range, as beyond 0.1 M, the plots were not linear.

outside the resealed ghosts, and outside for vesicles. The F values obtained were analyzed according to the Stern-Volmer equation [37], in order to assess the degree of accessibility of the various PLP-labeled sites to the collisional quencher acrylamide. As shown in Fig. 8 (upper), the plots were not linear over the entire range of concentration of quencher, probably because of the heterogeneity in the population of vesicles with respect to sidedness and because of osmotic effects which affect the scattering properties of the vesicles and possibly leakiness of quencher, as well. Nonetheless, when compared on a qualitative basis, it is evident that in cells labeled in the presence of DNDS, the degree of quenching was considerably lower when the quencher was applied on the inner surface of the membranes (i.e., in IOV's quenched from the outside) than when applied to the outer surface of membranes (i.e., in ROV's quenched from the outside). These differences were more significantly shown in experiments carried out with resealed ghosts (Fig. 8, lower). Here the plots were linear and the differences in the slopes and the Stern-Volmer constants (K_{SV}) indicate that PLP was virtually inaccessible to quencher acting from within the ghosts if the latter were derived from cells labeled in the presence of DNDS, but was maximally accessible when acting from without. Since in the presence of DNDS only the 65-kD fragment is labeled at a single *lys* residue, it can be deduced that the PLP tagged on that residue is exposed always at the outer aspects of the membrane. In order to assess the orientation of the PLP tagged on the *lys* residue in the 35-kD fragment [23], we assume that

$$F_t = F_{65} + F_{35} \tag{2}$$

and in the presence of DNDS

$$F_{tD} = F_{65} \tag{3}$$

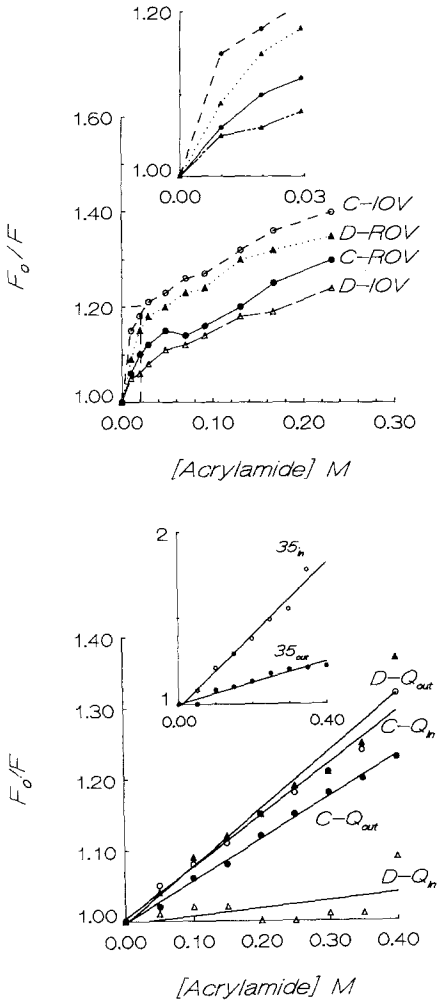

Fig. 8. Sidedness of acrylamide (AAm) quenching of right-side out or inside-out vesicles (*ROV* and *IOV*, respectively) and of resealed ghosts isolated from cells that were labeled with PLP-$NaBH_4$- either in the presence (*D*) or absence (*C*) of DNDS. *Upper:* ROV or IOV were prepared from cells labeled with PLP-$NaBH_4$ as described in the text and were analyzed for PLP-fluorescence (as described in previous figures) in the absence and presence of increasing concentrations of acrylamide (AAm). All samples were normalized for protein contents. *Lower:* Ghosts isolated from PLP-$NaBH_4$ labeled (± DNDS) were resealed in sorbitol media containing different proportions of acrylamide and were analyzed for fluorescence in the absence or presence of increasing concentrations of AAm. All samples were normalized for protein contents. The data obtained with the different preparations are given in terms of the Stern-Volmer plot where F represents the fluorescence intensities in the presence of quencher and F_o in the absence of quencher. AAm_{in} and AAm_{out} represent results of quenching by acrylamide either at the inner or outer surface of resealed ghosts. For resealed ghosts, regression analysis yielded the following Stern-Volmer constants K_{SV} (M^{-1}, r = corr. coeff.): *PLP*/AAm_{in} 0.80 (0.985), *PLP + DNDS*/AAm_{in} 0.10 (0.60), *PLP*/AAm_{out} 0.58 (0.998), *PLP + DNDS*/AAm_{out} 0.72 (0.992). *Lower inset:* Stern-Volmer plot of quenching of PLP fluorescence associated with the 35-kD fragment. The F of 35 kD_{in} was obtained by subtracting $F_t - F_{tD}$ for each AAm concentration placed inside the ghosts and F for 35 kD_{out} was the equivalent for AAm applied outside the ghosts. The K_{SV} values were: for 35 kD_{in} 2.33 M^{-1} (r = 0.98) and for 35 kD_{out} 0.35 (r = 0.97)

and therefore

$$F_{35} = F_t - F_{tD} \tag{4}$$

and from the Stern-Volmer plot of F_{35} we obtain K_{SV} for quenching inside and for outside, respectively. As seen in Fig. 8 (bottom, inset), the quenching yield was about fivefold higher from the inside than from the outside. These results indicate that PLP, which labeled the *lys* groups in the 35-kD fragment of band 3 from the outside, became largely exposed (ca. 55%) to the inner medium, while about 11% was also accessible from without. At present, one cannot eliminate the possibility that those 11% represent, at least in part, quenching from within either because of small leakage of the quencher or because of contamination with leaky ghosts, although as tested by centrifugation over a 30% metrizamide cushion, the degree of contamination was found to be lower than 5%. The same two factors could also contribute to underestimation of the quenching of the 35-kD fragment from within. However, the PLP-labeled *lys* on the 65-kD fragment was apparently accessible only from without and was completely inaccessible from the inside. We can rule out the possibility of leakage of quencher trapped in the ghosts based on the fact that in ghosts labeled in the absence of DNDS, quenching from within was even larger than from without. With two other collision quenchers, NaI and CsCl, the results were qualitatively similar to those obtained with acrylamide, but possible leakages of I and the relatively low quenching efficacy of Cs, precluded quantitative analysis. Some of these results were qualitatively similar to those observed with eosin-isothiocyanate labeling of band 3, except that the permeation properties of this probe and the residues labeled by it are still undefined and the quenching efficiencies of the agents used were considerably smaller [32].

Conclusions

Taken *in toto,* the present studies provide direct evidence for the functional involvement of sites on the 35-kD fragment of the AEP. This evidence reinforces previous studies which implied a similar involvement on the basis of correlations between papain cleavage of the 35-kD fragment and inhibition of transport [20] and correlations between disulfonic stilbene protection of the function and of labeling of that fragment by PLP [33, 34, 40] and by other amino reactive reagents [18, 35]. Moreover, the present studies indicate that the band-3 protein undergoes changes in conformation induced by transportable anions. The changes are reflected in

the availability at the outer membrane surface of binding sites to PLP, which might be the same sites that alternate between inward and outward conformations, i.e., the transport sites according to the *ping-pong* model of anion exchange [13, 16]. The protein domain that carries these sites comprises *lys* groups [35], as it has previously been shown that only *lys*-pyridoxal conjugates were isolated from amino acid analysis of PLP-$NaBH_4$ labeled membranes [4], and recent studies identified the *lys* in the 35-kD fragment as *lys* 869 (murine) or *lys* 852 (human) [42]. It is possible that the same *lys* on the 35-kD fragment, which becomes inaccessible to either PLP (this work) or to disulfonic stilbenes [10, 12] when administered to SO_4 cells placed in Cl media, is among those susceptible to PLP binding from the inner surface of resealed ghosts. As similar changes are observed with components of the 65-kD fragment, the present studies support the view that the transport domain is a mobile unit comprised by at least two noncontiguous transmembrane fragments of the band 3-protein, generated by chymotrypsin cleavage of the intact protein. Recent studies with Woodward's reagent K on band 3-mediated transport, have provided evidence for the involvement of a *glu* which confers the protein with the H-titratable and a H-cotransportable mobile moiety, which can alternate between inward and outward conformations [21]. Although the above-mentioned *lys* and *glu* groups as well as *arg* groups [43, 44] might reside in the functional domain of the protein, it remains to be shown whether or not they are directly involved in anion binding or translocation. The probes normally used for "specific" modification of these sites are anionic and rather bulky, so that the chemically modified groups might be just adjacent or in the vicinity of the critical, functionally relevant amino acids that recognize the "specific anionic" moiety of the probes. For example, in the process of substrate binding or translocation, sites are rearranged in such a way that the accessibility of various chemical groups in the transport domain changes accordingly. That the protein undergoes transport-related conformational changes is witnessed by the effects of anion gradients on binding of PLP to the 35-kD fragment and the resulting functional inhibition. Similar effects were also observed with DNDS, although in that case it is not clear whether binding occurred with components of the 65-kD fragment. The latter was assumed to be the case on the basis of the structural analogy of DNDS with the covalent reactive congener DIDS, which was shown to react with a *lys* in that fragment [38]. However, as deduced from quenching studies, binding of external PLP to sites on the 35-kD fragment appears to induce a shift in the position of the bound probe (transport site?) so that a (aqueous) path for quencher molecules opens from within the inner aspects of the protein, while becoming inaccessible to quenching from without. These conformational changes induced by bulky substrate analogs such as PLP, might be of relevance for understanding the accommodation of a large variety of anions by the transport site and the dynamics of translocation of the transportable anions. The conformational flexibility associated with substrate-induced changes in the transport domain of AEP, might be an intrinsic property of the anion exchange mechanism, which is consistent with many features of the anion exchange system and with the recently proposed (transition-state) theory which calls for a carrier conformational change in the process of substrate-binding stabilization [28, 29].

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Note Added in Proof

Recent studies of site-directed mutagenesis indicated that lys 539 of human band 3 and the equivalent lys 558 of murine band 3 were neither essential for anion transport nor for conferring susceptibility to inhibition by DNDS (Garcia and Lodish, *J. Biol. Chem.* **264:**19607–19613, 1989, and Bartel et al., *EMBO J.* **12:**3601–3609, 1989). However, conflicting results were reported regarding the possibility that the above residues are the actual targets of chemical modification by disulfonic stilbenes DIDS and its dihydro analog H_2DIDS.

In a recent series of studies conducted with [PLP] at concentrations $\leq$1 mM, it was found that symmetric changes in $[Cl]_{in}$ and $[Cl]_{out}$ significantly affected both PLP binding to AEP and PLP inhibition of anion exchange (S. Bar Noy and Z.I. Cabantchik, *unpublished*). At 37°C, the apparent first order rate constant of PLP binding to AEP was 0.37 $\pm$ 0.08 M^{-1} sec^{-1} at [Cl] = 150 mM and 0.93 $\pm$ 0.1 M^{-1} sec^{-1} at [Cl] = 10 mM. The k_{off} constants of the Schiff's bases formed between PLP and AEP were considerably slower and could not be determined with accuracy. However, after a 5-min reaction followed by fixation with $NaBH_4$, the reduction in binding of PLP observed at [Cl] = 150 mM as compared to [Cl] = 10 mM, was associated primarily with the 35-kD chymotryptic fragment of AEP, although in the presence of DNDS a slight increase in binding to the 65-kD fragment was consistently observed. The transport properties studied under reversible PLP binding conditions with PLP $\leq$ 1 mM show that the apparent K_s for Cl obtained from Hunter-Downes plots was 42 $\pm$ 15 mM. These data clearly indicate that in symmetric conditions there is an apparent competition between PLP and Cl for sites on the 35-kD fragment of AEP. They also provide an explanation for the apparent lack of competition found with relatively higher concentrations of PLP (Fig. 5), as in these conditions binding was probably maximal. However, at present we cannot exclude also possible allosteric effects elicited by the various ligands binding to different sites on both fragments of the AEP, as suggested by Salhany et al. [39, 40].