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Sequential Mechanism of the Malonate Transport System in Human Red Blood Cells

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Abstract

The kinetics of [2-^{14}C] malonate transport system by the human erythrocyte membranes have been examined. The stimulation of anion- exchange band 3 protein flux could be determined by measuring [2-^{14}C] malonate influx and efflux against its concentration gradient. The asymmetry of $K_{m(cis)}$, 21mM and $K_{m(trans)}$, 3.2 mM is of 7-fold. The $K_{i\ (cis)}$ of malonate-malonate homoexchange was also 21mM. These results are evaluated in terms of two broad categories of kinetic models, namely ping- pong and sequential mechanisms. We conclude that anion exchange is compatible with a sequential mechanism in which a random or specific order of malonate binding sites$_{cis\ \&\ trans}$ channel, congruently conformed asymmetrically with both inside- and outside facing orientations.

Lineweaver-Burk plots at varying [2-^{14}C] malonate and different fixed concentrations of malonate have shown a series of interesting lines that are contrasting the other anion transport system of ping-pong mechanism. ^{14}C-Malonate which is nonmetabolized in the erythrocyte is a useful kinetics probe for monitoring heteroexchange and self-inhibition.

Keywords: Sequential model, malonate, anion transport, erythrocytes, membrane, band 3

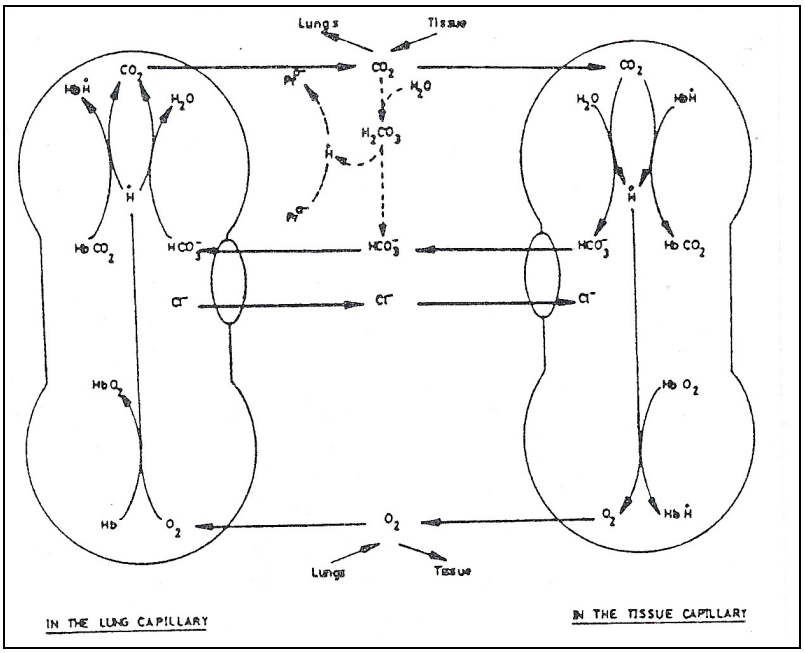
Abbreviations: SITS, 4-Acetamido-4'-isothiocyano-2, 2'-disulfonicstilbene; DIDS, 4,4'-Isothiocyanostilbene-2,2'-disulphonate; H_2DIDS,4,4'-Diisothio- cyano-dihydrostilbene-2,2'disulphonate; DBDS, 4,4'-Dibenzamidostilbene- 2,2'-disulphonate; DNDS, 4,4'-Dinitrostilbene-2,2'-disulphonate; BPS, Bathophenanthroline disulphonate; cis: Outward-facing; trans: Inward-facing; Hct: Haematocrit

Introduction

The anion exchanger-1 AE1 (synonymous with Band 3; capanophrin; gene SLC4A1) is the most abundant human red cell membrane protein. It is part of a family of anion exchange proteins found in the plasma membrane of various cell types (Gunn and Fröhlich,1979;Passow,1986; Jennings,1989;Donath et al, 1990; Salhany,1990; Reimeier,1993; Gibson and Ellory,2002; Knauf et al, 2002; Tanner,2002;miller and Nguitragool, 2009). The Band 3 (AE1) protein is a 95 kDa multipass membrane protein that is consistent with its classical function, electroneutral HCO_3^- - Cl^- exchange across the plasma membrane on a one-for-one basis (Fig. 1). The plasma membrane of most cells is relatively impermeable to chloride and bicarbonate ions. In contrast, a high concentration of Band 3

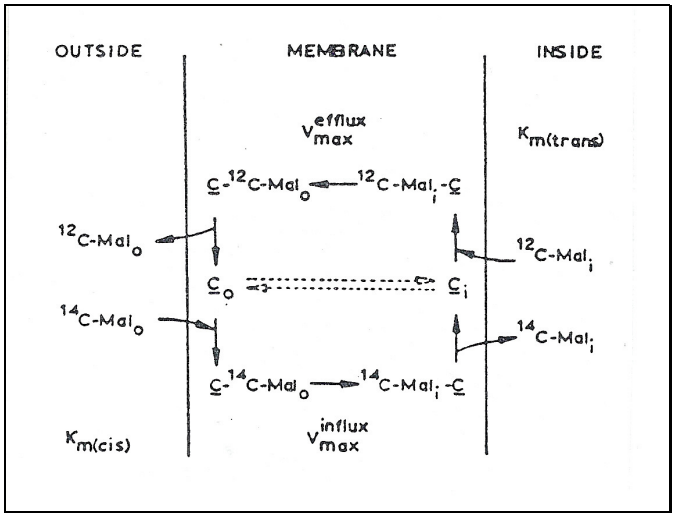
that is known to operate as an electroneutral anion exchanger, allows the red cell membrane to be very efficiently transport these anions, a process integral to their function in transporting CO_2 in blood. This protein is the predominant membrane transporter in red blood cells and plays a central role in structure and function of those cells and also in acid-secreting cells in the kidney. Hence, this is crucial for CO_2 uptake by the red cell and conversion by hydration catalyzed by carbonic anhydrase into a proton and a bicarbonate anion. The bicarbonate is then extruded from the cell by the Band 3 molecule 300-600ms period for which the red blood cell traverses the pulmonary circulation and hence, a high rate of anion flux is necessary (Salhany,1996 ; Ellory et al,2009). These transporters can also ferry a wide variety of other anions (Jennings, 1992; Guizouarn et al, 2001). Certain members of the anion exchange transporter family also have the secondary function of providing cytoskeletal and sytosolic protein binding sites via binding with ankyrin and protein 4.2 at the inner surface of plasma membrane in which the latter function is primarily assigned to an N-terminal cytoplasmic extension (Low, 1986; Salhany, 1990; Jannings, 2005). In recent years, our understanding of the anion exchange system of the human red blood cell has been greatly augmented by two sorts of information. On the one hand, kinetic studies have shown that the transport displays properties consistent with a mobile carrier model (Hajjawi and Hider, 1987; Jennings et al, 1990; Knauf et al, 2002; Hajjawi and Hider, 2009). On the other hand, the uses of the inhibitory chemical probes have lead to the identification of the 95,000-dalton polypeptide (Fairbanks et al, 1971). Band 3 makes up ~25% of the total red cell membrane protein and is present in the membrane as ~550,000 noncovalent dimmer (Verkman et al, 1983). In order to integrate these two kinds of information, additional knowledge is requited concerning the relationship between carrier sites postulated from kinetic analysis of transport and inhibitory sites in band 3 (Fig. 2), by the covalent reactions of chemical probes. Although the majority of studies associated with this protein have been centered on inorganic anions Schnell et al, 1978; Fievet et al, 1998; Jennings, 2005), organic anions are kinetically more informative (Hajjawi and Hider, 1987; Jennings and Adame, 1996; Hajjawi and Hider, 2009). The evaluation of the mechanism by which chemical probes inhibit anion transport can be greatly facilitated by the use of "bimodal" membrane topology inhibitors (Reithmeier, 1993).

Figure 1: A schematic diagram of chloride-bicarbonate exchange associated with O_2 and CO_2 transmembrane movement in the human erythrocyte.



Adopted from: Yamaguchi et al (2010)" Three-dimensional structural analysis of a membrane transport protein, band 3, existing in large amounts in human red blood cells, which carry oxygen ", Journal of Molecular Biology vol.397 (1), pp.179-189.

Figure 2: Schematic rationale for a possible transport mechanism for the exchange of intracellular malonate with extracellular malonate in the human red blood cell.

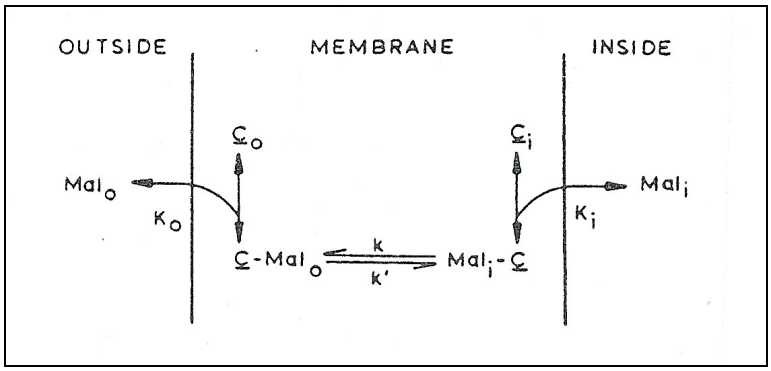


$\underline{C}_o$ and $\underline{C}_i$ represent the transport site which can complex with extracellular malonate anions $(Malonate)_o$ and the intracellular malonate anions $(Malonate)_i$, respectively. Since the exchange is one-to-one, no interconversations of the uncomplexed sites themselves' (Widdas mobile transporter) are allowed, i.e. mobility via the dashed lines is prohibited.

The combined findings of malonate/chloride exchange and malonate/proton cotransport for both malonate influx and efflux are best explained in terms of titratable transporter, in which the phenomena of both exchange diffusion and counter transport of malonate are described (Hajjawi and Hider, 2009). The concentration of extracellular malonate enhances the rate of efflux of intracellular ^{14}C-malonate. This implies that the mechanism of the type indicated in Fig.2 occurs although it can't distinguish between the schemes presented and that where the free transported is also able to cross the membrane (dashed lines). However, this latter possibility is excluded by the failure to induce "temporary uphill transport" by preincubation methods (Hajjawi and Hider, 1987). Thus, the malonate transporter cannot shuttle across the membrane except when loaded with an anion. The transporter acts therefore to exchange anions in a compulsory fashion and appreciable net flux cannot occur when the exchange is between the same ion species (Ussing, 1948).

Many anion transport studies in erythrocyte which used probe inhibitors have strongly suggested that the outward-facing and the inward-facing forms of the transporter are asymmetric for inorganic anions (Schnell et al, 1978; Gunn and Fröhlich, 1979; Jennings et al, 1990; Liu et al, 1996; Knauf et al, 2002), and for organic anions (Jennings and Adame, 1996; Hajjawi and Hider, 2009). Also, a specific class of inhibitors (such as:DIDS, H_2DIDS, SITS, BPS, DBDS, DNDS, and eosin) that is widely used group of inhibitors of anion exchange, was identified as competitive, since they blocked anion binding to Band 3, yet the inhibition is fully reversible for many of them (Hajjawi and Hider, 1987; Salhany, 1998). However, it is difficult for such inhibitor studies to be conclusive because of the topological asymmetry of the erythrocyte membrane. Nonetheless, there would seem to be no *priori* reason why the outward-facing and inward-facing forms of the transporter should have equal anion substrate affinities in the catalytic cycle of malonate transporter (Fig. 3).

Figure 3: Sequential model of the anion transport system.



The Band 3 protein can exist in a $\underline{C}_o$ form, with the transporter facing outward, and in $\underline{C}_i$ form, with the transporter facing inward. The malonate dissociation constants of the outward- and inward- facing forms are K_o and K_i , respectively. The rate constant for the conformational change from the inward-facing $\underline{C}$-$malonate_i$ form to the outward-facing $\underline{C}$-$malonate_o$ form is K, while the rate constant for the reverse conformational change is K'.

The probe of the study in Fig. 3 is a nonmetabolized permeant species, malonate, and thus in principle, such studies become more convincing. The rate of ^{14}C-malonate efflux into an extracellular medium containing ^{12}C-malonate was higher that that compared to efflux in the presence of an extracellular heterogeneous anion. Salhany et al (2003) and Jennings (2005) reported similar effects on sulphate, phosphate and chloride efflux. In a sequential mechanism (Cook and Cleland, 2007), both $malonate_i$ and $malonate_o$ anions bind either in a random or ordered fashion prior the anion exchange into opposite cellular compartment at a rate concentration –dependent.

The present paper is concerned with providing more kinetics data about the malonate anion exchange mechanism in the human red blood cell anion exchange protein, AE1.

Materials and Methods

[2-^{14}C]Malonate and [U-3H]inulin were purchased from Radiochemical Centre, Amersham, Bucks, and UK; Silicone oil was purchased from Hopkin and Williams, Romford, Essex, UK. All other materials were supplied by Sigma (London) Chemical Co., Poole, Dorset, UK, or by BDH Ltd., Poole, Dorset, UK, and were AnalaR grade whenever possible. All solutions were prepared with glass-distilled water.

Erythrocyte Preparation

Freshly outdated blood containing citric acid, sodium citrate, dextrose and adenosine was collected from Essex County Hospital, Colchester, UK. Human erythrocytes were separated by sedimentation at 2500 g (at r_{av} 13 cm) and 4 ^{o}C for 5 min using MSE Mistral 4L. The plasma and buffy coat were removed by aspiration. The erythrocytes were then washed three times by resuspension and sedimentation in ice-cold NaCl (152mM). Finally, erythrocytes were suspended in Krebs-Ringer bicarbonate (Umbreit et al, 1946) (pH 7.4), 1:1 (v/v) for isotope uptake studies, and kept on ice. Erythrocytes were always used for experiments immediately after preparation.

The volume of erythrocyte intracellular water (68.8 %) was determined with the use of [^{3}H]inulin as described by Winter and Christensen (1964). The standard dry weight of erythrocyte suspension (typically = 30%) was used to calculate the intracellular fluid for each experiment.

[2-^{14}C] Malonate Uptake Studies

[^{14}C] Malonate influx was measured using a slight modification of the procedure reported by Eavenson and Christensen (1967) for pigeon erythrocytes. An isoosmotic malonate/sucrose solution (1 ml, pH 7.4) containing 0.05 – 0.25 µCi/mi was pipetted into a test tube and preincubated at 37 ^{o}C for 15 min.

An erythrocyte suspension (packed cells: Krebs-Ringer bicarbonate buffer, 1:1 by vol, 1 ml) was then added, oxygenated and incubated. The media was shaken mechanically throughout the incubation period. At predetermined intervals 0.5 ml of incubation medium was added to polypropylene conical tubes (1.5 ml) containing 0.5 ml of silicone oil (ρ 1.07) and centrifuged for 20 s in a Beckmann Microfuge B (Rosenberg and Rafaelsen, 1979). Aliquots of the supernatant fluid (0.1 ml) were taken for scintillation counting, the remainder being removed by aspiration. Extra care was taken not to aspirate portions of the pellet while removing the silicone oil. H_2O (0.25 ml) was then added to the packed cells with vigorous mixing. The resulting haemolyzed erythrocytes were bleached with 10% trichloroacetic acid (0.25 ml). The denatured material was sedimented by centrifugation and a 0.1 ml sample of supernatant fluid was taken for scintillation counting.

Under these incubation conditions, with extracellular chloride = 40 mM, chloride ions efflux from the cell at the onset of the incubation due to the chloride concentration gradient across the erythrocyte membrane. This net efflux creates a diffusion potential which is positive inside. This diffusion potential increases both the initial rate and the distribution ratio of malonate uptake (unpublished observations). These incubation conditions were used throughout the study. Similar results were obtained by substituting Krebs Ringer bicarbonate buffer by phosphate/citrate, Tris-HCl, Sorenson's phosphate or glycine/NaOH buffers (pH 7.4).

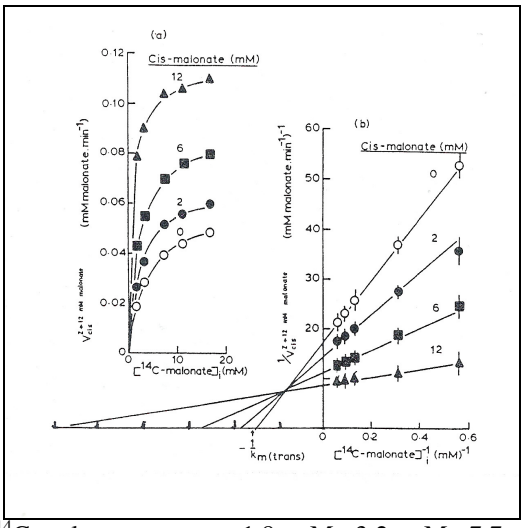
The rate of uptake of malonate anions (1 to 20 mM) was linear for time period to 5 min. Fixed time assays were run either for 1 min or 2 min intervals.

Results

Malonate has been established to be nonmetabolized in human erythrocytes and permeate these cells rapidly (Hajjawi and Hider, 1987, 2009). As a result it was selected as a probe for isotope uptake studies. The concentration of malonate was limited to the range 1-20 mM, because under such conditions the transmembrane gradient of malonate failed to change the shape of erythrocytes as demonstrated by light scattering studies and 'dry weight' measurements (Hajjawi and Hider, 1987). Uptake was analysed in terms of Michaelis – Menten kinetics. Under the adopted incubations conditions, the kinetic parameters {($K_{m(cis)}$) and ($K_{m(trans)}$)} for malonate influx are appearant values, accommodating the inhibitory influence of chloride. However, as the extracellular chloride concentration (40 mM) was maintained throughout the entire range of experiments, the appearant kinetic constants are useful in the analysis of comparative inhibitory potencies of anions. Hajjawi and Hider (2009) reported the asymmetry in the malonate transport in human red blood cell membranes. Using the Lineweaver-Burk, Dixon, Eisenthal-Cornish-Bowden, and Wolf-Hofstee plots values of $K_{m(cis)}$ = 21 mM and of $K_{m(trans)}$ = 3.2 mM were obtained. The effect of extracellular (cis) ^{12}C-malonate concentration on the rate of intracellular (trans) ^{14}C-malonate efflux was further examined. The objective of this experiment was to provide information concerning the chemical malonate fluxes across the erythrocyte membranes *in vivo*. Since washed intact cells closely approximate to the *in vivo* system, a number of experimental restrictions however were imposed when using intact cells; the main restriction being the relative difficulty of manipulating the cytoplasmic milieu. More often than not, intracellular constituents are indirectly estimated. Nonetheless, an attempt was made to monitor the efflux kinetics of ^{14}C-malonate as a function of the contents of the incubation medium. Erythrocytes (25% Hct.) were incubated at 37 ^{o}C for 40 min in isoosmotic ^{14}C-malonate solutions (pH 7.4)). The incubation medium was plunged into ice-cold Krebs-Ringer bicarbonate buffer and the cells were centrifuged (r_{av} 13 cm, 2500 rpm) at 4 ^{o}C for 5 min. The supernatant was aspirated and the cells were then washed by re-suspension, centrifugation and aspiration three times in Krebs-Ringer bicarbonate buffer and finally resuspended in Krebs-Ringer bicarbonate buffer 1:1 (v/v).Hence, erythrocytes (25% Hct.) of different fixed concentrations of intracellular ^{14}C-malonate$_{trans}$ were incubated at 37 ^{o}C in isoosmotic unlabelled ^{12}C-malonate solutions (pH 7.4). The rate of ^{14}C-malonate efflux was monitored as a function of extracellular ^{12}C-malonate$_{cis}$ concentration. It was shown that the efflux of ^{14}C-malonate was increased with increasing extracellular ^{12}C-malonate$_{cis}$ concentration (Fig. 4 a) and

linearity was observed by the use of Lineweaver-Burk plots (Fig. 4 b). The family of lines intersect to the left of $1/V_{trans}$ –axis and above $1/[^{14}C\text{-malonate}]_{i(trans)}$ –axis.

Figure 4: Recruiting outward –facing malonate transporters.



The concentration of intracellular $^{14}C\text{-malonate}_{trans}$ was 1.8 mM, 3.2 mM, 7.7 mM, 11.1 mM and 16.6 mM. The concentration of extracellular $^{12}C\text{-malonate}_{cis}$ was 0 mM, 6 mM, 12 mM, and 18 mM. (a) Initial rate of $^{14}C\text{-malonate}_{trans}$ efflux as a function of $^{12}C\text{-malonate}_{cis}$ concentration. (b) Lineweaver-Burk plot for double-reciprocal of initial rate of $^{14}C\text{-malonate}_{trans}$ efflux vs $^{12}C\text{-malonate}_{cis}$ concentration.

Discussion

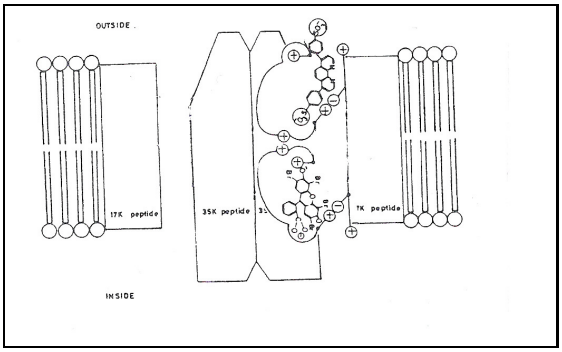
The experiments reported in this paper were performed to test certain aspects of kinetics entity model for malonate anion transporting system. In reality, Band 3 that traverses the membrane 14 times with 911 amino acids or so, is the main transport pathway for organic and inorganic anions in human red blood cells (Giebel and Passow ,1960; Motais ,1975; Cousin and Motais, 1979;Hajjawi and Hider,1987,2009; Jennings and Adame ,1996; Ellory et al,2009; and Yamaguchi et al ,2010). Band 3 protein has more than one substrate (A, B) and more than one product (P, Q). So, how do we do Band 3 kinetics on complicated anion exchange system? The answer is fairly straightforward. We keep one $malonate_{cis}$ (substrate A) fixed, and vary the other $malonate_{trans}$ (substrate B) and obtain a series of hyperbolic plots of V_o vs A at different fixed B concentration and in the absence of allostric behaviour obeys Michaelis-Menten kinetics. This would give a series of linear 1/V vs 1/A Lineweaver-Burk double-reciprocal plots. The pattern of the plots depends on how the $malonate_{cis}$ and $malonate_{trans}$ interact with the transporter (Cook and Cleland, 2007).Hence, Cleland (1963) considered two specific kinetic models for anion-exchange, namely the sequential (simultaneous) and the ping-pong model. In the sequential model, the unloaded transporter contains two sites, one facing outward and one inward. Only after a labelled malonate from the outside and an unlabelled malonate from the inside are bound to AE1 system does a conformational change take place that causes the anion binding sites to exchange position. Malonate anions are then released into solutions at the opposite side of the membrane from their starting point. In ping-pong model, a labelled malonate in the outside solution bind to the outward-facing form of the transport system. A conformational reorientation then occurs giving rise to the inward-facing form of the AE1 system Malonate is then released into the inside solution, and an unlabelled malonate from inside is bound to AE1 system. Reorientation of the AE1 system to the outward- facing form now occurs, and the malonate is then released into the outer solution. The AE1 must be assumed that its conformational change takes place very infrequently if there is no anion bound to the transport system.

There are two camps for an asymmetric anion-exchange mechanism. Salhany (1998), Tanner (2002) and Salhany et al (2003) reported sequential mechanism, where as Jennings et al (1990), Knauf

et al (2002) and Jennings (2005) reported that anion exchange flux through Ban 3 is believed to take place by way of a ping-pong mechanism. One of the most striking aspects of the profile presented in Fig. 4 above is that the intersecting lines on Lineweaver-Burk plots as a function of the $malonate_{trans(intracellular)}$ concentration. Therefore, we suggest a sequential model that may be operative for Band 3, while at the same time seeing evidence for transport site recruitment, which is not consistent with a simple ping-pong model. There are two interacting AE1 sites and an essential ternary complex occurs within the malonate cycle, between an influxing and effluxing ($K_{m(cis)}$ = 21 mM, $K_{m(trans)}$ = 3.2mM). Thus, malonate or competitive inhibitor binding to one site accelerates the release of the transported malonate at the other site of the same membrane surface within such ternary complexes. Thus, the model explains the self -malonate inhibition by the high malonate concentrations (either on the outward site or on the inward site competitively) (Hajjawi and Hider, 1987, 2009).

This is consistent with a binding model where two malonate binding transport sites per Band 3 subunit (Fig. 5). A large anionic probe such as BPS and eosin (intramolecular charge distance, 11.4 Å and 8.8 Å ,respectively) bind the transporter, but they do not need to do so. BPS, eosin and other stilbenesulphonates are anchored to block access to the anion binding site on the subunit; the allosteric site-site interactions enables malonate to unblock the site at the outer surface of the membrane and malonate can displace stilbenesulphonates (Hajjawi and Hider, 1987).

Figure 5: Binding of large anionic probes of the transport anion sites of a Band 3 dimer.



Eosin is shown binding the inward-facing transporter, occupying both open and a closed "modifier" sites. Also, bathophnanthroline disulphonate is shown binding to the outward facing transporter, occupying only the open (outside) site through blocking the channel.

Within the context of a single site ping-pong model, it is impossible for malonate or its competitive inhibitor to accelerate the release of a bound substrate when it approaches the complex from the same membrane surface. This is because the inhibitor and the substrate are competitively proposed to bind to a single mutually exclusive transport site. Jenings (2005) reported the presence of a second binding/transport site for chloride anion transporter AE1. Hence, the single- site ping-pong model cannot explain such flux acceleration!

Dicarboxylates with an intramolecular charge distance less than 4 Å are rapidly transported even if they possess considerable bulk like phthalic acid, though isomers m-phthalate (6.0 Å) and p-phthalate (7.0 Å) inhibit malonate competitively but are not transported. Similarly, the rate of cis-butenedioic acid (maleate, intramolecular charge distance, 3.7 Å) membrane permeation is markedly greater than trans-butenedioic acid (fumarate, intramolecular charge distance, 5.7 Å). Also, larger hydrated inorganic anions (Ganong, 1977) such as Cl^- (3.8 Å), HCO_3^- (6.6 Å), SO_4^{2-} (7.4 Å), and HPO_4^{2-} (10.3 Å) bind to Band 3 transporter to compete competitively with malonate and permeate the cell membranes too. Therefore, a relationship between the physico-stereochemical properties of organic dianions and their relative binding site of Band 3 conform to complex formation {[$dianion_i$ – $transporter_i$] and [$dianion_o$ – $transporter_o$]} for specific flux cycle (Hajjawi and Hider, 1987, 2009), i.e. a simple ping-pong anion mechanism is not feasible.

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