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Chloride–bicarbonate exchange in red blood cells: physiology of transport and chemical modification of binding sites

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About 80% of the CO_2 formed by metabolism is transported from tissues to lungs as bicarbonate ions dissolved in the water phases of red cells and plasma. The catalysed hydration of CO_2 to bicarbonate takes place in the erythrocytes but most of the bicarbonate thus formed must be exchanged with extracellular chloride to make full use of the carbon dioxide transporting capacity of the blood. The anion transport capacity of the red cell membrane is among the largest ionic transport capacities of any biological membrane. Exchange diffusion of chloride and bicarbonate is nevertheless a rate-limiting step for the transfer of CO_2 from tissues to lungs. Measurements of chloride and bicarbonate self-exchange form the basis for calculations that demonstrate that the ionic exchange processes cannot run to complete equilibration at capillary transit times less than about 0.5 s.

The anion exchange diffusion is mediated by a large transmembrane protein, constituting almost 30% of the total membrane protein. The kinetics of exchange diffusion must depend on conformational changes of the protein molecule, associated with the binding and subsequent translocation of the transported anion.

We have characterized the nature of anion-binding sites facing the extracellular medium by acid–base titration of the transport function and modification of the transport protein *in situ* with group-specific amino acid reagents. Anion binding and translocation depend on the integrity and the degree of protonation of two sets of exofacial groups with apparent pK values of 12 and 5, respectively. From the chemical reactivities towards amino acid reagents it appears that the groups whose pK = 12 are guanidino groups of arginyl residues, while the groups whose pK = 5 are likely to be carboxylates of glutamic or aspartic acid.

Our studies suggest that the characteristics of anion recognition sites in water-soluble proteins and in the integral transport proteins are closely related.

1. Introduction

The tightly coupled exchange diffusion of anions across red cell membranes is mediated by an integral membrane protein, known as band 3 from its location by polyacrylamide gel electrophoresis of solubilized membrane proteins (Fairbanks *et al.* 1971). Band 3 constitutes almost 30% of the membrane protein with about 10^6 molecules per cell. This abundance and the ease with which the cell membrane can be isolated have made the anion exchange system a very suitable subject for studies of the molecular basis of a specific passive transport process.

The transport system mediates the physiologically important exchange of chloride for bicarbonate across the erythrocyte membrane. This anion shift constitutes an important rate-

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limiting step in the overall transfer of carbon dioxide from tissues to lungs, in spite of the abundance of the transport protein.

We shall first report on the tracer exchange of chloride and bicarbonate across red cell membranes under conditions that simulate as closely as possible those of arterial blood. Next we evaluate the role of the anion transport in determining the time course of CO_2 uptake into the blood. Finally we present some recent attempts to identify functionally essential amino acid residues as building parts of the exofacial anion-recognition site of the transport system.

TABLE 1. UNIDIRECTIONAL CHLORIDE AND BICARBONATE FLUXES IN HUMAN RED CELLS

(The rate coefficients of efflux of $^{36}Cl^-$ and $H^{14}CO_3^-$ from labelled red cells into a large extracellular volume were determined by the continuous-flow method (Brahm 1977). Before the measurement of tracer efflux, erythrocytes were equilibrated at 38 °C, pH 7.4, in a medium containing 110 mM KCl, 25 mM $KHCO_3$, 1 mM acetazolamide. The experiments were performed in duplicate and the rate coefficients of tracer efflux shown in the table are the means of two determinations. The unidirectional chloride and bicarbonate fluxes were calculated from the relation

$$J_{uni} = k(V/A)C_i \text{ mol cm}^{-2}\text{ s}^{-1},$$

where k/s^{-1} is the rate coefficient of tracer efflux, $(V/A)/cm$ is the ratio of cellular solvent volume to membrane surface area (4.4×10^{-5} cm, s.d. 0.1, $n = 6$), and $C_i/(\text{mol cm}^{-3})$ is the intracellular concentrations of chloride or bicarbonate. The chloride distribution ratio $^{36}Cl^-_{(\text{cell water})}/^{36}Cl^-_{(\text{medium})}$ was 0.61 (s.d. 0.02; $n = 6$).)

rate coefficients of tracer efflux/s^{-1}			unidirectional fluxes/(10^9 mol cm^{-2} s^{-1})		
donor	$^{36}Cl^-$	$H^{14}CO_3^-$	chloride	bicarbonate	chloride plus bicarbonate
A	12.0	15.6	34.8	10.3	45.1
B	12.2	15.0	35.1	9.8	44.9
C	10.2	14.3	30.9	9.8	40.7
C	10.9	13.1	32.0	8.7	40.7
D	11.6	14.1	34.5	9.5	44.0
D	12.4	16.4	35.9	10.8	46.7

2. UNIDIRECTIONAL CHLORIDE AND BICARBONATE FLUXES

The rapid self-exchange of chloride and bicarbonate is shown in table 1. The unidirectional fluxes were calculated from the efflux rates of radioactive tracers, determined by a continuous flow method under conditions with no net flux of anions across the membranes (Brahm 1977). The results obtained with erythrocytes from four donors illustrate the small interindividual variations of the anion fluxes as well as the reproducibility of repeated determinations on cells from the same donor. The sum of chloride and bicarbonate fluxes is close to 45 nmol cm^{-2} s^{-1}, similar to the maximum chloride flux at 38 °C in a bicarbonate-free system (Brahm 1977).

Chloride and bicarbonate compete for the transport system, the affinity of bicarbonate being higher (Wieth 1979). Under the conditions used for the experiments of table 1, bicarbonate exchanges at a rate that is almost 50% higher than chloride. The average values of the rate coefficients of the exchange processes correspond to half times of 45 ms (bicarbonate) and 60 ms (chloride). As expounded below, these rates cannot be directly applied to evaluate the time course of the physiological net fluxes of chloride and bicarbonate occurring in tissue and lung capillaries.

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3. Physiology of chloride–bicarbonate exchange

(a) *Transport capacity*

The transit time of a red cell through a human pulmonary capillary is about 0.7 s at rest, and decreases to about 0.3 s when the cardiac output is maximally increased (Wagner 1977). Similar, or slightly longer, transit times are believed to apply to peripheral tissue capillaries. To evaluate whether the CO_2 transport capacity of the blood can be made full use of when blood is exposed to the tissue or alveolar p_{CO_2} for such brief periods, it is necessary to consider the distribution of CO_2 between the different forms in which it is transported and the time course of the events that follow a step change in p_{CO_2}.

Table 2. CO_2 uptake by arterial blood

(Values in millimoles per litre of blood; numbers in parenthesis denote concentrations in millimoles per litre of H_2O. The data for equilibrated venous blood are calculated from the parameters listed in the legend to table 3, and assuming that the uncatalysed hydration of CO_2 is complete. 1 Torr ≈ 133 Pa.)

arterial blood (p_{CO_2} = 40 Torr; S_{O_2} = 98 %)	
plasma [HCO_3^-]	13.20 (25.0)
red cell [HCO_3^-]	5.20 (16.5)
dissolved [CO_2]	1.05
carbamino [CO_2]	0.52
equilibrated venous blood (p_{CO_2} = 46 Torr; S_{O_2} = 75 %)	
plasma [HCO_3^-]	14.19 (27.1)
red cell [HCO_3^-]	5.76 (18.1)
dissolved [CO_2]	1.20
carbamino [CO_2]	0.76
CO_2 *uptake*	
Δ (plasma [HCO_3^-])	0.99; 51 %
Δ (red cell [HCO_3^-])	0.56; 29 %
Δ (dissolved [CO_2])	0.15; 8 %
Δ (carbamino [CO_2])	0.24; 12 %
total	1.94; 100 %

At rest, with a cardiac output of 5 l min^{-1}, about 2 mmol CO_2 is taken up per litre of blood. The distribution of CO_2 is shown in table 2. By far the largest fraction of CO_2 is transported as HCO_3^-, and we shall focus on this aspect of CO_2 transport. The steps involved in the transport of CO_2 as HCO_3^- are illustrated schematically in figure 1*a*. When arterial blood is exposed to the higher tissue p_{CO_2}, CO_2 enters the plasma by diffusion through the endothelial cell membranes. This rapid process takes place with a minimal p_{CO_2} gradient. Bicarbonate formation in plasma, by the uncatalysed hydration of CO_2, is too slow to be of significance for capillary CO_2 uptake. The continued uptake of CO_2 by the blood is therefore critically dependent on the intracellular catalysed hydration. The activity of the red cell carbonic anhydrase is sufficient to accelerate HCO_3^- formation up to 20000 times (Wistrand 1981). Within the red cell the overall reaction

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \qquad (1)$$

continues rapidly from left to right as long as the reaction products are removed. The removal of H^+ is very efficient, because the buffer properties of haemoglobin are amplified by the O_2-linked pK shifts that accompany the transition from oxyhaemoglobin to deoxyhaemoglobin

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(Edsall 1980), the so-called Haldane effect. The removal of HCO_3^- is likewise efficient, because the HCO_3^- is exhanged for extracellular Cl^-. This exchange leads to a net efflux of HCO_3^- as long as

$$[HCO_3^-]_{cell}/[HCO_3^-]_{plasma} > [Cl^-]_{cell}/[Cl^-]_{plasma}. \quad (2)$$

The importance of the Haldane effect and the anion exchange for CO_2 transport is illustrated in table 3, where we have calculated the CO_2 uptake into arterial blood as it is exposed to a step change of p_{CO_2} from 40 to 47 Torr† on entering the tissue capillary. The calculations assume

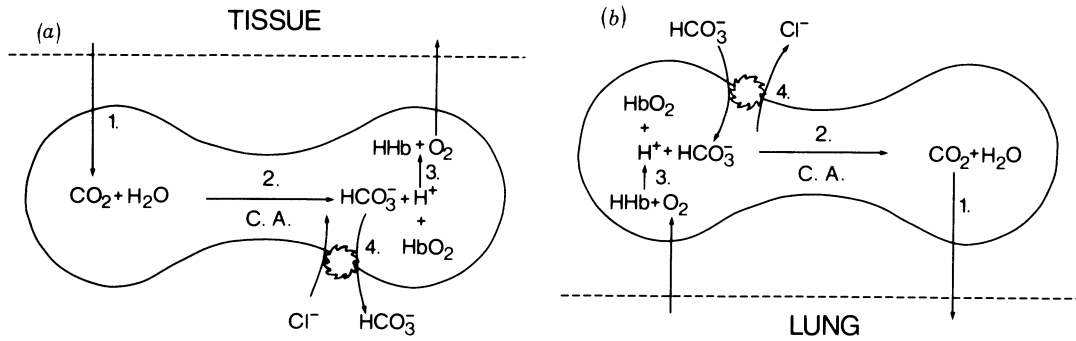

FIGURE 1. Schematic representation of the partial processes involved in the transport of CO_2 as HCO_3^- in blood. (*a*) The sequence of events in a tissue capiliary: 1, diffusion of CO_2 into the red cell; 2, carbonic anhydrase (C.A.)-catalysed hydration of CO_2 to carbonic acid and subsequent dissociation into HCO_3^- and H^+; 3, buffering by haemoglobin (Hb) of the protons thus produced. The buffering is in part achieved by the oxygen-linked protonation of Hb accompanying O_2 unloading; 4, exchange of intracellular HCO_3^- for extracellular Cl^- mediated by the anion exchange system. (*b*) The reverse sequence of events in a lung capillary. See text for further explanation.

that the transit time is long enough to ensure equilibration of all reactions but the slow extracellular hydration of CO_2, which was included in the calculations of table 2. The calculations are based on the value for Haldane coefficient of Siggaard-Andersen (1971), the carbamino data of Bauer & Schröder (1972), and 'standard' values for the other parameters; details of the calculations will be presented elsewhere.

The figures in table 3 illustrate the importance of both the Haldane effect and anion exchange for CO_2 uptake by the blood. In the absence of both Haldane effect and anion exchange (upper left panel), CO_2 uptake is only about 40% of the uptake seen when both mechanisms are used (lower right panel). The relative contributions of the Haldane effect and the anion exchange to HCO_3^- formation are about equal. A comparison of the four panels in table 3, either horizontally or vertically, shows that HCO_3^- uptake is increased by about 65% when either process is activated. Absence of either will thus lead to a considerably decreased ability to load CO_2 into capillary blood. The consequences of extracellular CO_2 hydration and buffering are seen by comparing the figures in the lower right panel in table 3 with the figures for equilibrated venous blood in table 2. Note, in particular, that venous blood has a lower p_{CO_2} than capillary blood (Bidani *et al.* 1978).

A table similar to table 3 can be constructed for passage through the pulmonary capillaries, where the CO_2 release follows the sequence illustrated in figure 1*b*. The mirror-image reactions of loading and unloading CO_2 are driven by the difference in p_{CO_2}. The transport of CO_2 from tissues to lungs can thus be visualized as being driven by the overall p_{CO_2} difference between

† 1 Torr ≈ 133 Pa.

the tissues and the alveolar air. Increased resistance to CO_2 transport will thus be reflected in an increased tissue–lung p_{CO_2} difference to ensure elimination of the metabolically produced CO_2. The largest of the resistances involved in CO_2 transport is associated with anion exchange (Wieth & Brahm (1980); Crandall & Bidani 1981). Inhibition of the anion exchange, e.g. by salicylate ingestion (Wieth & Brahm 1978; Crandall *et al.* 1982), must therefore be accompanied by an increased tissue–lung p_{CO_2} difference. The sequence of events can be understood by

TABLE 3. THE IMPORTANCE OF THE HALDANE EFFECT AND ANION EXCHANGE FOR CO_2 UPTAKE IN BLOOD

(Values in millimoles per litre of blood; numbers in parentheses denote concentrations in millimoles per litre of H_2O. The following parameters were used: haematocrit, 0.45; intracellular H_2O volume, 0.35 per litre of blood; extracellular H_2O volume, 0.528 per litre of blood; anion distribution ratio, 0.66; osmolality, 300 mosmol kg^{-1}; plasma Cl^- concentration, 110 mm†; plasma HCO^- concentration, 25 mm; intracellular buffer capacity, 50 mmol H^+ per litre of red cells per pH unit; extracellular buffer capacity, 7 mmol H^+ per litre of plasma per pH unit; CO_2 solubility coefficient, 0.03125 mmol per litre of H_2O per torr; pK_{CO_2}, 6.10; haemoglobin concentration, 22 meq per litre of red cells. Carbamino dissociation constants (overall values): arterial blood, p_{CO_2} = 40 Torr, $K_{carb}^{HbO_2}$ = 23.3 mm; K_{carb}^{Hb} = 7 mm; all other blood samples, $K_{carb}^{HbO_2}$ = 26 mm, K_{carb}^{Hb} = 8 mm.)

		without anion exchange	with anion exchange
CO_2-loaded arterial blood (p_{CO_2} = 47 Torr; S_{O_2} = 98 %)			
	plasma $[HCO_3^-]$	13.20 (25.1)	13.72 (26.1)
	red cell $[HCO_3^-]$	5.75 (18.2)	5.58 (17.5)
	dissolved $[CO_2]$	1.24	1.24
	carbamino $[CO_2]$	0.55	0.55
uptake	Δ (plasma $[HCO_3^-]$)	0; 0 %	0.52; 46 %
uptake	Δ (red cell $[HCO_3^-]$)	0.55; 71 %	0.38; 34 %
uptake	Δ (dissolved $[CO_2]$)	0.19; 25 %	0.19; 17 %
uptake	Δ (carbamino $[CO_2]$)	0.03; 4 %	0.03; 3 %
uptake	total	0.77; 100 %	1.12; 100 %
end-capillary blood (p_{CO_2} = 47 Torr; S_{O_2} = 75 %)			
	plasma $[HCO_3^-]$	13.20 (25.1)	14.05 (26.9)
	red cell $[HCO_3^-]$	6.12 (19.3)	5.84 (18.2)
	dissolved $[CO_2]$	1.24	1.24
	carbamino $[CO_2]$	0.78	0.78
uptake	Δ (plasma $[HCO_3^-]$)	0; 0 %	0.85; 44 %
uptake	Δ (red cell $[HCO_3^-]$)	0.92; 67 %	0.64; 33 %
uptake	Δ (dissolved $[CO_2]$)	0.19; 14 %	0.19; 10 %
uptake	Δ (carbamino $[CO_2]$)	0.26; 19 %	0.26; 13 %
uptake	total	1.37; 100 %	1.94; 100 %

† In this context the symbol mm is used to represent millimoles per kilogram (millimolal).

considering the consequences of a sudden increase in the resistance to CO_2 uptake in the tissue capillaries: tissue p_{CO_2} rises as long as the rate of CO_2 production exceeds the rate of CO_2 uptake in the blood; the increased p_{CO_2} will not only act to increase the rate of CO_2 uptake in the blood, but will also act as a strong stimulus for the central chemoreceptors and thus elicit a hyperventilatory response and lower the alveolar p_{CO_2}. The result is a new steady state where CO_2 elimination again equals production – at the expense of an increased tissue–alveolar p_{CO_2} difference. A similar series of events will, of course, be seen with other manoeuvres that impede the uptake and release of CO_2 from blood, e.g. almost complete inhibition of carbonic anhydrase (Wistrand 1981).

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(b) Time course of bicarbonate–chloride exchange

The time course of CO_2 uptake and release has been the subject of several numerical analyses, but data on anion transport that can form the basis for the evaluation of the role of anion exchange as a rate-limiting factor have only recently become available (Chow *et al.* 1976; Wieth & Brahm 1980). The limitations imposed by the anion exchange depends on the time course of the net anion shifts in relation to the capillary transit time. It is therefore important to be able to estimate the rate constant of the net anion shifts that occur in capillary blood. It is indeed possible to obtain an approximate expression for this rate constant, because the net HCO_3^- flux through the anion exchange system is an approximately linear function of the intracellular $[HCO_3^-]$ over the physiological concentration range (Wieth & Brahm 1980; see also the discussion below). Anion exchange can therefore be approximated as a first-order process, if we assume that $[HCO_3^-]$ at time $t = 0$ jumps from its initial value in arterial blood to a new, constant value (the end-capillary concentration). This assumption implies, among other things, that the delays related to diffusion, CO_2 hydration and H^+ buffering are disregarded (steps 1–3 in figure 1). The rate constant for this idealized anion shift, k', can be expressed as (Wieth & Brahm 1980):

$$k' = J(0)/M_{\text{total}}, \tag{3}$$

where $J(0)$ denotes the initial net efflux of HCO_3^- at $t = 0$ (in moles per square centimetre per second), and M_{total} denotes the total amount of HCO_3^- that leaves the cells during the exchange (in moles per square centimetre membrane area).

The magnitude of $J(0)$ can be estimated from the unidirectional flux data in table 1 and information about the relative affinities of HCO_3^- and Cl^- for the exchange mechanism. Net HCO_3^- flux occurs during the transient anion disequilibrium imposed by the p_{CO_2} increase (see (2)). The disequilibrium is imposed on the intracellular fluid and will not initially change the unidirectional influxes of HCO_3^- and Cl^-. A sudden increase in intracellular HCO_3^- concentration by X mm will consequently lead to a net HCO_3^- efflux equal to the difference between the new and the pre-existing unidirectional effluxes:

$$J(0) = J_{\max}\left\{\frac{([HCO_3^-]+X)/K_{HCO_3^-}}{1+[Cl^-]/K_{Cl^-}+([HCO_3^-]+X)/K_{HCO_3^-}} - \frac{[HCO_3^-]/K_{HCO_3^-}}{1+[Cl^-]/K_{Cl^-}+[HCO_3^-]/K_{HCO_3^-}}\right\} \tag{4}$$

$$\text{or}\quad J(0) = X\left\{\frac{(J_{\max}/K_{HCO_3^-})(1+[Cl^-]/K_{Cl^-})}{(1+[Cl^-]/K_{Cl^-}+([HCO_3^-]+X)/K_{HCO_3^-})(1+[Cl^-]/K_{Cl^-}+[HCO_3^-]/K_{HCO_3^-})}\right\}, \tag{5}$$

where $J_{\max}$ is the maximal unidirectional exchange flux (assumed to be the same for HCO_3^- and Cl^-), and K_{Cl^-} and $K_{HCO_3^-}$ denote the intracellular concentrations of either Cl^- or HCO_3^- necessary for half-maximal exchange. Reasonable 38 °C estimates for $J_{\max}$, K_{Cl^-} and $K_{HCO_3^-}$ are 45×10^{-9} mol cm^{-1} s^{-1}, and 45 and 30 mm respectively, while $[Cl^-]$ and $[HCO_3^-]$ are 72.6 and 16.5 mm respectively. The expression within the curly brackets in (5) has the dimensions centimetres per second and can formally be visualized as a permeability coefficient for net HCO_3^- efflux (equal to net Cl^- influx) through the exchange pathway. This analogy is strengthened by noting that the numerical value of the expression is essentially independent of the magnitude of X (the variation is less than 6% for concentration changes less than 6 mm). Formally we can thus write

$$J(0) = X.P_{\text{ex}} \tag{6}$$

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where P_{ex} denotes the permeability coefficient for the net HCO_3^- flux. Using the numbers above we find that P_{ex} is about 3.9×10^{-4} cm s^{-1}, in good agreement with the data for *in vitro* net HCO_3^- efflux given by Chow *et al.* (1976). We stress that the permeability coefficient in (6) is a purely phenomenological parameter with no mechanistic implications, except that we operate in a concentration range where the net HCO_3^- flux varies linearly with the intracellular HCO_3^- concentration. We also stress that the net HCO_3^- efflux arises as a relatively small difference between two large unidirectional fluxes.

The amount of HCO_3^- per unit membrane area that leaves the red cells during the anion shift is, disregarding H_2O shifts and changes in the anion distribution ratio,

$$M_{total} = (X/r_{Cl^-})V_{pl}/A, \tag{7a}$$

where r_{Cl^-} denotes the anion distribution ratio, and V_{pl} and A denote the plasma H_2O volume and total red cell membrane area per litre of blood, respectively. Equation (7*a*) is a rough approximation, as the small H_2O shifts and the increase of the anion distribution ratio that accompany the intracellular HCO_3^- production have surprisingly large effects on the distribution of the HCO_3^- produced during capillary passage. If we correct for these complications M_{total} becomes

$$M_{total} = [\{X(1-H)0.96\}/(A'Hr_{Cl^-})]/f_{cor}, \tag{7b}$$

where the correction factor f_{cor} denotes how much (7*a*) overestimates the anion shift. The exact expression for f_{cor} is difficult to obtain in closed form, but it is possible to obtain a linearized approximation:

$$f_{cor} = \frac{1 + [HCO_3^-]\{0.0033(1/r_{Cl^-} - 1) + \alpha_H(1-H)0.96/r_{Cl^-}^2\}}{(1-0.0033[HCO_3^-]) - [HCO_3^-]\ (0.7H/\{0.96(1-H)\})\{0.0033 + \alpha_H(1-H)0.96/r_{Cl^-}\}}, \tag{8}$$

where α_H corrects for the change in r_{Cl^-},

$$\alpha_H = \{0.01058 - r_{Cl^-}(0.00471 - 0.00127H)/(1-H)\}/H, \tag{9}$$

A' is the surface-area : volume ratio of red cells (1.63×10^4 cm^{-1}), H is the haematocrit, and the factors 0.96 and 0.7 are used to convert from total plasma or cell volumes to the respective H_2O volumes. We finally obtain our estimate for k' as

$$k' = \{6.6Hr_{Cl^-}/(1-H)\}f_{cor}, \tag{10}$$

where we have used the values for P_{ex} and A' listed above. The correction factor, f_{cor}, is appreciable. If $H = 0.45$, $r_{Cl^-} = 0.66$ and $[HCO_3^-] = 16.5$ mm we find that $f_{cor} = 1.57$ and $k' = 5.6$ s^{-1}. If $H = 0.3$, on the other hand, and the other parameters are unchanged we find that $f_{cor} = 1.95$ and $k' = 3.6$ s^{-1}. We thus find that the anion exchange under normal conditions should be 90 % completed after 400–500 ms, while 90 % completion will not be reached before 600–700 ms for the anaemic individual. The total HCO_3^- uptake is 90 % completed somewhat earlier than one would estimate from k', as a significant fraction of the HCO_3^- is transported in the red cells (see table 3). We can thus conclude that the anion exchange mechanism, despite its great capacity is barely able to handle the load put upon it under resting conditions and that the bicarbonate distribution will not reach equilibrium in the blood at transit times below 0.5 s. This conclusion is in agreement with the $^{13}CO_2$ rebreathing data of Piiper *et al.* (1980), where it was shown that the overall resistance to CO_2 elimination is slight at rest, but becomes appreciable during exercise.

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4. The nature of exofacial anion-binding sites

Current models of the anion exchange mechanism assume that the unidirectional translocation of an anion (i.e. one half cycle of the 1 : 1 exchange process) is triggered when the anion binds to a 'site' (Jennings 1982). Passage of the ion through the transport protein is associated with a conformational change, which makes the transport system ready to transport another anion only from that side of the membrane from which the last transported anion has been

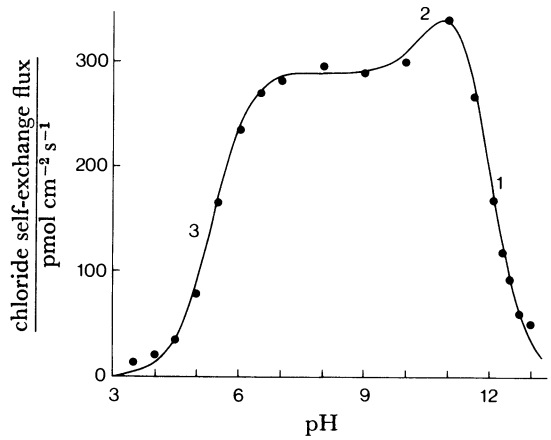

Figure 2. Extracellular titration of chloride self-exchange fluxes in resealed human erythrocyte ghosts between pH 3.5 and 13 (0 °C, 165 mM KCl). Chloride transport was determined with ghosts having an initial pH of 7.2–8. Three sets of titratable groups were identified; (1) groups with a pK of 12, (2) the purported modifier sites with a pK of about 11 (Wieth & Bjerrum 1982), and (3) an acid group that is titrated from the external side of the membrane with an apparent pK of about 5.2 (Milanick & Gunn 1982; Wieth & Brahm 1982).

released. The transport system is thus locked in a stable configuration until another transportable anion binds to the exchange gate. Rapid interconversions (*ca.* 10^5 s^{-1}) between these two configurations are triggered by rapidly permeating anions (chloride and bicarbonate). The strict alternating exposure of the opening of the exchange gate to the two sides of the membrane accounts for the 'ping-pong carrier kinetics' of the transport system (Gunn & Fröhlich 1979), for its ability to remember the direction of the last transport event, and for its inability to mediate large anion net fluxes.

Studies on the kinetics of anion transport show that the apparent affinities of halides for the transport system are about 15-fold higher at the outside than at the inside of the membrane (Gunn & Fröhlich 1979); but it is not known whether this results from a true structural asymmetry of the anion-binding regions at the two sides of the membrane. To gain more information about the characteristics of anion binding, we have examined the properties of exofacial anion recognition sites with methods used to characterize anion-binding sites in enzymes. As a working hypothesis we assume that the interactions between a transported anion and the transport protein are similar to those that determine the binding of anions to water-soluble proteins like haemoglobin and anion-binding enzymes.

Functional anion-binding sites in the active centres of enzymes are composed of many different amino acid residues, which act in concert to modulate substrate binding and the subsequent catalytic process. Functionally essential amino acid residues have been identified by chemical modification with group-specific amino acid reagents designed to modify groups,

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which are likely to be critical for function, as judged for example from the pH dependence of catalysis (Means & Feeney 1971). We have used a similar strategy in a search for functionally important amino acid residues at the exofacial anion-binding site of the transport system.

(*a*) *Extracellular titration of the anion transport system*

First, we examined the acid–base properties of critical groups exposed to the medium by studying chloride self-exchange as a function of extracellular pH (figure 2). These experiments were based on our observation that extracellular pH at 0 °C can be varied between 3 and 13 without any irreversible effects on the transport system, provided that the intracellular pH is

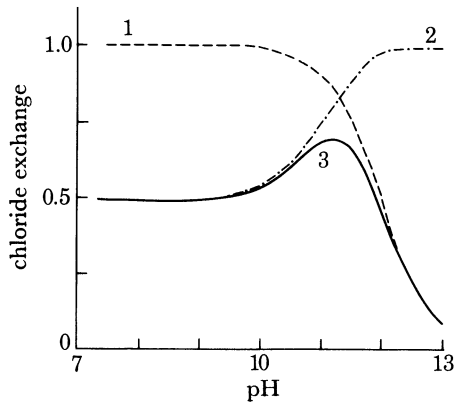

FIGURE 3. Schematic diagram interpreting the alkaline pH dependence of chloride self-exchange (3) as a result of two pH-dependent functions: (1) titration of a group that permits transport only when protonated, and (2) titration of an inhibitory modifier site, inhibiting transport only when anions are bound to the protonated form. (From Wieth & Bjerrum 1982.)

between 7 and 8 (Wieth & Bjerrum 1982; Wieth & Brahm 1982). The transport function is affected by the titration of at least three classes of groups, two in the alkaline range (marked 1 and 2 in figure 2) and a third group in the acid range (marked 3). The inhibited transport is activated when the titration moves from alkaline towards acid pH, as if transport depends on the protonation of groups with an apparent pK of 12. The pK is chloride-dependent, decreasing linearly with $\log_{10}[Cl^-]_{out}$ between an extracellular chloride concentration, $[Cl^-]_{out}$, of 100 and 15 mM. Continuing the titration a small hump is observed at pH 10–11 (marked 2 in figure 2). This maximum, which is more pronounced at higher chloride concentrations, is ascribed to the titration of a so-called modifier site, characterized kinetically by Dalmark (1976). Anion binding to this site is supposed to inhibit the exchange. We have found that the inhibitory effects of chloride, iodide and *N*-(4-azido-2-nitrophenyl)-2-aminoethanesulphonic acid (NAP-taurine) are all abolished by the deprotonation of groups with a pK of about 11 (Wieth & Bjerrum 1982). Figure 3 shows that the titration of the transport function in the alkaline pH range is adequately accounted for by assuming the existence of two classes of titratable, transport-regulating groups: one set with a pK of 12, which must be protonated to facilitate transport, and another set with a pK of about 11, which only exerts an inhibitory modifier effect when protonated.

The third class of titratable groups (marked 3 in figure 2) has an apparent pK of 5.2–5.4 at an extracellular chloride concentration of 165 mM (Wieth & Brahm 1982). The pK decreases

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moderately with extracellular chloride, being 4.8 at a concentration of 2 mM. It is likely that protonation of this set of groups converts the transport system from a form transporting monovalent anions into a different form facilitating the transport of divalent ions, a reaction already proposed in the first model of chloride and sulphate transport by a titratable carrier (Gunn 1972). Milanick & Gunn (1982) have shown that the pK of the group, which was 5 at 6 mM chloride in the absence of sulphate, increases to 5.95 at saturating sulphate concentrations. The

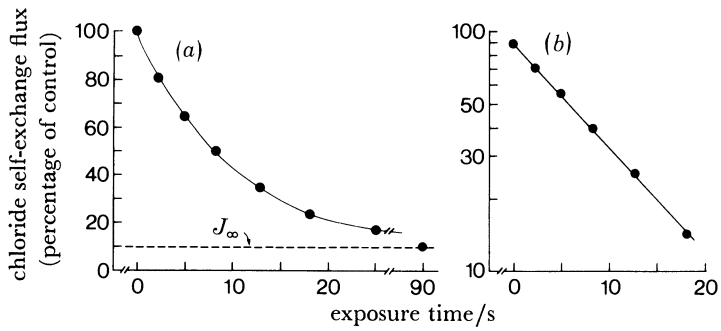

FIGURE 4. Rate of irreversible inactivation of the chloride exchange system with phenylglyoxal (PG). (*a*) Chloride self-exchange flux (as a percentage of the transport capacity of an untreated control sample) as a function of the duration of exposure to 18 mM PG (38 °C, 165 mM KCl, extracellular pH 10). PG treatment was carried out on resealed ghosts with an initial intracellular pH of 6.6. Subsequent flux determinations were carried out at pH 7.2 at 0 °C. Maximum inactivation after prolonged exposure (J_∞) was 10 % of control flux (J_0). The inhibitable flux is therefore (100 − 10) % = 90 %. (*b*) The irreversible inactivation of the inhibitable flux ($J_0 - J_\infty$) proceeds as an apparent first-order reaction at fixed concentrations of PG and extracellular chloride and at fixed pH. (From Wieth *et al.* (1982).)

transport protein thus contains two sets of groups whose proton affinities are affected by anionic ligands. It is an interesting possibility that the acid group may be involved as a proton donor in the co-transport of hydrogen ions with sulphate (Jennings 1976) and with chloride (Jennings 1978), since both processes are mediated by the fully protonated form of the transport system.

(*b*) *Chemical modification of amino acid side chains that regulate transport*

The information that monovalent anion transport depends critically on at least two classes of titratable groups exposed to the extracellular phase, one set with a pK of about 12 and another set with a pK of about 5, allows us to speculate about which amino acid side chains could be involved.

(i) *Arginyl modification with phenylglyoxal*

The guanidino group of an arginyl residue is the likely candidate for an amino acid side chain with a pK of 12. Typical pK values of other positively charged residues (α-amino groups, ε-amino groups of lysine, and imidazoles of histidine) are all significantly lower.

Phenylglyoxal (PG) reacts preferentially with the guanidino groups of arginyl residues at alkaline pH and only slowly with α- or ε-amino groups (Takahashi 1968). Moreover, ^{14}C-labelled PG is stable, and can be used to locate modified residues in proteins. Figure 4 shows that PG is, in fact, an irreversible inhibitor of anion exchange under conditions where we only expect to modify extracellularly exposed arginyl residues (Wieth *et al.* 1982). PG readily permeates the red cell membrane: the half-time of equilibration is 40 ms at 25 °C. When the

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intracellular pH is neutral, however, only those arginyl residues that are exposed to the alkaline medium react with PG. The time course of the irreversible inactivation of the anion transport system, which accompanies the chemical modification of red cell membranes, is shown in figure 4. By varying the pH and anionic composition of the reaction medium we found that the kinetics of transport inactivation was fully compatible with the assumption that PG only reacts with the deprotonated form of the functionally essential arginyl residues, which, in agreement with our titration studies, were found to have a chloride-dependent pK that reaches a maximum value of 11.7 (cf. figure 6 of Wieth *et al.* 1982). Two reversible inhibitors of anion transport, salicylate and DNDS (4,4-dinitrostilbene-2,2′-disulphonic acid), both of which inhibit the transport function in a competitive fashion (Crandall *et al.* 1982; Fröhlich 1982), decrease the rate coefficient for inactivation, further evidence that the reactivity of the groups is determined by anion binding. Our conclusion, that arginyl residues are critical for the function of the anion transport system, is supported by the finding of Zaki (1981, 1982) that sulphate transport is irreversibly inhibited after membrane modification with a different arginine-specific reagent 1,2-cyclohexanedione.

A puzzling, but persistent, observation is that complete inactivation of anion transport was not obtained even after prolonged treatment with PG (figure 4). The residual flux was 9.1% (s.e.m. 0.3, $n = 20$). A possible explanation would be that the maximally modified transport system – by analogy with findings for certain enzymes (Takahashi 1968) – operates at one-tenth of the rate of the intact system. We can exclude this possibility, because the residual flux of the transport system is completely inhibited after covalent reaction of the modified membranes with a number of molecules of 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) that is sufficient to label only 10–15% of the transport protein molecules (Wieth *et al.* 1982). Complete inhibition (98–99%) can also be attained when PG-treated cells are washed free of PG at room temperature and are later subjected to a second PG treatment. We conclude that a fraction of 10–15% of the band 3 molecules are refractory to the first PG treatment and that 90% of this unreacted fraction can be modified during a second exposure to the reagent. The number of band 3 molecules that do not react on the first treatment is similar to the number of band 3 molecules that are anchored to the cytoskeleton by means of an intermediate protein, ankyrin (Bennett & Stenbuck 1980). If indeed the cytoskeleton-bound band 3 molecules are refractory to treatment with PG, our results show that there is a dynamic equilibrium between bound and free band 3 molecules, equilibration being obtained by washing the cells at room temperature. If so, some kind of transmembrane cooperativity exists in band 3, since the reactivity of an exofacial group is abolished by the immobilization of the protein molecule at an intracellular site.

We have examined the stoichiometric relation between PG binding and the location of PG in band 3 by using ^{14}C-labelled PG, as reported in preliminary communications (Bjerrum *et al.* 1982*a*, *b*): maximal inactivation of the anion transport system can be achieved by the binding of enough PG to modify only 2 out of the 44 arginyl residues in band 3 when the reaction is performed in a medium containing 165 mM KCl. When chloride is replaced with sucrose or sucrose–citrate, the rate of inactivation is substantially increased and maximum transport inactivation is obtained after binding enough PG to modify only one arginyl residue in band 3. Importantly, PG binds to a peptide segment different from that to which DIDS binds covalently. Extracellular treatment of the membranes with chymotrypsin produces two fragments from band 3, a DIDS-binding 65 kDa peptide and a 35 kDa fragment, which is the

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one that is labelled with PG. We conclude that both these peptide fragments contribute to form the functional exofacial anion-binding site. The singular reactivity of only one or two arginyl residues in band 3 may reflect the localization of these critical groups in a mixed hydrophobic–hydrophilic environment, thus lowering the pK of the critical groups relative to that of other groups facing the extracellular medium.

After the modification of arginyl residues, only 50 % of the band 3 molecules will bind DIDS, when the DIDS treatment is carried out at DIDS concentrations below 10 μM (30 min, 38 °C). We believe this 'half of the sites' reactivity is related to the presence of band 3 as dimers or higher oligomers in the membrane (see below).

(ii) *Chemical modification of membranes with carbodiimides*

In our search for the identity of the amino acid residue responsible for the titratable transport function with a pK of about 5 (see figure 2), we first thought of the carboxyl group of a glutamic or aspartic acid residue. An imidazole group cannot be excluded with certainty, but its pK would be unusually low, and the pK of the transport function is not changed significantly when the temperature is raised from 0 to 10 °C. This result is expected for a carboxyl group, which has an insignificant ionization enthalpy, whereas the pK of a histidyl residue with an ionization enthalpy of 29.3–33.5 kJ mol^{-1} (Cohn & Edsall 1943) should decrease measurably with the temperature increase.

Carboxylates can be modified with water-soluble carbodiimides (Means & Feeney 1971), and partial inhibition of sulphate transport has been obtained after reacting red cells with carbodiimides (Deuticke 1977). Further exploration of transport modification is difficult because the membranes are destroyed by prolonged treatment, lysis being observed after 15 min incubation with a permeating carbodiimide (1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulphonate; CMC) at 38 °C. The membrane damage may be due to modification of the intracellularly exposed proteins in the cytoskeleton. We consequently decided to attempt selective modification at the external side of the membrane. We have used 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)-carbodiimide iodide (EAC), a water-soluble carbodiimide (George & Borders 1979) that carries a quaternary ammonium group, which should prevent the reagent from permeating the cation-tight membrane. The structure of the cationic form of EAC is

$$CH_3CH_2—N{=}C{=}N—(CH_2)_3\overset{+}{N}(CH_3)_3.$$

EAC does indeed inactivate the anion exchange irreversibly. The time course of irreversible transport inactivation is shown in figure 5. Irreversible inhibition of about 50 % of the transport capacity was obtained with a rate of 0.35 min^{-1}, with 30 mM EAC, but subsequent inactivation of the remaining transport function occurred at a rate that was at least 100-fold slower. The decrease in the rate of inactivation found after 10 min exposure in the experiments of figure 5 is not due to decomposition of the reagent, because only the slow component of the modification is observed when the membranes are subjected to a second treatment with freshly prepared solutions of EAC. We are thus again faced with the problem whether a residual transport activity is caused by a decreased transport rate affecting all transport molecules, or whether only a fraction of the transporters have been successfully modified.

Anion transport is irreversibly inhibited in intact cells after covalent binding of one DIDS molecule per band 3 molecule (Ship *et al.* 1977). It was possible to demonstrate that EAC modifies 50 % of the transporters by comparing the effect of covalent binding of DIDS on

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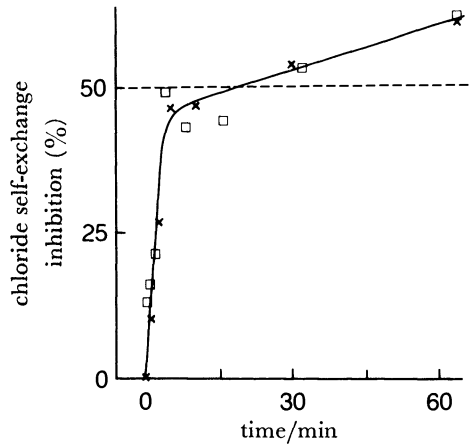

FIGURE 5. Time course of the irreversible inactivation by EAC of chloride self-exchange in resealed human erythrocyte ghosts. Resealed ghosts, washed in unbuffered 165 mM KCl, were injected into a 38 °C solution of 30 mM EAC, 165 mM KCl 165, buffered at pH 5.75 with 5 mM 2-(*N*-morpholino)-ethanesulphonic acid (MES). The pH increased to 6 after the addition of ghosts. The reaction was stopped by diluting samples of the suspension into a large volume of ice-cold 165 mM KCl buffered with 2 mM tris-(hydroxymethyl)aminomethane (Tris). The final pH of the mixture was above 9 at 0 °C. The cells were washed repeatedly in the flux medium (165 mM KCl, 2 mM phosphate, pH 7.3, at 0 °C) before the measurement of the unidirectional $^{36}Cl^-$ efflux; EAC treatment had no effect on cell volume, so the fractional inhibition (I) shown on the ordinate could be calculated from the rate coefficients of chloride efflux for the inhibited ghosts (k_i) and for an untreated control sample, $k_0 : I = (1 - k_i/k_0) \times 100$. The results from two experiments are denoted by different symbols. Further explanation is given in the text.

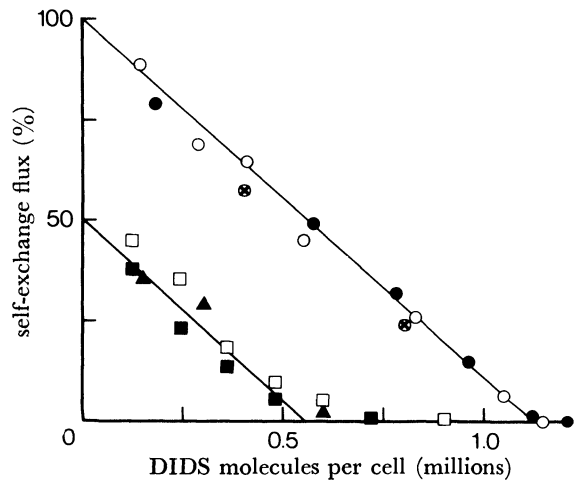

FIGURE 6. Effect of binding of DIDS on anion exchange in intact and EAC-treated erythrocyte membranes. DIDS treatment was carried out as described by Wieth (1979) on intact (circles) and EAC-treated resealed erythrocyte ghosts (squares and triangles). The ordinate shows the fractional self-exchange flux as a percentage of the flux of a control sample that had not been treated with either DIDS or EAC. Symbols: ○ and ● show results obtained by Wieth (1979) in measurements of chloride (○) and bicarbonate (●) self-exchange. The results denoted ⊗ were obtained with ghosts prepared from red cells of the same donor who delivered the red cells used for EAC treatment. The lower line of the figure shows results obtained with ghosts treated with EAC for 30 min under conditions as in figure 5 before exposure to DIDS. Anion transport was 50% inactivated after the carbodiimide treatment (see text). The symbols □, ■ and ▲ indicate three experiments with cells from the same donor. The number of DIDS molecules that inactivated the anion transport completely in the EAC-treated membranes (6×10^5 per cell) was one-half of the number needed to inactivate anion exchange in red cell membranes that had not been treated with EAC before the exposure to DIDS.

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anion exchange in intact and in EAC-modified membranes. The results are shown in figure 6. The transport capacity decreases linearly with DIDS binding in the intact cells, and complete inhibition of the transport is obtained with 1.2×10^6 DIDS molecules per cell. After EAC treatment the transport capacity was reduced by 50 % ($\bar{x} = 50.6$, s.e.m. 1.2, $n = 8$). The transport decreased linearly with DIDS binding, but complete inhibition was obtained with 6×10^5 DIDS molecules per cell, i.e. half the number needed to label all band 3 molecules in the control cells. This provides strong evidence that EAC modifies only half of the band 3 molecules during the rapid phase of inactivation.

In the membrane, band 3 exists in the form of dimers (Steck 1974) or even higher oligomers (Weinstein *et al.* 1980). We interpret our results as follows: only the first protomer of band 3 reacts readily with EAC. The unreacted protomers of EAC-treated membranes continue to transport chloride at an unchanged rate, and bind DIDS preferentially when cells are exposed to low concentrations of DIDS as in the experiments of figure 6.

Although the transport system is an oligomeric protein, kinetic studies of chloride transport have not shown evidence for cooperativity between the transport function of the protomers. Macara & Cantley (1981*a*), on the other hand, have shown that binding of a bulky stilbene disulphonate to one protomer of band 3 impedes the access of inhibitor molecules to the second protomer. The data of Nigg *et al.* (1979) likewise show that pretreatment of cells with H_2DIDS (4,4′-diisothiocyano-1,2′-diphenylethane-2,2′-disulphonic acid) reduces the covalent binding of eosin isothiocyanate by 50 %, from about 10^6 to 5×10^5 molecules per cell, and Zaki (1981) has reported a 50 % reduction of H_2DIDS-binding capacity after modification of red cells with cyclohexanedione. In addition we now report (i) that PG treatment can reduce DIDS binding by 50%, (ii) that EAC treatment inactivates only 50% of the transporters at a rapid rate, and (iii) that the remaining half of the sites in EAC-treated membranes react readily with DIDS. Thus, there is ample evidence that steric or allosteric interdependence (or both) between the protomers of band 3 is discernible by means of inhibitor molecules that bind covalently to the extracellularly exposed anion-binding region of the transport system. Although steric or electrostatic interactions cannot be excluded, it seems likely that specific ligation at one protomer of the dimer induces changes in the reactivity of groups in the adjacent protomer, transmitted through one or more interprotomeric contact domains.

Although our studies are not conclusive, they suggest that the titratable groups with a pK of about 5 are carboxylates that can be modified with water-soluble carbodiimides. This is supported by our unpublished observation that transport inactivation with CMC is accelerated in the presence of the nucleophile L-tyrosine ethyl ester, which potentiates the modification by CMC of an essential carboxyl group in yeast hexokinase (Pho *et al.* 1977).

5. Anion-binding sites in proteins

Our findings suggest that positively charged guanidino groups and negatively charged carboxylates are both essential for the transport for monovalent anions. The anion-recognition site in the transport system thus shares properties with anion-binding sites in other biomacromolecules, which bind anionic ligands during the performance of their biological functions.

There are numerous examples that demonstrate that arginyl residues are essential components of recognition sites for negatively charged substrates and cofactors at enzyme active sites (Riordan *et al.* 1977). Indeed, a survey of the literature on proteins even concluded, as an

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important generality, that 'arginyl residues constitute positively charged binding sites for enzymes acting on anionic substrates and for all proteins that interact with anions' (Riordan 1979). Our finding that a single arginyl residue seems critical for the functioning of the anion transport protein is similar to the observation that many enzymes can be inactivated by the reaction with PG of a single particularly reactive residue per enzyme protomer or active site (cf. Borders *et al.* 1979; Riordan 1979).

That negatively charged carboxylate groups also may be essential for anion transport function seems surprising at first. Examples of the apparent paradoxical existence of functionally critical carboxyl groups at anion-binding sites are found, however, in the active centre of yeast hexokinase (Pho *et al.* 1977) and in the oxygen-affinity regulating bicarbonate-binding site of crocodilian haemoglobin (Perutz *et al.* 1981). The functional role of carboxyl groups in anion transport is obscure, but it is possible that the conformational changes accompanying anion translocation involve electrostatic interactions between the transported anion and charged groups in the transport pathway. The rapid oscillations between the 'in' and 'out' configurations of an exchange gate may thus be due to the rupture and re-establishment of stabilizing intramolecular salt bridges between positively and negatively charged amino acid side chains (Macara & Cantley 1981*b*; Wieth 1981). The chloride-induced and sulphate-induced pK shifts of the titratable groups in the transport protein are valuable indicators of configurational changes that accompany the interactions with negatively charged 'transport substrates'. This is so, even if the coupling between anion and proton binding should be allosteric, like the oxygen-linked proton release from a haemoglobin molecule, where the pK shifts of a few amino acid side chains far from the haem form the molecular basis for the Bohr and Haldane effects (Edsall 1980).

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