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# Kinetic independence between red cell anion exchange and urea transport

O. Fröhlich and S.C. Jones

*Emory University School of Medicine, Atlanta, GA (U.S.A.)*

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**Urea equilibrium exchange fluxes were measured in human red cells under conditions which recruit the anion transporter into an outward-facing or an inward-facing state (with respect to the anion transport site). Regardless of these conditions, urea transport always occurred at the same rate: $41 \pm 2$ mol · (kg cell solids · min)$^{-1}$ with 1.5 M urea at 0°C. These data suggest that the pathway on the band-3 protein which mediates anion transport is kinetically uncoupled from urea transport and is probably not involved in the transport of urea across the red cell membrane.**

Solomon and coworkers [1] have suggested that the band-3 protein of the red cell membrane is not only involved in anion transport but also mediates the transport of water and urea. This is an interesting suggestion if one considers the high rates of water and urea transport and the small number of intrinsic proteins that are present in significant quantities in the erythrocyte membrane. However, the experimental evidence in favor of this notion is not very strong, particularly for the specific suggestion that the transport mechanisms for chloride, urea and water physically share a channel-like pathway through the band-3 protein. In a comparative studies of chloride and urea transport in red cells from different species, Brahm and Wieth [2] demonstrated that avian red cells possess an anion exchange mechanism but no urea transporter. Furthermore, there are virtually no inhibitors which are common to anion and urea transport and inhibit with similar inhibitory potencies. The anion transport-inhibiting stilbene disulfonates have no effect on urea transport [3]. *p*-Chloromercuribenzenesulfonate (pCMBS) which inhibits urea transport with a high affinity and water transport with a lower affinity [4,5], has no effect on anion transport [6,7]. Phloretin inhibits both chloride and urea transport, but it acts noncompetitively with respect to chloride transport [8] and competitively with respect to urea transport [3]. The only drug known to inhibit both chloride and urea movements with comparable potencies is a hybrid compound containing both stilbenedisulfonate and chloromercuric residues [9].]

In this study, we used a more direct to search for possible kinetic interactions among the different transported substrates, by testing whether transportable anions have an effect on urea transport. The rationale of the experiments is that the anion transport mechanism shifts between two conformations in which the anion binding/transport site faces either the intracellular or the extracellular space [10,11]. These two states differ in their potential energy profile of the transport pathway at least for chloride ions, as judged from the different conductance rates of monovalent an-

Correspondence: O. Fröhlich, Department of Physiology, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.

ions through the inward-facing and the ouward-facing state [12]. If urea and chloride share the same physical pathway, one might expect that the two conformational states should be also sufficiently different with respect to urea. This should result in different rates of urea transport when the anion transporter exists in these two different states.

Urea transport is much more rapid than chloride transport [3]. At physiological concentrations, chloride equilibrium exchange has a time constant in the range of 10–20 s at 0 °C [13]. For comparison, urea exchange at comparable concentrations is essentially completion after 1–2 s at 0 °C [3]. We therefore measured tracer urea fluxes as equilibrium exchange under conditions where the urea transporter is nearly saturated (1.0–1.5 M urea) to slow down the time course of tracer equilibration. In addition, we modified the existing filtration technique [13] to permit more rapid sampling. For this we switched from handling each syringe individually to a more mechanized setup. Filter holders were held in a thermostatted metal block and their input was connected to tubings which brought the efflux solution from the thermostatted efflux chamber to the filter. The filtrate was guided through a valve into collecting syringes, pulled by a vacuum in the syringes. By sequentially opening and closing the valves it was possible to collect five time points within 5 s. This method is slightly faster than the quenching method of Ku et al. [14]. It also has the advantage that it does not require a potent and complete transport inhibitor to stop the tracer efflux until the cells are separated from the medium. This greatly helps in situations such as the present one, where the substrate can permeate the membrane by more than one pathway and not all pathways are inhibitable (diffusion through the lipid bilayer), and where no rapidly acting, high-potency inhibitors are available to completely stop the flux.

The procedures in preparing the cells and media and in calculating the fluxes were the same as those applied in tracer chloride transport experiments [13]. Blood was drawn into heparinized containers and plasma and white cells were removed after centrifugation and by repeated washing in 150 mM KCl, 30 mM Hepes (pH 7.6) ('buffered saline'). To load the cells with urea, they were incubated and washed several times in buffered saline plus the desired urea concentration (1.5 or 1.0 M). In the studies where we examined the effect of extracellular chloride, we used as efflux media either buffered saline ('high-Cl') or 25 mM tripotassium citrate, 200 mM sucrose, 30 mM Hepes ('zero-Cl') (pH 7.6) both containing the desired urea concentration. To lower the intracellular chloride concentration to essentially zero, we replaced it with the very slowly transported sulfate. For this, we incubated the cells, at a hematocrit of 5–10%, three times for 30 min at 37 °C in 110 mM $K_2SO_4$, 30 mM Hepes (pH 7.6), with centrifugations to remove and renew the incubating medium. The calculations of the urea fluxes were performed in the same way as tracer chloride exchange fluxes [13] from the rate of appearance of tracer in the filtrates and the urea content of the cells. All flux experiments were performed at 0 °C.

In the first series of experiments we examined the effect of outward-recruitment of the anion transport site on urea transport. For this we measured the rate of urea equilibrium exchange in three different efflux media: (1) high-Cl buffer, (2) zero-Cl buffer, and (3) 100 μM DNDS (2,2′-dinitro-4,4′-stilbenedisulfonate), a reversible anion exchange inhibitor [10], in high-Cl buffer. In high-Cl media, the anion transport site is mostly inward-facing [15], due to the intrinsic kinetic asymmetry of the anion transport mechanism. It is recruited outward by the absence of extracellular Cl as well as by the presence of extracellular DNDS [15,16]. In the zero-$Cl_0$ medium, the (outward-facing) anion binding site is not occupied, in the DNDS medium it is presumed to be occupied by the inhibitor. The data in Part A of Table I indicate that neither the outward shift of the transport site relative to the physiological condition nor its occupation by DNDS had any effect on urea transport. The absence of a DNDS effect confirms the observation by Brahm [3] that reaction with the irreversibly acting stilbene derivative DIDS (2,2′-diisothiocyano-4,4′-stilbenedisulfonate) did not inhibit urea transport.

The second series of experiments examined the condition in which virtually all anion transport sites are recruited into the inward-facing state, as opposed to 80–90% under physiological ionic con-

TABLE I

EFFECT OF DIFFERENT ANION GRADIENTS ON UREA EQUILIBRIUM EXCHANGE

Urea transport was measured as tracer efflux from cells pre-equilibrated wth 1.5 M, and with 1.5 M urea in the efflux medium. pH = 7.6 and $T = 0\,^{\circ}C$. The flux units are mmol·(kg Hb·min)$^{-1}$. Indicated error estimates are S.D. of 4–5 flux determinations.

Part A: Recruitment by Cl gradient and DNDS (normal $Cl_i$).

| Efflux medium | Flux |
|---|---|
| $Cl_0 = 150$ mM | 40.7±2.5 |
| $Cl_0 = 0$ mM | 38.8±1.8 |
| $(DNDS)_0 = 100\ \mu M$ | 40.4±3.1 |

Part B: Recruitment by Cl-$SO_4$ gradients

| Intracellular | Extracellular | Flux |
|---|---|---|
| Cl | Cl | 38.9±3.1 |
| Cl | $SO_4$ | 41.6±1.7 |
| $SO_4$ | Cl | 44.1±1.2 |
| $SO_4$ | $SO_4$ | 43.0±3.4 |

ditions [15]. This was accomplished by replacing the intracellular chloride with the very slowly transported sulfate through pre-incubation in sulfate medium. Part B of Table I shows that again there was no significant difference in the rates of urea equilibrium exchange in the presence of the different anion gradients. The data in these two experimental series therefore demonstrate that urea transport is not influenced by the conformational state of the anion transporter. They also show that it does not depend on the rate with which the conformational states of the anion transporter interconvert. In the presence of chloride, this rate is approx. 400 $s^{-1}$ at 0 °C [17], and in the presence of sulfate it is less than 1 $s^{-1}$ [18], while it is arrested altogether in the presence of the competitive inhibitor DNDS.

In summary, this study revealed no kinetic connection between the anion transport and the urea transport pathways. If any transport elements on the band-3 protein were shared by the two pathways, one would have expected an effect of the conformational state of the anion transporter or of its turnover rate on urea permeation. We observed no such effect. This observation is similar to that made by Brahm and Galey [19] who found no coupling between anion or urea fluxes and water movements. Strictly spoken, our data do not exclude the possibility that the two pathways are located in different parts of the same band-3 protein, as long as they are not kinetically coupled. Neither can we exclude at this point that the urea transport pathway is created by the association of several band-3 molecules. However, it is not obvious how the membrane-intrinsic part of band 3 could be sufficiently large to form two separate, kinetically uncoupled transport domains. The data are therefore most easily explained by two physically separate pathways (proteins) for anions and urea. Furthermore, the notion of two independent transport proteins has recently been strengthened by the observation that human erythrocytes of a certain blood group are devoid of mediated urea transport but possess normal anion and water permeabilities [19].

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