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THE CHOLINE TRANSPORT SYSTEM OF ERYTHROCYTES

DISTRIBUTION OF THE FREE CARRIER IN THE MEMBRANE

R.M. KRUPKA and R. DEVÉS

Research Institute, Canada Agriculture, London, Ontario N6A 5B7 and Department of Biochemistry, University of Western Ontario, London, Ontario N6A 5C1 (Canada)

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Summary

A method is described, based on the kinetics of transport, for determining the equilibrium distribution of the carrier site on the inner and outer surfaces of the cell membrane, and this method is applied to the choline carrier of human erythrocytes. This method depends on measurement of flux ratios for both entry and exit, i.e., the transport rates of a low concentration of labeled substrate into a solution which contains either no substrate or a saturating concentration of unlabeled substrate. The concentrations of inward-facing and outward-facing carrier are found to be nearly equal, and therefore the 5-fold difference in choline affinity on the inner and outer surfaces of the membrane cannot be explained by an unequal carrier distribution. It is also shown that both reorientation and dissociation of the carrier-substrate complex are far more rapid than reorientation of the free carrier.

In the most familiar model for membrane transport the carrier site alternately faces either the inner or outer surface of the cell membrane, and in a perfectly symmetrical system the free carrier would be equally likely to face either way. However, experiment shows that transport systems may be unsymmetrical with respect to their substrates. For example, the apparent substrate affinities on the inner and outer surfaces of the membrane differ substantially in both the glucose [1,2] and choline [3] facilitated transport systems of erythrocytes. We say 'apparent' because with identical binding constants the affinities would appear to differ if the free carrier partitions unequally across the membrane and is more abundant on one side.

The truth of this proposition is evident from the general expressions for

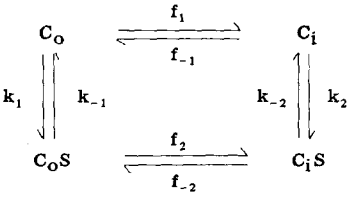

Fig. 1. Transport scheme for the reorienting carrier model in which the carrier site faces either inward (C_i) or outward (C_o). Substrate in the external solution adds to C_o to form C_oS, and internal substrate forms a complex with C_i.

experimental half-saturation constants in terms of individual rate constants in a transport scheme (Fig. 1). The ratio of zero-*trans* affinity constants for the outer and inner surfaces of the cell membrane is as follows [4]:

$$\frac{\bar{K}_{S_o}}{\bar{K}_{S_i}} = \frac{k_2\left\{f_1 + k_{-1}\left(\frac{f_1 + f_{-2}}{f_2 + f_{-2}}\right)\right\}}{k_1\left\{f_{-1} + k_{-2}\left(\frac{f_{-1} + f_2}{f_2 + f_{-2}}\right)\right\}} \tag{1}$$

and clearly this ratio depends on the partition of free carrier, f_1/f_{-1}. Substrates such as glucose and choline are of special interest in this connection because they exhibit *trans* acceleration by unlabeled substrate on the opposite side of the membrane [1—3,6]. For this to happen (see below), the rate-limiting step in zero-*trans* flux must be reorientation of the free carrier, f_1 and f_{-1}, and in the limiting case where these constants are far smaller than the other rate constants in Fig. 1, the ratio of measured affinities becomes:

$$\bar{K}_{S_o}/\bar{K}_{S_i} = f_{-1}/f_1 \tag{2}$$

Surprisingly, the ratio now depends only on the partition of free carrier, and is actually independent of the true dissociation constants, k_{-1}/k_1 and k_{-2}/k_2. This represents the maximum effect of f_{-1}/f_1 on relative affinities. Eqn. 2 was derived with the aid of the following relationship among the constants, which is required by the principle of microscopic reversibility in an equilibrating system:

$$f_1 f_{-2} k_{-1} k_2 = f_{-1} f_2 k_1 k_{-2} \tag{3}$$

Because unequal substrate affinities could result from an imbalance in the distribution of carrier sites on the two membrane surfaces rather than from differences in their structure, it is of interest to measure this distribution, and the general treatment of transport kinetics which we recently presented [4] suggests a particularly simple way of doing so. Here we apply the method to choline transport in erythrocytes, as part of a larger study of this system.

An earlier analysis of transport kinetics by Lieb and Stein [5] showed that this ratio can be calculated from the maximum rates of transport measured in three different experiments: equilibrium exchange ($\bar{V}_S$), zero-

trans entry ($\overline{V}_{S_o}$), and zero-*trans* exit ($\overline{V}_{S_i}$). The relationship was shown to be:

$$(\overline{V}_{S_i}/\overline{V}_{S_o} - \overline{V}_{S_i}/\overline{\overline{V}}_S) < f_{-1}/f_1 < (\overline{V}_{S_o}/\overline{V}_{S_i} - \overline{V}_{S_i}/\overline{\overline{V}}_S) \quad (4)$$

The method which we now employ has the advantage of being experimentally simpler and as a result more accurate than that mentioned above. It involves only two experimental parameters which set upper and lower limits for f_{-1}/f_1 directly. The method depends on the *trans* acceleration observed with choline, and in particular on measurement of $(\tilde{v}^S/\bar{v})_{S_i \to 0}$ and $(\tilde{v}^S/\bar{v})_{S_o \to 0}$, which are ratios of infinite-*trans* and zero-*trans* flux [6]. The first is the ratio of the rates at which labeled substrate, S, is transported out of the cell, either into a solution of unlabeled substrate at a saturating concentration ($\tilde{v}^S$), or into a solution containing no substrate at all ($\bar{v}$). In the required experiment, the labeled substrate within the cell, S_i, is present at a concentration well below its half-saturation constant. The second flux ratio, $(\tilde{v}^S/\bar{v})_{S_o \to 0}$, is the reverse of this, namely the rate at which labeled substrate outside the cell, at a very low concentration, enters cells already loaded with a high concentration of unlabeled substrate, or cells free of substrate.

The general expressions for the flux ratios in terms of individual constants in the scheme in Fig. 1 are as follows:

$$(\tilde{v}^S/\bar{v})_{S_i \to 0} = \frac{1 + f_{-1}/f_1}{1 + \frac{f_{-1}}{f_2} + \frac{f_{-1}}{k_{-2}}\left(1 + \frac{f_{-2}}{f_2}\right)} \quad (5)$$

$$(\tilde{v}^S/\bar{v})_{S_o \to 0} = \frac{1 + f_1/f_{-1}}{1 + \frac{f_1}{f_{-2}} + \frac{f_1}{k_{.1}}\left(1 + \frac{f_2}{f_{-2}}\right)} \quad (6)$$

From these equations the maximum values of the flux ratios must obviously be:

$$(\tilde{v}^S/\bar{v})_{S_i \to 0} \leqslant 1 + f_{-1}/f_1 \quad (7)$$

$$(\tilde{v}^S/\bar{v})_{S_o \to 0} \leqslant 1 + f_1/f_{-1} \quad (8)$$

Where there is *trans* acceleration the flux ratios are greater than unity, and if $f_{-1} = f_1$ the maximum possible value is 2. The two experimental ratios are seen to set limits on the magnitude of f_1/f_{-1}.

(i) $(\tilde{v}^S/\bar{v})_{S_i \to 0}$. The methods for measuring this constant were described before, and a value of 1.93 ± 0.043 was reported [6]. By substitution into Eqn. 7, the lower limit for f_{-1}/f_1 is found to be 0.93 ± 0.043.

(ii) $(\tilde{v}^S/\bar{v})_{S_o \to 0}$. Washed cells were loaded with 333 μM choline chloride for 20 h at 37°C, with changes of suspending medium after 2 and 4 h. The cell suspension (40% packed cells) included 0.1% glucose and 0.02% chloramphenicol, as in previous studies. The cells initially contain endogenous choline, approx. 13 μM [7]. At equilibrium, the internal choline concentration should be over 230 μM. Previous experiments with [^{14}C]choline showed that 60 μM choline attained equilibrium in less than 16 h; with 140 μM choline the internal concentration rose to nearly 80% of the equilibrium

value in this time (Devés, R. and Krupka, R.M., unpublished results). The internal choline concentration in the present experiment should therefore exceed 200 μM, which is to be compared with the internal half-saturation concentration of 33 μM (Devés, R. and Krupka, R.M., unpublished results). Control cells were washed free of endogenous choline by incubation at 37°C in buffered salt solution (10% packed cells during the first 4 h, with one change of buffer, and 40% packed cells for 16 h).

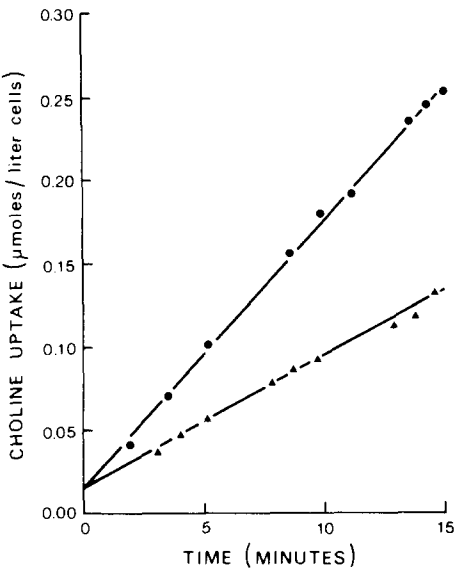

Fig. 2. Rate of uptake of 0.23 μM [^{14}C]choline into cells free of choline (lower line) or containing a near-saturating concentration of choline (upper line). The ratio of the two rates, $(\tilde{v}^S/\bar{v})_{S_o \to 0}$, is 2.07 ± 0.048 (see text).

After incubation the cells were washed free of external choline at 0°C (control cells were similarly treated) and rates of uptake of 0.23 μM [^{14}C]-choline were determined by methods already described [8]. The results are plotted in Fig. 2. From a least-squares analysis, the rates in the presence and absence of internal choline were 16.65 ± 0.193 and 8.02 ± 0.162 nmol/min per l packed cells, respectively; and the ratio of these rates, which is equal to $(\tilde{v}^S/\bar{v})_{S_o \to 0}$, is 2.07 ± 0.048. From Eqn. 8, f_1/f_{-1} is 1.07, and f_{-1}/f_1 has an upper limit of 0.93 ± 0.042.

(iii) $[C_o]/[C_i]$. In the absence of substrates or inhibitors the relative abundance of carrier sites outside and inside, $[C_o]/[C_i]$, is equal to f_{-1}/f_1. From the results given above, this ratio is not less than 0.93 ± 0.043 and not more than 0.93 ± 0.042. It follows that $f_{-1}/f_1 = 0.93 \pm 0.03$. The exact value obtained for this ratio has been found to vary somewhat with different samples of cells. Occasionally, it is found to be as low as 0.5 or as high as 1.5 but most often it is nearer the value found here. Consequently, large differences in apparent substrate affinities cannot be explained by an imbalance in the f_{-1}/f_1 ratio (the experimental values of $\bar{K}_{S_o}$ and $\bar{K}_{S_i}$ are 6.3 and 33 μM, respectively).

Cabantchick and Ginsburg [9] studied the uridine transport system of

erythrocytes and applied the analysis of Lieb and Stein [5] to their observations. The calculated f_{-1}/f_1 ratio was 0.2—0.3, and $\bar{K}_{S_o}/\bar{K}_{S_i}$ was equal to 0.18—0.25. Hence, in this case, the apparent difference in affinities may be explained by an asymmetric distribution of the free carrier.

(iv) Rate-limiting steps in transport. The subject of rate-limiting steps in relation to exchange flux and zero-*trans* flux has been discussed by Hoare [10], by Regen and Tarpley [11], and by Lieb and Stein [5]; and the observation that the maximum rate of equilibrium exchange of choline is 5-fold greater than that for zero-*trans* entry [3] indicates that the rates of reorientation and dissociation of the carrier-substrate complex must be more than 4-fold greater than the rate of reorientation of the free carrier. The present measurements of flux ratios confirm and extend this conclusion. As seen above, the two flux ratios $(\tilde{v}^S/\bar{v})_{S_i \to 0}$ and $(\tilde{v}^S/\bar{v})_{S_o \to 0}$ give identical estimates of f_{-1}/f_1, and this can only happen if the denominators of Eqns. 5 and 6 have a value very close to unity. It follows that:

$$\frac{f_{-1}}{f_2} + \frac{f_{-1}}{k_{-2}}\left(1 + \frac{f_{-2}}{f_2}\right) \ll 1 \tag{9}$$

$$\frac{f_1}{f_{-2}} + \frac{f_1}{k_{-1}}\left(1 + \frac{f_2}{f_{-2}}\right) \ll 1 \tag{10}$$

Therefore, in choline transport, movement of the substrate complex (governed by f_2 and f_{-2}) and dissociation of choline from this complex (k_{-1} and k_{-2}) are far more rapid processes than movement of the free carrier (f_1 and f_{-1}).

(v) Other transport models. Our analysis has been based on the familiar one-site reorienting carrier model, even though there are other possibilities, for example, models with substrate sites simultaneously exposed on both sides of the membrane [12,13]. Here, only one form of the free carrier exists, and an interpretation involving two different carrier states would obviously be inappropriate. However, evidence is available from experiments with both reversible and irreversible inhibitors which rules out such models for choline transport and which is entirely consistent with the one-site mechanism [14]. Our analysis is therefore required by the evidence at hand.

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