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Original article

Malonate transport in human red blood cells

Omar S. Hajjawi and Robert C. Hider[1]
Department of Chemistry, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, Essex, UK
([1]address for offprints)

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Abstract

Kinetic parameters of [2-^{14}C]malonate uptake by the human erythrocyte membrane have been determined as K_m, 24 mM and turnover number, 5×10^4 s^{-1}. The translocation of this organic dianion is concentration, pH and temperature dependent. Competitive inhibition of malonate uptake by eosin and inorganic anions, strongly implies that a common route exists for both inorganic anions and organic dianions, namely the anion-exchange Band 3 protein. ^{14}C-Malonate which is nonmetabolized in the erythrocyte, could be a useful probe for monitoring anion-exchange in reconstituted Band 3 systems.

Introduction

Cl^-/HCO_3 exchange across erythrocyte membranes plays a central role in the elimination of carbon dioxide from the lungs (1–3). Erythrocytes experience rapid transit (0.5 s) through the pulmonary capillaries (4) and hence, a high rate of anion flux is necessary. This transport process has been associated with the Band 3 protein (5–8), and in view of the high unit fluxes, diffusion via a channel appears likely (9). Although the majority of studies associated with this protein have been centred on inorganic anions (10), organic anions are potentially more informative. By virtue of their differing stereochemistry, they offer a means of investigating the size and specificity of the postulated channel (11). In early studies with organic anions, osmotically induced haemolysis of bovine erythrocytes was implemented in order to monitor anion transport (12, 13). On the basis of these findings, it was suggested that whereas hydrophilic anions are capable of crossing the erythrocyte membrane rapidly through pores, the rate of permeation of hydrophobic anions appeared to be largely dependent on their lipid solubility. Aubert and Motais (14) proposed a 'three point attachment' hypothesis for hydrophilic anions and it was concluded that a selectivity filter of diameter 7.6–9.0 Å was a major determinant of permeability. More recently, it has been demonstrated that organotin-induced anion exchange in human erythrocytes is electrosilent (15). Also, Hoffman *et al.* (16) have reported that the contribution of anion exchange to the conductance of Salamander erythrocyte membranes is negligibly small. These experiments suggest that a tightly coupled 1:1 anion exchange cannot occur via a simple water-filled channel and that a more complicated process occurs probably involving a protein conformational change (8, 17, 18).

The work reported in this paper is directed towards providing more information concerning the specificity of the anion transporter present in

human erythrocytes. Surprisingly, relatively little attention has been devoted towards organic dianions, and although such studies have been reported for bovine erythrocytes, no kinetic parameters are available. By assuming that a facilitated process operates in the membranes (19, 20), we have determined K_m and the turnover number for malonate together with K_j values for the effect of a range of organic and inorganic anions on malonate uptake.

Materials and methods

[2-^{14}C]malonate and [U-^{3}H]inulin were purchased from Radiochemical Centre, Amersham, Bucks, UK; Silicon oil was purchased from Hopkin and Williams, Romford, Essex, UK. All other chemicals were supplied by Sigma (London) Chemical Co., Poole, Dorset, UK, or by BDH Ltd., Poole, Dorset, UK, and were of 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. Human erythrocytes were separated by sedimentation at 2500 g (at r_{av} 13 cm) and 4 °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 (152 mM). Finally, erythrocytes were suspended in Krebs-Ringer bicarbonate (21) (pH 7.4), either 1:10 (v/v) for haemolysis studies or 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 (22). The standard dry weight of erythrocyte suspension (typically $\approx$ 30%) was used to calculate the intracellular fluid for each experiment.

Incubation conditions

Light scattering studies. A slightly modified procedure based on that used by Aubert and Motais (14) for ox erythrocytes was implemented. 1 ml of an isoosmotic dicarboxylate ammonium salt (pH 7.4) was pipetted into a 1 ml silica cuvette, capped and allowed to reach 37 °C. 10 μl of erythrocytes, Krebs Ringer bicarbonate buffer (1:10, v/v) was then added and mixed gently. The change in optical density was monitored at 500 nm on a Varian 634 spectrophotometer, and was recorded graphically. The resulting change of light scattering with time is represented by plotting $(OD_\infty - OD_0) - (OD_t - OD_0)/(OD_\infty - OD_0)$ vs time. This fraction is identical to $1-(OD_t - OD_0)/(OD_\infty - OD_0)$, i.e. $(1 - S_t/S_\infty)$. During experiments of long duration, the incubation medium was mixed manually at 10 min intervals in order to minimise artifacts associated with sedimentation.

[2-^{14}C]malonate uptake studies. [^{14}C]Malonate influx was measured using a slight modification of the procedure reported by Eavenson and Christensen (23) for pigeon erythrocytes. An isoosmotic malonate/sucrose solution (1 ml, pH 7.4) containing 0.05 – 0.25 μCi/ml was pipetted into a test tube and preincubated at 37 °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 silicon oil (ρ 1.07) and centrifuged for 20 s in a Beckmann Microfuge B (24). 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 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 supernatent 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 periods up to 5 min. Fixed time assays were run either for 1 min or 2 min intervals.

Results

Light scattering studies

The influx of malonate was monitored by the change in light scattering as a function of time (Fig. 1). The initial part of this biphasic process was most likely associated with the loss of the erythrocyte discoid character and did not result in the release of haemoglobin. The second part of the progress curve was associated with haemolysis. Both the initial rate of swelling and the rate of haemolysis were found to be proportional to the concentration of malonate (20–100 mM) and were pH dependent with the optimum rate swelling occuring at pH 7.2 ± 0.2. When erythrocytes were incubated in a range of isoosmotic diammonium carboxylates (100 mM, pH 7.4), the rate of swelling was found to be strongly dependent on the structure of the permeant species (Table 1). The sequence of the observed rates reflects a similar permeation specificity to that previously reported for bovine erythrocytes, the more strongly permeable species, causing rapid swelling.

Bathophenanthrolinedisulphonate inhibits the malonate-induced erythrocyte swelling and haemolysis (Fig. 1). In the presence of this anionic

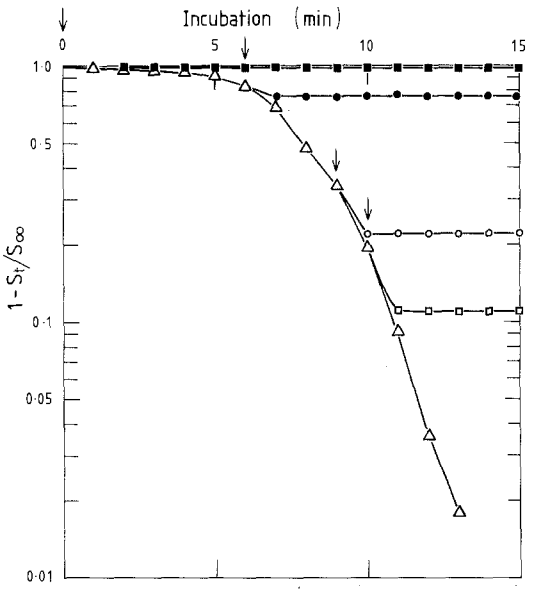

Fig. 1. The influence of bathophenanthroline disulphonate on malonate-induced erythrocyte swelling and lysis. 10 μL of erythrocyte suspension was added to an isoosmotic diammonium malonate (63 nm/sucrose solution (1 ml) at 37 °C. The change in optical density at 500 mM was monitored. A progress curve of log ($1-S_t/S_\infty$) vs time was plotted, where S_t is light scattering at time t and S_∞ is light scattering at infinite time. The arrows indicate when bathophenanthroline disulphonate (1 mM) was added to the erythrocyte suspension. The corresponding values of ($1-S_t/S_\infty$) are given by ■, 0 min; ●, 6 min; ○, 9 min; □, 10 min and △, no addition.

Table 1. Dicarboxylate induced swelling of human erythrocytes.

Dicarboxylate	Mol. Wt.	Intercharge distance (Å)	(k_I (min^{-1})
Oxalate	92	2.7	1.2
Malonate	108	3.5	1.1×10^{-1}
Maleate	116	3.7	5.2×10^{-2}
Fumarate	116	5.7	2.2×10^{-3}
Succinate	118	5.6	1.7×10^{-3}
Glutarate	132	6.1	$<2\times10^{-4}$
Malate	134	5.2	$<2\times10^{-4}$
Adipate	146	6.7	$<2\times10^{-4}$
Tartrate	150	6.1	$<2\times10^{-4}$
Isophthalate	166	6.0	$<2\times10^{-4}$
Phthalate	166	3.7	5.1×10^{-2}
Terephthalate	166	7.0	6.1×10^{-4}

Human erythrocytes suspended in Krebs-Ringer bicarbonate buffer (10 μL 1:10, v/v) were added to 1 ml of diammonium carboxylate (100 mM, pH 7.4, 37 °C). Dicarboxylate induced light scattering was recorded graphically as a function of time. The initial slope (k_I) of the biphasic progress curve was calculated).

probe, light scattering by the erythrocyte remained virtually constant. Because relatively high concentrations of organic dianions (≥20 mM) are necessary to generate measurable light scattering changes, classical Michaelis-Menten Kinetic constants proved difficult to determine.

[2-^{14}C]Malonate uptake studies

Malonate has been established to be nonmetabolized in human erythrocytes (data not presented) and permeates these cells rapidly. As a result it was selected as a probe for isotope uptake studies. The

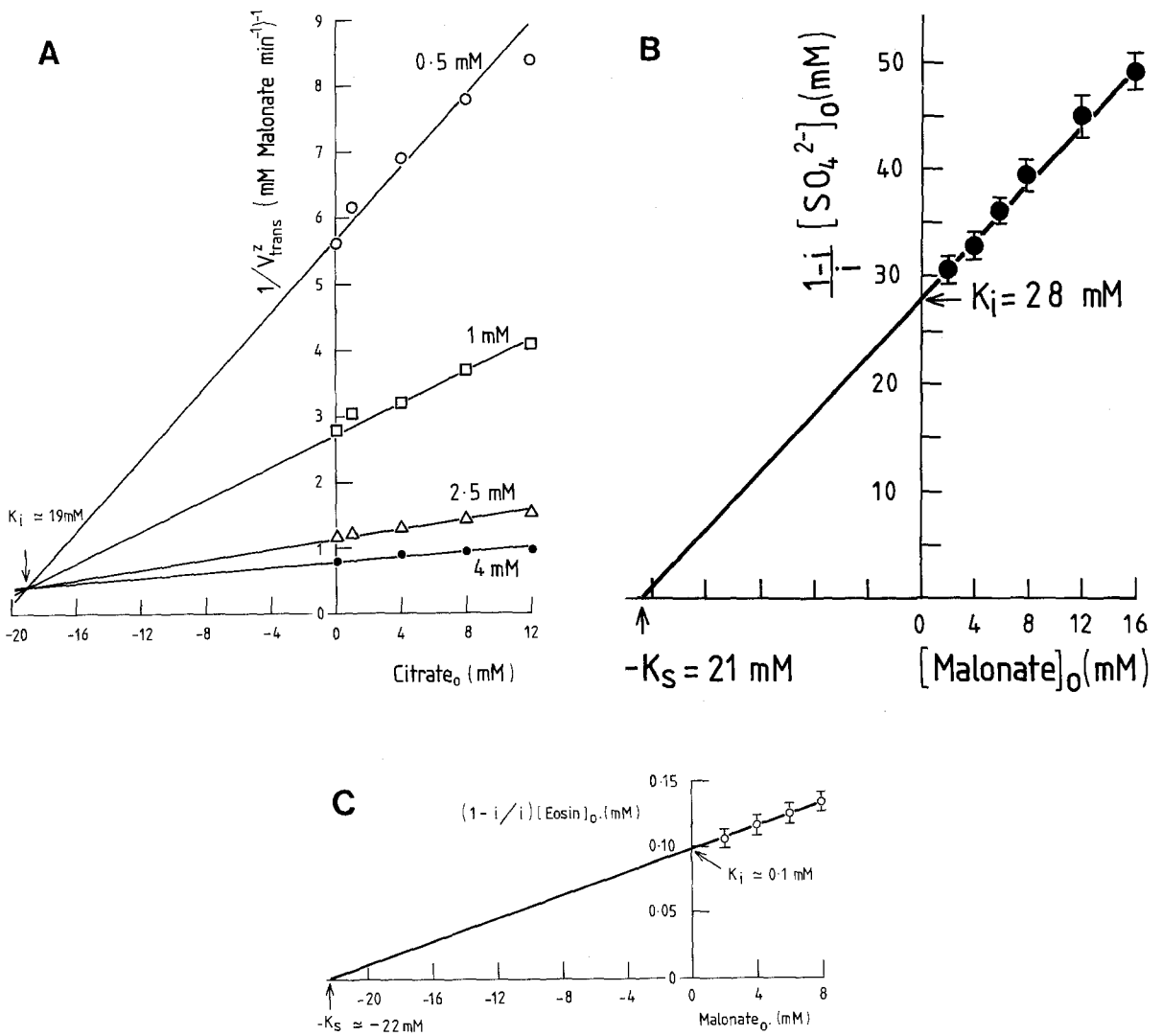

Fig. 2. The effect of inhibitory anions on erythrocyte uptake of ^{14}C-malonate. Erythrocyte Krebs-Ringer bicarbonate buffer suspension (1 ml, 1:1 w/v) was incubated at 37 °C in ^{14}C-malonate sucrose solutions (1 ml) containing a fixed concentration of inhibitory anion. The rate of zero-trans influx of malonate was calculated as a function of concentration of medium anion for the determination of apparent K_I value. (A) Dixon plot for the reciprocal of rate of malonate uptake vs concentration of medium citrate. (B) Hunter-Downs plot for $\frac{(1-i)}{i}$ $[SO_4^{2-}]_{out}$ vs concentration of medium malonate, where i is the fractional inhibition of malonate uptake. (C) Hunter-Downs plot for $\frac{(1-i)}{i}[Eosin]_{out}$ vs concentration of medium malonate.

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. Uptake was analysed in terms of Michaelis-Menten Kinetics. Under the adopted incubation conditions, the Kinetic parameter ($K_{m(out)}$) for malonate influx is an apparent value, accomodating the inhibitory influence of chloride. However, as the extracellular chloride concentration (40 mM) was maintained throughout the entire range of experiments, the apparent Kinetic constants are useful in the analysis of comparative inhibitory potencies of anions. Using the Lineweaver-Burk and Hanes-Woolf plots a K_m value of 24 ± 0.2 mM was obtained. The turnover number, 5×10^4 s^{-1} was determined graphically from the maximum rate of malonate uptake as a function of the estimated number of Band 3 protein molecules per erythrocyte.

The uptake of ^{14}C-malonate was further examined by measuring the inhibitory effect of a range of ammonium salts of mono-, di- and tricarboxylic acids. Pyruvate, oxaloacetate, oxalate, maleate, phthalate and citrate (Fig. 2A) were shown to be powerful competitive inhibitors (Table 2). Other carboxylates, for instance glutarate and tartrate, proved to be weaker inhibitors. Inorganic anions were found to inhibit malonate uptake in a competitive manner (Fig. 2B; Table 2). Furthermore, eosin which is known to bind the external portion of the anion exchange Band 3 protein (25) was also found to behave as a competitive inhibitor (Fig. 2C).

The influence of medium pH on ^{14}C-malonate uptake was monitored over the pH range 6.5–9.5, a range where malonate exists predominantly as a dianion (pK, 2.7; pK_2, 5.3). Changes in the apparent values of both $K_{m(out)}$ and $V_{max}^{zero\text{-}trans}$ were observed. A bell-shaped curve of log V_{max}/K_m versus pH_{out} resulted with maximum rate of malonate uptake in the vicinity of pH 7.4. The influence of temperature (2–40 °C) on ^{14}C-malonate uptake was monitored at pH 7.4. The apparent $K_{m(out)}$ value for malonate remained virtually constant over this temperature range. The corresponding Arrhenius plot for apparent values of $V_{max}^{zero\text{-}trans}$ was linear, yielding an activation energy of 74 ± 3 KJ mol^{-1}.

Discussion

The two experimental techniques reported in this study complement each other. Those dicarboxylates which are capable of rapidly permeating the human erythrocyte, as demonstrated by light scattering (Table 1), possess a high inhibition potency for ^{14}C-malonate uptake (Table 2). As sulphate (Fig. 2B) and chloride (not presented) act as com-

Table 2. K_i Values for anionic inhibitors of erythrocyte malonate uptake.

Inhibitory anion	Molecular weight	K_i, mM	Inhibitory anion	Molecular weight	K_i, mM
Pyruvate	88	16	Isophthalate	166	16
Oxalate	92	6	Phthalate	166	10
Maleate	116	26	Terephthalate	166	10
Fumarate	116	40	Citrate	210	19
Succinate	118	35	HPO_4^{2-}	96	15
Oxaloacetate	132	6	SO_4^{2-}	96	28
Glutarate	132	45	Bathophenanthroline disulphonate	591	0.7
Malate	134	43			
Adipate	146	45	Eosin	692	0.1
Tartarate	150	48			

Conditions of incubation were as reported in Fig. 2. The initial rate of ^{14}C-malonate uptake was monitored in the presence of a fixed concentration of anionic inhibitor (1 – 100 mM). The apparent K_I values were determined from both Dixon and Hunter-Downs plots.

petitive inhibitors of ^{14}C-malonate uptake, it seems most probable that malonate, together with the other carboxylate anions used in this study, are substrates for the Band 3 anion exchange protein. This conclusion is supported by the finding that both eosin and bathophenanthroline-disulphonate (a structural analogue of the stilbene-2,2-disulphonates) inhibit ^{14}C-malonate transport, (Figs. 1 and 2C, Table 2). Furthermore, the observations of a pH optimum of 7.4 and an E_a value of 74 ± 3 KJ mol^{-1} in this study compare well with the values obtained using inorganic anions (17, 26–28). These results consolidate the view that inorganic anions and small organic dianions share the same transport mechanism. This being the case, ^{14}C-malonate uptake could well prove to form the basis of a useful kinetic assay for anion translocation activity in reconstituted systems.

The structure of dicarboxylate anions governs their ability to penetrate erythrocyte membranes, although both permeant and impermeant species compete with ^{14}C-malonate for the anion exchange protein; for example, terephthalate does not permeate the cell rapidly (Table 1) and yet it is a potent inhibitor of ^{14}C-malonate uptake (Table 2). Thus, the simultaneous use of the two techniques reported in this study renders it possible to distinguish between different classes of competitive inhibitor; permeant and nonpermeant.

The anion transport process shows a clear specificity. Dicarboxylates with an intercharge distance less than 4 Å are rapidly transported (Table 1) even if they possess considerable bulk, for instance phthalic acid. Dianionic isomers are processed differently, for instance, although isophthalate, terephthalate and phthalate all inhibit malonate uptake (Table 2) only the isomer with adjacent carboxylate functions, phthalate, is transported. Similarly, the rate of maleate (*cis*, intercharge distance 3.7 Å) uptake is markedly greater than fumarate (*trans*, intercharge distance 5.7 Å). This specificity of dicarboxylate uptake by human erythrocytes is qualitatively similar to that reported for bovine erythrocytes (13, 14).

The critical intercharge distance probably enables the dicarboxylate species to bind to the anion channel in a specific manner, indicating that a complex is formed between the anion and the channel protein during at least one stage of the flux cycle. Clearly, the permeation mechanism of anions is not simply diffusion through a positively charged molecular sieve. Significantly, all the dicarboxylates capable of rapid permeation of human erythrocyte membranes are planar (Fig. 3) and in view of their small intercharge distance, are capable of forming tight bidentate hydrogen bonded ion pairs with the side chain of arginine. Similar complexes are formed between both sulphate, phosphate and arginine and such interactions are quite stable in aqueous solution (29, 30). Treatment of red cells with arginine-selective reagents, such as phenylglyoxal and cyclohexanedione, leads to the inhibition of anion translocation (31–34). These observations suggest that complex formation between arginine and the permeating anion is an essential step in the translocation process.

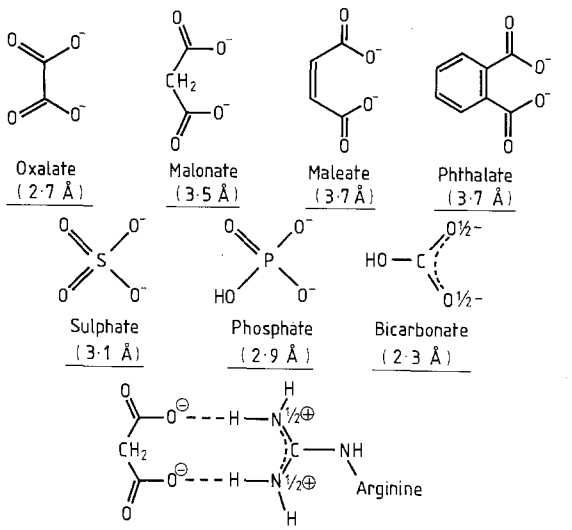

Fig. 3. Structure of permeant dianions. Each dianion is capable of forming a stable (hydrogen bonded + electrostatic) interaction with a guaninidine function, as indicated with the malonate dianion.

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