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The Choline Carrier of Erythrocytes: Location of the NEM-Reactive Thiol Group in the Inner Gated Channel

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Summary. Choline transport across the human erythrocyte membrane is irreversibly inhibited when N-ethylmaleimide (NEM) reacts with a carrier SH group which is located outside the substrate site, and which is exposed in the inward-facing form of the carrier but prevented from reacting in the outward-facing form. The location of the SH group with respect to the membrane has now been determined by studying the dependence of the NEM-alkylation rate on the intracellular and extracellular pH. The results show that the reactive SH group equilibrates with hydrogen ions in the cytoplasm, but is completely isolated from hydrogen ions in the external medium. With this added evidence it becomes possible to conclude that the SH group is located in the inner gated channel of the choline carrier.

Key Words N-ethylmaleimide · carrier SH-group · gated channel · carrier mechanism · intracellular pH · thiol

Introduction

To explain how an integral membrane-spanning protein can have the properties of the carrier model, with a substrate site alternately exposed on opposite sides, it is necessary to postulate that the transport protein contains channels which alternately connect a substrate binding region with the solution at the two membrane faces. In such a mechanism, which is illustrated in Fig. 1 (right), the channels on the two sides necessarily open and close in a coordinated manner, allowing the substrate site to be accessible from only one compartment—inside or outside the cell—at a time.[1] As the channels exist in these two states, open and closed, they are said to contain gates. Thus the carrier protein alternates between two different conformations, one inward-facing, with the substrate site exposed inside the cell, and one outward-facing, with the site exposed to the external medium. Evidence showing that the choline transport system of erythrocytes operates in this way was summarized earlier [5].

Any investigation of the structure and action of the gated channels could be facilitated by reagents specifically bound in these regions of the protein. We have previously shown that such reagents should have characteristic properties, by which they could be identified [9]. In particular, we can predict that (1) a reagent of this kind would be bound outside the substrate site; (2) it would, when bound, interfere with substrate translocation; and (3) it would be able to bind, on one side of the membrane or the other, only when the substrate site is exposed on the same side (*see* Fig. 1). Here we wish to show that N-ethylmaleimide (NEM), which irreversibly inhibits the choline carrier in erythrocytes, has exactly the characteristics predicted for a molecule binding in the inner gated channel.

NEM reacts with a thiol group in the carrier, as shown by Martin [10]. The evidence is as follows: cystamine, a disulfide, also inhibits, and the inhibition is reversed by dithiothreitol; furthermore, pretreatment of the system with cystamine protects

[1] In the choline system, diffusion of a substrate molecule from its binding site into the bulk solution (i.e. dissociation from the substrate site) is a rapid (nonrate-limiting) step in transport [3]. Passage through the channels may therefore be regarded as being relatively unhindered, and as in the model in Fig. 1, the channels may be represented as being wide and as having smooth surfaces that do not interact with the substrate. This minimal physical model possesses the essential kinetic properties of the transport system: a rate-limiting carrier conformational change that alternately exposes substrate binding regions on opposite sides of the membrane, and relatively unimpeded diffusion of the substrate from the carrier site into the surrounding medium. The diagram in Fig. 1 should not be taken to imply that the actual carrier protein is perfectly symmetrical, that a single defined region in the carrier interacts with the substrate, or that the barrier described as a "gate" covers more than a fraction of the channel length. Evidence that the channel surfaces are partly nonpolar and are large enough to bind nonpolar inhibitors was discussed earlier [9].

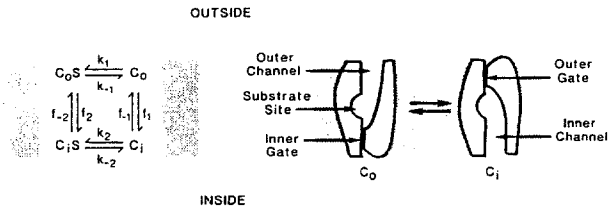

Fig. 1. The carrier model and the gated channel mechanism, in relation to the cell membrane. The carrier model (left) is a purely kinetic scheme defining the functional properties of the transporter but leaving the physical counterpart unspecified; C_o and C_i are carrier states having a substrate site exposed to the outer or inner compartment, respectively, and forming a complex (C_iS and C_oS) with the substrate in the same compartment. The gated channel mechanism (right) is a diagrammatic representation of a transport protein having functional properties defined by the kinetic scheme on the left (i.e., by the carrier model); C_o and C_i are outward-facing and inward-facing conformations of the transmembrane protein. The inner and outer channels contain reciprocating gates, in the sense that the channels alternately open and close. Molecules that bind in the gated section of the inner or outer channel are expected to behave in identifiable ways (*see* text)

against the irreversible inhibition by NEM. It is of interest that certain highly polar disulfides—dithiocholine, cystine, and 6,6′dithionicotinic acid—fail to inhibit. It has also been shown that NEM reacts outside the substrate site, and reacts only with the inward-facing carrier form [3, 4, 7, 10, 11]. What remains to be determined is whether the reactive thiol group is exposed to the solution inside the cell, outside the cell, both, or conceivably neither, as in the case where it is buried within the transport protein. NEM, since it is soluble in a nonpolar environment and freely penetrates a lipid bilayer by simple diffusion, would be present both inside and outside the cell as well as within the membrane, and it should also be able to penetrate into nonpolar regions of the protein structure. Hence it should be able to react with almost any part of the carrier.

If NEM reacts specifically in the inner gated channel, as the previous findings could suggest, then the reaction site should be exposed to the cell interior but never to the external solution. Our test of the site's location depends on the far greater reactivity of the ionized form of a thiol group, RS^-, than of the protonated form, RSH, and the consequent increased rate of alkylation at rising pH. In the erythrocyte system, reaction of NEM with the choline carrier is indeed pH dependent; and furthermore, what is essential for our purpose, the pH inside the cell and in the suspending medium can be controlled independently. Hence it should be possible to determine whether the carrier thiol group is sensitive to, and therefore in equilibrium with, the internal or external pH, and so to decide on its location.

This experimental procedure, of varying the pH, entails no disruption of the membrane structure, and is preferable to any approach to the problem involving the preparation of resealed ghosts, for example experiments with impermeant maleimides. Equilibration of hydrogen ions across the red cell membrane depends on the operation of the anion exchange carrier, where HCO_3^-, after being exchanged for Cl^-, takes up a H^+ to give CO_2 and water. By inhibiting the anion exchanger with the specific reagent 4,4-diisothiocyanostilbene-2,2-disulfonic acid (DIDS), the internal and external compartments may be isolated with respect to pH, as has been shown by Deuticke, Bayer and Forst [1].

Materials and Methods

Human blood, stored for under 4 weeks before use, was obtained from a blood bank. The red cells were washed several times in isotonic saline and packed by centrifugation.

The method for determining the rate of inactivation of the choline transport system by NEM was described previously [3]. In brief, cells were incubated with 1 mM NEM at 37°C for various periods of time, and the reaction was stopped by adding mercaptoethanol (final concentration 10.6 mM). The cells were washed and packed by centrifugation, and the residual transport activity was determined from the rate of uptake of 1.6 μM [methyl-^{14}C]-choline. Plots were constructed of the natural logarithm of the fractional transport activity v/v_0, versus the time of treatment t (where v is the activity at time t and v_0 the original activity). From the slope of the predicted straight line in this plot, found by a least-squares analysis, the inactivation rate constant k was calculated, according to the following equation:

$$\ln(v/v_0) = -kt. \tag{1}$$

To set the pH, cells were suspended and washed several times in buffered saline solutions. These solutions contained 15 mM $NaHPO_4$ and 145 mM NaCl, and the pH was adjusted by addition of 280 mM NaOH. In experiments in which the internal and external pH were in equilibrium, the buffers contained 5 mM $NaHCO_3$ (as well as 140 mM NaCl and 15 mM phosphate), in order to facilitate pH equilibration.

At equilibrium, the relationship between the internal and external pH, pH_i and pH_o, respectively, is given by Eq. (2):

$$pH_i = pH_o + \log([Cl^-_{in}]/[Cl^-_{out}]). \tag{2}$$

The ratio of internal and external concentrations of chloride ion $[Cl^-_{in}]/[Cl^-_{out}]$ sets the membrane potential and therefore determines the concentration ratio for other permeant ions, such as H^+. The chloride ratio as a function of the external pH was found from the published values of Gunn et al. [8].

To fix the internal pH, DIDS was added to the buffered cell suspension at a final concentration of 208 μM. The pH of the suspension was measured before and after addition of DIDS, and was found not to change. The internal pH was calculated from that in the external solution, using Eq. (2).

After having been incubated with DIDS for 1 hr at room temperature, the cells were packed by centrifugation and suspended in buffered saline solutions containing 16 μM DIDS (as a precaution to ensure that the anion exchanger remained inhib-

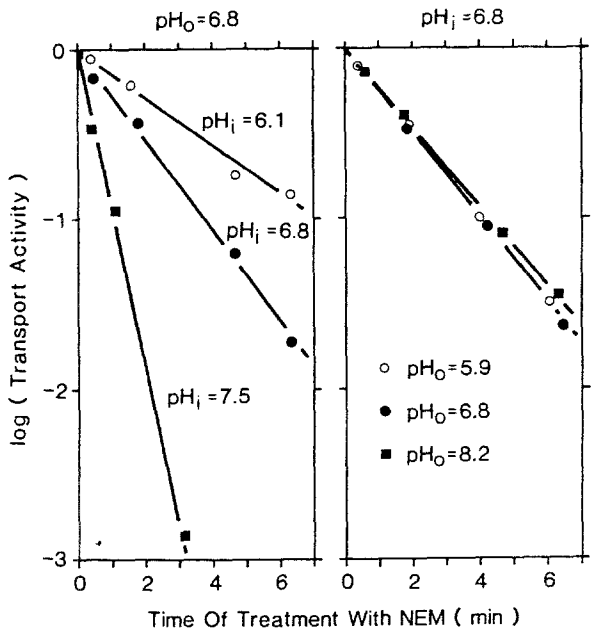

Fig. 2. The dependence of the reaction of N-ethylmaleimide (NEM) on the pH of the suspending medium (pH_o) and on the pH inside the cell (pH_i). The suspending medium contained 385 μM choline and 1 mM NEM. The natural logarithm of the fractional transport activity is plotted against the time of treatment of the cells with NEM, according to Eq. (1); the rate constants for carrier inactivation were calculated from the slopes and are listed in Table 1 (Experiments 3 and 4)

ited; at this stage the buffers were prepared with boiled water in order to minimize the HCO_3^- concentration). The cell suspension, pre-equilibrated at 37°C, was then treated with NEM, and the pH of the suspension was determined.

Results

The decline in transport activity during treatment of the cells with NEM is shown in Fig. 2 for two cases: first the external pH is held constant and the internal pH varied; and second, the internal pH is held constant and the external pH varied. It is seen that the reaction of NEM depends on the internal but not the external pH. A summary of experiments involving these and other conditions is given in Tables 1 and 2. In the experiments listed in Table 1, a saturating concentration of choline was present in the suspending medium while the cells were being treated with NEM; in those listed in Table 2, choline was absent. The effect of choline on the inactivation rate at 6.8 and 7.6 is given in Table 3, where the ratio of the two inactivation rates is seen to agree with the flux ratio; $(\bar{v}/\bar{v})_{Si\to 0}$, at the same pH, as predicted by the carrier model [3].

At pH 6.8 and below, inactivation rates were determined with fair precision, but at pH above 7.5, especially in the presence of external choline, some

Table 1. NEM-inactivation rate constants (k) measured in the presence of external choline (385 μM)[a]

Experiment	DIDS	External pH	Internal pH	k (min^{-1})[b]
1	–	6.0	6.1	0.13 ± 0.009
	–	6.7	6.8	0.30 ± 0.015
	–	7.7	7.6	0.9
2	+	5.9	6.1	0.15 ± 0.006
	+	6.7	6.8	0.30 ± 0.005
	+	8.1	7.6	1.1
3	+	5.9	6.8	0.25 ± 0.004
	+	6.8	6.8	0.25 ± 0.003
	+	8.2	6.8	0.23 ± 0.005
4	+	6.8	6.1	0.14 ± 0.012
	+	6.8	6.8	0.27 ± 0.012
	+	6.8	7.5	0.9

[a] In experiments 2, 3 and 4 cells were pretreated with 208 μM DIDS to lock in the internal pH, which was calculated from Eq. (2) (*see* Materials and Methods). The cells were then exposed to 1 mM NEM in buffered saline at various pH values containing 385 μM choline. Inactivation rate constants were calculated, according to Eq. (1), from log plots (*see* Fig. 2).
[b] The measurements at high internal pH give minimum values, as explained in the text.

Table 2. NEM-inactivation rate constants (k_{obs}) measured in the absence of external choline[a]

Exp.	DIDS	External pH	Internal pH	k_{obs} (min^{-1})	Calculated k (min^{-1})[b]
1	–	6.2	6.3	0.10 ± 0.028	0.13
	–	6.9	6.9	0.16 ± 0.003	0.30
	–	7.7	7.6	0.55 ± 0.068	1.3
2	+	6.1	6.7	0.12 ± 0.018	0.22
	+	6.8	6.7	0.11 ± 0.024	0.20
	+	7.7	6.7	0.11 ± 0.010	0.20
3	+	7.1	6.3	0.10 ± 0.020	0.13
	+	7.1	6.8	0.19 ± 0.004	0.34
	+	7.1	7.7	0.36 ± 0.025	0.95

[a] The experiments were done as described in Table 1 and in Materials and Methods.
[b] The observed rate constant is dependent on the partition of the carrier between inner and outer forms, which is itself pH dependent; the partition-independent constant was calculated from Eq. (3), using values of $(\bar{v}/\bar{v})_{Si\to 0}$ taken from reference [6].

Table 3. Effect of external choline on the NEM-inactivation rate

External pH	k_{obs} (min^{-1})		Ratio	$(\bar{v}/\bar{v})_{Si\to 0}$[a]
	minus choline	plus choline (385 μM)		
6.8	0.19 ± 0.023[b]	0.33 ± 0.019[b]	1.74	1.8
7.6	0.50 ± 0.029	1.11	2.2	2.4
	0.46 ± 0.057	1.06	2.3	

[a] Values taken from reference [6].
[b] Values taken from reference [3].

uncertainty is introduced by the rapidity of the reaction, which causes the transport activity to become very low and hard to measure accurately. The difficulty is aggravated by a slight leakiness possibly induced in the cell membrane by the combined treatment with NEM and elevated pH. This degree of leakiness would normally be unimportant but may be significant in relation to strongly inhibited rates of transport, where it could account for the curvature which is sometimes seen in plots according to Eq. (1), with an approach to an apparently limiting inhibition. The calculated rates at high pH in the presence of choline are therefore to be regarded as minimum values, and on account of this uncertainty we have refrained from listing standard errors for these measurements.

Discussion

The rate constant for NEM reaction, we find, increases as the internal pH rises. This is true when the external pH is held constant and also when the internal and external pH are in equilibrium (experiments 1, 2 and 4 in Table 1; experiments 1 and 3 in Table 2). By contrast, when the internal pH is constant, changes in the external pH have no effect whatever on the rate of NEM reaction (experiment 3 in Table 1; experiment 2 in Table 2). DIDS, which we have used in isolating the internal and external compartments, and which as noted above irreversibly inhibits the anion exhanger, does not inhibit choline transport, and does not affect the reaction of NEM with the choline carrier or the pH dependence of this reaction (*compare* experiments 1 and 2, Table 1).

Inactivation rates are higher in the presence than in the absence of external choline (Tables 1 and 2), and a correction is required to bring the two measurements into line. It has previously been shown that the carrier-substrate complex moves much faster than the free carrier (based on the observation of accelerated exchange [3, 11]); hence, in the presence of external choline inward translocation of the carrier, in the form of the carrier-substrate complex, is much faster than its return in the free form ($f_2 \gg f_{-1}$ in Fig. 1), and as a result the carrier accumulates almost entirely in the inward-facing form C_i. As C_i is the carrier form with which NEM reacts, the inactivation rate constant found in the presence of external choline is a direct measure of the reaction under investigation:

$$\text{NEM} + C_i \xrightarrow{k} C_{\text{inactive}}. \tag{3}$$

The rate constant measured without substrate in the external solution should be lower than this, because here the carrier exists partly in the outward-facing form C_o, which does not react with NEM. As a further complication, the partition of the carrier between these forms, C_o and C_i, is dependent not only on the presence of the substrate but on the internal pH [6]. The required correction is found in the following way.

As NEM reacts only with the inward-facing carrier form C_i (Eq. 3), the observed rate constant for NEM reaction may be written as

$$k_{\text{obs}} = \frac{k[C_i]}{C_t} = \frac{k[C_i]}{[C_i] + [C_o]} = \frac{k}{1 + [C_o]/[C_i]} = \frac{k}{1 + f_{-1}/f_1} \tag{4}$$

where $[C_o]/[C_i]$, the carrier partition, is equal to f_{-1}/f_1 in Fig. 1, and C_t is the total carrier concentration, $[C_o] + [C_i]$. The required correction factor, $1 + f_{-1}/f_1$, can be determined by experiment, for the flux ratio may be shown to have exactly the same dependence on the equilibrium carrier partition, f_{-1}/f_1 [2]:

$$(\bar{v}/\bar{v})_{Si\to 0} = 1 + f_{-1}/f_1. \tag{5}$$

$(\bar{v}/\bar{v})_{Si\to 0}$ is the ratio of exit rates of a low concentration of labeled choline into a medium containing saturating unlabeled choline or no choline, i.e., exchange flux relative to zero trans flux. Another way of expressing these relationships is to say that the ratio of inactivation rates in the presence and absence of external choline is in theory equal to the flux ratio $(\bar{v}/\bar{v})_{Si\to 0}$, in agreement with observations on various substrates [3]. The results in Table 3 show that the expected equivalence is found at different pH values as well.

From Eqs. (4) and (5) the partition-independent NEM-inactivation constant k is given by

$$k = k_{\text{obs}} \cdot (\bar{v}/\bar{v})_{Si\to 0}. \tag{6}$$

Corrected values of the inactivation rates are listed in Table 2, based on estimates of the flux ratios reported earlier [6]; as noted above, the flux ratio varies with the internal pH, but is independent of the external pH. The two sets of estimates of k, either in the presence or absence of external choline (Tables 1 and 2), are seen to be in reasonable agreement, and the trends are without any question the same. Clearly, the carrier thiol group reacting with NEM equilibrates with hydrogen ions in the internal solution, but is completely isolated from hydrogen ions in the solution outside the cell.

The question posed at the beginning was this: is the NEM reaction site located in the inner gated channel of the choline carrier? By hypothesis, the

gate in the channel is that section that closes tightly enough to prevent even small molecules from passing through, and opens wide enough to make an easy passage for the substrate (Fig. 1)[1]. As we have already noted, NEM reacts outside the substrate site, the evidence being that the reaction rate increases rather than decreases when a substrate analog is bound [4]. Further, NEM reacts with the inward-facing but not the outward-facing form of the carrier, as shown by various observations [3, 4, 7, 10, 11]: (i) nontransported substrate analogs bound on the outer surface of the membrane protect against NEM, and those bound inside increase the rate of the reaction; (ii) the rate of the NEM reaction is related in an exactly predictable way to the distribution of a substrate across the membrane and to its maximum transport rate (where a series of substrates is tested). We now find that the NEM site is exposed to the solution inside the cell but not outside. Thus all the criteria noted in the Introduction for distinguishing a site in the inner gated channel are fulfilled, and we may conclude that this is probably where the reactive thiol group is located. NEM can reach this site because, being lipid-soluble, it enters the cell by simple diffusion. The reason highly polar disulfides fail to inhibit [10] now becomes clear; they cannot cross the cell membrane and are therefore restricted to the external solution. The disulfide, cystamine, though predominantly an ion in the protonated form, exists partly as a neutral amine capable of entering the cell in the same way as NEM, and it therefore inhibits [10].

One last point is that the pH dependence of the alkylation reaction is distinguishable from that of an ionizing group, of pK_a 6.8, previously shown to affect the mobility of the free carrier [6]. At pH 6.1, 6.8 and 7.6, the amounts of the group of pK_a 6.8 in the unprotonated form would be in the ratio 0.33, 1.0 and 1.7, respectively, while the relative rates of alkylation at these pH values are 0.5, 1.0 and 3. That the same ionizing group, a thiol, could be involved in both effects is therefore unlikely, a reasonable conclusion considering that a pK_a of 6.8 would be unusual for a protein thiol group, with a typical value of over 9.

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