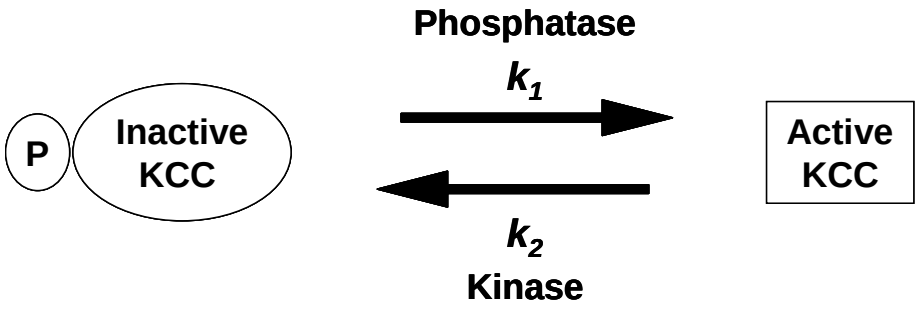
KCC activity is regulated by phosphatase/kinase equilibrium and activation is associated with a net dephosphorylation event of the transporter or an associated regulator. Although a number of complex models have been suggested [2,3], a simplified two state model of KCC activation can be represented as:



The rate constants k_1 and k_2 represent the rates of the phosphatase and kinase reactions, respectively. The relative activation of the transporter (V/Vmax) is a function of these two rate constants[4,5]:

$$V/Vmax = k_1/(k_1 + k_2). \quad (1)$$

Likewise, the delay time (τ) for activation of KCC after stimulation is defined as [5,6]:

$$\tau = 1/(k_1 + k_2). \quad (2)$$

KCC activity is low in the steady state, even in SS RBC, indicating that $k_2 > k_1$ and the equilibrium is normally shifted to the left, maintaining the transporter in its inactive, "phosphorylated" state (see below). Swelling, acid pH and sulfhydryl alkylation with N-ethylmaleimide (NEM) increase both activity and delay time, which can only be accomplished algebraically by a decrease in k_2. (An increase in k_1 will increase activity, but will decrease delay time[4,5].) Thus swelling (as well as other stimuli) appears to activate KCC by inhibiting the regulatory kinase, presumably due to a change in macromolecular crowding resulting from dilution of hemoglobin[7].

The effect of urea on reticulocyte volume reduction mediated by KCC can be interpreted in light of the two state kinetic model of KCC activation. An example will illustrate how inhibition of the regulatory kinase of KCC could have a profound effect on volume reduction to produce a

dramatically different CHC. Given that V/Vmax = $k_1/(k_1+k_2)$ [5], and for the whole RBC population, KCC is approximately 12% activated at CHC = 34 (Figure 1 of [1]), it follows that

$$V/Vmax = 0.12 = k_1/(k_1+k_2), \text{ so that}$$

$$k_2 \cong 7\, k_1. \qquad (3)$$

If the activity of the regulatory kinase (k_2) is suddenly inhibited 90 % to give a new value, $[k_2]_I$, that is 0.1 of its original value (and assuming k_1 remains unchanged), then

$$[k_2]_I = 0.10\, k_2 = 0.7\, k_1, \qquad (4)$$

and activation of KCC will be defined by

$$\begin{aligned} V/Vmax &= k_1/(k_1+[k_2]_I) \\ &= k_1/(k_1+0.7\,k_1) \\ &= k_1/(1.7\,k_1) \\ &= 0.59 \end{aligned}$$

representing about 60 % maximal activation of KCC. The cell will shrink until KCC flux returns to its original steady state level, of about 12% activation, assuming influx pathways are unchanged, which is associated with return to a kinase activity equal to the original, uninhibited value (k_2) . As the cell shrinks, kinase activity will increase, in response to increasing CHC, and kinase activity at the new steady state, $[k_2]_{SS}$, will be defined by:

$$[k_2]_{SS} = k_2 = 10\,[k_2]_I$$

In other words, the cell will have to shrink to a new steady state CHC that would produce a ten fold increase in residual kinase activity to restore the cell to its original kinase activity, and therefore return to its original KCC flux rate, to re-establish a stable (albeit reduced) volume. Now we may ask: what change in CHC would bring about such an increase in kinase activity?

To relate CHC to k_1 and k_2 , we start by using the published data on relationship between V/Vmax and MCHC[1], which is similar for SS RBC [filled symbols] and AA RBC [open symbols], as shown in Fig. A1. The data can be fit well by the 4th order polynomial [$V/Vmax = -0.0077(MCHC)^4 + 1.0813(MCHC)^3 - 55.972(MCHC)^2 + 1254.8(MCHC) - 101.92$] although the equation has no theoretical significance. (See Figure S1.)

Thus, for any given CHC a value of V/Vmax can be calculated using this equation. Then, using to Eq. (1), V/Vmax can be related to k_1 and k_2 as follows:

$$V/Vmax = k_1/(k_1 + k_2) \quad (1)$$
$$= (k_1/k_2)/(k_1/k_2 + 1)$$
$$V/Vmax(k_1/k^2) + V/Vmax = (k_1/k_2)$$
$$V/Vmax = (k_1/k_2) - V/Vmax(k_1/k_2)$$
$$V/Vmax = (k_1/k_2)(1 - V/Vmax)$$

$$V/Vmax/(1 - V/Vmax) = (k_1/k_2) \quad (5)$$

This permits the calculation of a value of (k_1/ k_2) for each CHC, via the associated V/Vmax value. If we normalize these (k_1/ k_2) values to the value of (k_1/ k_2) at MCHC 34 gm/dl (and assume that k_1 is independent of MCHC[4,5]), these normalized values at various MCHC represent the values of k_2 relative to that at MCHC = 34 gm/dl (or rk_2). These values of rk_2 are plotted vs. MCHC in Fig. A2; the dashed line represents rk_2 = 1. From Fig. A2, it is apparent that a ten fold increase in residual kinase activity $(k_2)_I$ (to compensate for a 90% inhibition of the kinase) would require a change in CHC from 34 gm/dl to around 36.4 gm/dl. These calculations are subject to the caveat the relationship between KCC activation and CHC was determined for CHC of whole blood samples, not reticulocytes, so the actual CHC values are not comparable to the results of volume reduction experiments in reticulocytes. Nevertheless, the exercise illustrates how inhibition of the regulatory kinase of KCC by urea can have such a profound effect on reticulocyte volume reduction and resultant CHC.

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