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Reaction of Internal Forms of the Choline Carrier of Erythrocytes with N-Ethylmaleimide: Evidence for a Carrier Conformational Change on Complex Formation

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Summary. The choline carrier of human erythrocyte membranes exists in distinguishable outward-facing and inward-facing conformations, and previous studies demonstrated that only the latter reacts with N-ethylmaleimide, producing an irreversible inhibition of transport. We now report experiments to determine the individual reaction rates for the two inward-facing forms: the free carrier and the complex. The pseudo-first-order rate constant for the complex with a substrate analog, di-*n*-butylaminoethanol, is found to be nearly double that for the free carrier, showing that the carrier conformation is altered following addition of a ligand (with 1 mM N-ethylmaleimide at pH 6.8, 37 °C, the constants are 0.57 ± 0.05 min^{-1} and 0.33 ± 0.02 min^{-1}, respectively). Hence three different conformational states have been distinguished by experiment: (1) the inward-facing free carrier; (2) the inward-facing complex; and (3) the outward-facing carrier.

Key words: carrier, conformational change, choline transport, substrate complex, inhibition, N-ethylmaleimide

Evidence for a carrier conformational change which occurs during the process of transport was obtained in previous studies of irreversible inhibition of the choline system by N-ethylmaleimide (NEM) [4, 5, 11]. The carrier exists as an equilibrium mixture of two interconvertible forms, one in which the substrate binding site is exposed to the solution outside the cell and the other in which it is exposed inside. These two forms are distinguished experimentally by the fact that the inward-facing carrier reacts with NEM while the outward-facing carrier does not. In an earlier study Edwards [5] considered that the internal free carrier and the internal complex have essentially the same conformation and react with NEM at the same rate, though the experimental observations were not conclusive in this respect. The two rate constants have now been determined separately and are found to be different: the complex reacts nearly twice as fast as the free carrier.

A knowledge of the inactivation rate constants for the various carrier forms is important in several ways. First, it can provide evidence for a conformational change in the carrier both upon reorientation between the inner and outer surfaces of the membrane and upon binding of the substrate. It can also provide a means of measuring the partition of the carrier across the membrane under various conditions, and from the effects of membrane perturbation on this distribution the physical relationship between the carrier and the membrane enclosing it may be investigated. In addition, if the carrier distribution is measured in the presence of a substrate analog at equilibrium across the membrane, then the ratio of true instead of apparent affinity constants on the inner and outer surfaces, and hence the specificity of the inner site in relation to the outer, may be determined. With this information inferences may be drawn about possible changes in the configuration of the transfer site during carrier reorientation, which bears on the question of whether the same or different sites are exposed on either side.

Materials and Methods

Choline chloride (methyl-^{14}C, 30 Ci/mol) was obtained from New England Nuclear. The isotope was stored at −10 °C and its purity examined by paper chromatography [butanol/HCl/H_2O (4:1:1)],

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with choline and trimethylamine hydrochloride as standards. The tertiary amines were distilled at reduced pressure before use. All chemicals were reagent grade. Red cells were prepared from outdated human blood from a blood bank.

Inactivation Rates

Choline-free cells (2.5% hematocrit) were treated with 1 mM NEM for varying lengths of time at 37 °C, as previously described [4]. Sodium phosphate buffer, 5 mM and pH 6.8, containing 154 mM NaCl was used throughout the experiments. The residual transport activity was estimated from the rates of uptake of 2.5 μM ^{14}C-choline. The uptake assay was described elsewhere [3]. Plots were constructed of the natural logarithm of the rate of choline entry, as determined in the uptake assay, against the time of treatment with N-ethylmaleimide. The pseudo-first-order inactivation rate constant was obtained from the slope of the line by a least-squares analysis.

In determining inactivation rates in the presence of substrate analogs at equal concentrations across the cell membrane, washed cells were pre-equilibrated with the analogs before being treated with NEM. In the case of the tertiary amines, which come to equilibrium in less than 5 min [7][1] incubation was for 1 hr, while with choline the cells were incubated for at least 10 hr in a suspension containing 0.02% chloramphenicol [1].

Affinity of Analogs from Effects on Inactivation Rates

The half-saturating concentration of a substrate analog ($\bar{K}_{Ti}$) may be determined from the concentration dependence of the inactivation rate constant Q_{app}. The results are plotted according to the following linear relationship found by rearrangement of a general equation derived previously [4]:

$$Q_{app}=Q_{Ti}-\frac{\bar{K}_{Ti}}{[Ti]}(Q_{app}-Q) \tag{1}$$

where Q_{Ti} is the inactivation constant when the analog is present at a saturating concentration, and Q that in its absence. Eq. (1) is written for an analog which is present inside the cell but not outside, or which is present on both sides but only binds to the inward-facing carrier. An expression of exactly the same form could be written for an analog outside the cell.

Results

Reactivity of the Internal Free Carrier with NEM (q_1)

The simplest way of measuring the reactivity of C_i (q_1) is to push the carrier into this form by means of external choline, as suggested by Martin [11] and Edwards [5]. To have the desired effect the transport mechanism of the substrate choline would have to meet the following two conditions: (i) the carrier-substrate complex must move inward across the membrane far more rapidly than the free-carrier returns,

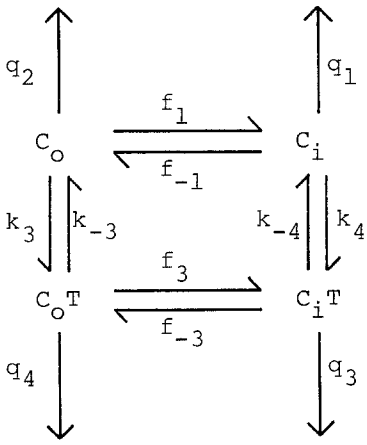

Fig. 1. A cyclic carrier mechanism involving inner and outer forms of the free carrier (C_i and C_o, respectively) and of the complex with substrate (C_iT and C_oT). Each carrier species reacts with an irreversible inhibitor at a rate governed by the constants q_1 to q_4. Rate constants for carrier-reorientation are indicated by the letter f, and those for complex formation by the letter k

and (ii) the substrate complex must dissociate rapidly on the inner membrane surface, relative to the rate of carrier reorientation. These requirements are made apparent in an equation giving the proportion of the carrier in the free inward-facing form in the steady-state, when there is a saturating concentration of substrate outside the cells and no substrate inside:

$$\frac{[C_i]}{C_t}=\frac{1}{1+\frac{f_{-1}}{f_3}+\frac{f_{-1}}{k_{-4}}\left(1+\frac{f_{-3}}{f_3}\right)}. \tag{2}$$

Eq. (2) is found from a general treatment [2] of the carrier scheme in Fig. 1; C_i is the inward-facing free carrier and C_t the total carrier concentration in all forms. From the rates of accelerated exchange of choline in both entry and exit, f_3, f_{-3}, k_{-3} and k_{-4} were previously shown to be far larger than f_1 or f_{-1}, which is to say that much the slowest step in the transport cycle is reorientation of the free carrier [8]. As a result the expression in the denominator of Eq. (2) is approximately equal to unity and the equation reduces to $[C_i]\simeq C_t$. Hence the inactivation rate under these conditions is that for C_i, which is q_1 (*see* Fig. 1). The same point, that in the presence of saturating external choline the observed inactivation rate constant (Q_{To}) equals q_1, may be proven by inserting the above inequalities into the general expression for Q_{To}. The equation, derived previously [4], is as follows:

$$Q_{To}=\frac{q_1+\frac{f_{-1}q_3}{k_{-4}}+\frac{f_{-1}}{f_3}\left(1+\frac{f_{-3}}{k_{-4}}\right)q_4}{1+\frac{f_{-1}}{f_3}+\frac{f_{-1}}{k_{-4}}\left(1+\frac{f_{-3}}{f_3}\right)}. \tag{3}$$

[1] Devés, R., Krupka, R.M. Apparent noncompetitive inhibition of transport by inhibitors bound at the substrate site: A test of the carrier mechanism involved in erythrocyte choline transport. (*submitted*)

Table 1. Rates of inactivation of transport by 1 mM N-ethylmaleimide (NEM), with saturating choline in the external solution[a]

Choline concentration[b] (μM)	Q_{To}
25	0.348 ± 0.012
25	0.300 ± 0.003
25	0.341 ± 0.240
25	0.332 ± 0.035
50	0.357 ± 0.026
50	0.348 ± 0.063
50	0.316 ± 0.018
50	0.322 ± 0.015

[a] The reaction was started by the addition of an isotonic solution of choline and N-ethylmaleimide to a suspension of washed cells. Reaction conditions: 37 °C, pH 6.8. The inactivation rate constant Q_{To} and its standard deviation were found in a least-squares analysis of a plot of the logarithm of the residual transport activity *versus* the treatment time. Each determination was carried out with a sample of cells from a different donor. From Eqs. (2) and (3) the measured inactivation rate Q_{To} closely approaches q_1 when the external substrate is choline. The mean value of Q_{To} is 0.333 ± 0.019 min^{-1}.

[b] The binding constant for choline influx, $\bar{K}_{To}$, is 6.3 ± 0.6 μM.

Rate constants for inactivation of transport by 1 mM NEM with saturating choline in the external solution are recorded in Table 1. The mean value of q_1 measured in this way is 0.333 ± 0.019 min^{-1}.

Reactivity of the Internal Carrier-Substrate Complex with NEM (q_3)

To achieve the condition in which the carrier is entirely in the form of the internal complex, the cells might be loaded with a nontransported analog at a saturating concentration. The difficulty here is that an analog is needed which penetrates the cell membrane by another route, for example because of its lipid solubility, and yet is retained during the NEM treatment. In his earlier study Edwards [5] employed the secondary amine metanephrine in such an experiment, but because its exit through the membrane is rapid (half-time 2 min) very short reaction periods and unusually high NEM concentrations were required, and this together with a continuously declining metanephrine concentration could lead to some uncertainty about the measured rates. Metanephrine was shown not to undergo transport on the choline carrier, but it was not determined whether or not its inhibition of choline transport was competitive. In any event the inactivation rate constant found with saturating internal metanephrine was higher than that with saturating external choline by a factor of 1.50 ± 0.09, while the combined effect of external choline and internal metanephrine was the same as the effect of internal metanephrine alone. Possibly because of the uncertainties which we have noted, Edwards concluded that the reactivities and therefore the conformations of the free and complexed carrier could be taken as essentially the same.

Table 2. Rates of inactivation by 1 mM N-ethylmaleimide (NEM) in the presence or absence of an equilibrated substrate analog at a saturating concentration[a]

Substrate analog	Binding constant (μM)	Concentration (μM)	Q_{app} (min^{-1})	Average value of Q_{app}
–			0.16 ± 0.014	
			0.19 ± 0.020	0.19
			0.22 ± 0.008	± 0.023
			0.17 ± 0.06	
Choline	6.3	50	0.25 ± 0.02	
	± 0.6	50	0.21 ± 0.003	0.23
		50	0.21 ± 0.007	± 0.020
		50	0.25 ± 0.007	
2-Dimethyl	18.7	91	0.19 ± 0.002	
amino-	± 0.1	100	0.23 ± 0.010	0.23
ethanol		250	0.25 ± 0.010	± 0.023
		500	0.24 ± 0.008	
2-Di-*n*-	1,130	5,000	0.54 ± 0.008	
butyl-	± 180	10,000	0.66 ± 0.006	0.57
amino-		10,000	0.54 ± 0.010	± 0.046
ethanol		10,000	0.55 ± 0.018	
		–	0.55 ± 0.023[b]	

[a] Conditions, 37 °C, pH 6.8. The inactivation rate constant Q_{app} together with its standard deviation was found from a least-squares analysis of a plot of the logarithm of the residual transport activity *versus* the time of treatment with N-ethylmaleimide [4]. Determinations are reported for cell samples from different donors.

[b] From the intercept in Fig. 2.

Another method of determining q_3 which avoids the difficulties encountered with an inhibitor such as metanephrine, and which we have used in the present study, involves a choline analog specific for the internal carrier site. Di-*n*-butylaminoethanol is such an analog, for (i) it enters the cells by passive diffusion, coming to equilibrium in 3–4 min; (ii) it competes with choline for the carrier site without undergoing transport on the carrier; and (iii) it becomes bound almost exclusively to the inward-facing form of the carrier rather than the outward-facing form[2]. In this case the cells may be treated with NEM when the analog is at equilibrium across the cell membrane.

Inactivation rates were therefore determined in the presence of dibutylaminoethanol at a near-saturating concentration, after preincubation of the cells with the amine for a period of 1 hr to ensure equilibri-

[2] *See* Footnote 1, p. 100.

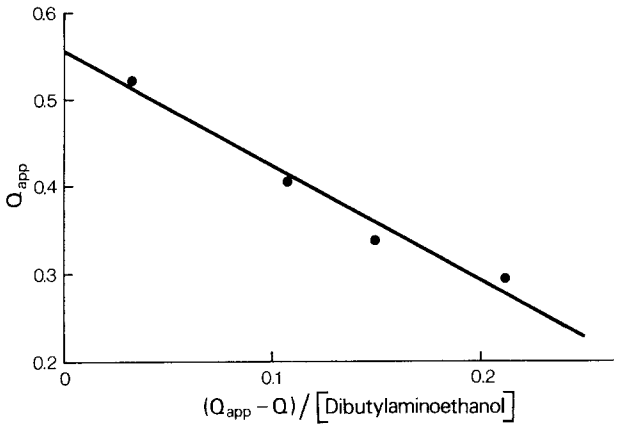

Fig. 2. Dependence of the inactivation rate Q_{app} on the concentration of 2-di-*n*-butylaminoethanol. The results are plotted according to Eq. (1). The half-saturation constant for the amine, obtained from the slope, is 1.30 ± 0.16 mM; and Q_{Ti} $(=q_3)$, obtained from the intercept, is 0.551 ± 0.023 min^{-1} (as found in a least-squares analysis of the experimental points). Units of Q_{app}, min^{-1}, and of concentration, mM. Conditions: 1 mM N-ethylmaleimide; pH 6.8; 37 °C

um. The pH of the solution as always was 6.8, where the amine exists almost entirely in the cationic form. Another choline analog, dimethylaminoethanol, was also tested in order to find the effect of a tertiary amine which is similar to choline in size and which should therefore add to both the internal and external carrier sites, as choline does. Experimental inactivation rate constants are recorded in Table 2 for cells treated with NEM either in the absence of a substrate analog or in the presence of equilibrated dibutylaminoethanol, dimethylaminoethanol, or choline. The pseudo-first-order rate constants in choline and dimethylaminoethanol are identical, 0.23 min^{-1}, showing that a tertiary amine in itself has no unusual effect on the rate. This is slightly higher than in the absence of a substrate analog, 0.19 min^{-1}. With dibutylaminoethanol the rate constant is considerably higher, 0.57 min^{-1}, and this represents the reactivity of the internal complex. By contrast, other inhibitory substrate analogs which are bound externally rather than internally almost completely protect the system against inactivation. Quaternary amines unable to penetrate the membrane, such as dimethyl-*n*-butyl(2-hydroxyethyl) ammonium and diethyl-*n*-decyl(2-hydroxyethyl) ammonium ions, may be cited as examples [4].

In agreement with these results intermediate inactivation rates, lying between those for the inner and outer carrier forms, are expected for the free carrier and also for the system equilibrated with a substrate analog which is bound on both sides of the membrane. This is because both carrier forms are present under these conditions, but only the inner form reacts. The constants given in Table 2 conform with this prediction: in pure buffer the constant is 0.19 min^{-1} compared to 0.33 min^{-1} for the internal free carrier, and in the presence of choline or dimethylaminoethanol it is 0.23 min^{-1} compared to 0.57 min^{-1} for the internal complex.

To find the half-saturation constant of dibutylaminoethanol, inactivation rates were measured in the presence of varying concentrations of the amine. The results are plotted in Fig. 2, and the line of best fit to the data gives a half-saturation constant of 1.30 ± 0.16 mM. The competitive inhibition constant in transport (exit) was previously[3] shown to be 1.13 ± 0.18 mM. If our hypothesis and kinetic treatment are correct, the same experimental parameter $\bar{K}_{Ti}$ should be found in both experiments (Eq. (1); ref. [2] and Footnote 1), and the agreement in the two measurements therefore supports the interpretation. Edwards [5] reported a similar agreement for choline and for an externally bound inhibitor, decamethonium.

Discussion

The experiments on NEM inactivation rates show that the internal free carrier reacts more slowly than the complex, the rate constants being 0.33 ± 0.02 min^{-1} for the free carrier compared to 0.57 ± 0.05 min^{-1} for the complex with dibutylaminoethanol. This conclusion is supported by the data of Edwards [5] on metanephrine, as discussed above. The results prove that the carrier conformation is altered when a nontransported substrate analog, dibutylaminoethanol, is bound, but they do not provide unequivocal evidence about the effect of choline. Such evidence is not easily obtained because the steady-state level of the substrate complex is extremely low when choline is present inside the cell and not outside [owing to rate-limiting reorientation of the free carrier; *see* Eq. (2) above]. However, other evidence indicates that a similar change occurs. First, a conformational change has been detected with substrates other than choline. We have found (*manuscript in preparation*) that diethylaminoethanol and dimethylamino-1-propanol are transported substrates, and that when they are present at equilibrium inside and outside the cell, the inactivation rate, though slower than with dibutylaminoethanol, is substantially faster than that of the internal free carrier (q_1). It follows that both these substrates have a higher affinity on the inside than on the outside, and that the conformation of the carrier is altered when they are bound. We have seen above that this is not a necessary property of tertiary amines, since dimethylaminoethanol has no such effect. It has also been found that the rate constant for inactivation of the external complex (q_4)

[3] *See* Footnote 1, p. 100.

is the same (near zero) for all members of a series of substrates having varying transport rates and for nontransported substrate analogs [4], suggesting that the inactivation rate constant for the complex is independent of the rate at which it undergoes reorientation in the membrane.

All the results on NEM inactivation rates are in quantitative agreement with a simple two-state mechanism in which the carrier assumes either an outward-facing or an inward-facing orientation, and in which the conformations of the free and complexed carrier on one side of the membrane are similar to one another but markedly different from those on the other side. These conclusions rest on the finding [4, 5] that the inward-facing forms of the carrier react with NEM while the outward-facing forms do not (q_1, $q_3 \gg q_2$, q_4). In other studies [9] we showed that the transport mechanism is cyclic, in that a substrate transfer site is exposed alternately on one side of the membrane and then the other rather than on both sides at once, as in linear mechanisms. In the latter, reversible competitive inhibitors should add to the carrier simultaneously on the inner and outer surfaces of the membrane, while in cyclic mechanisms they could only add on one side at a time, and these alternatives were clearly distinguished by the concentration dependence of inhibition by substrate analogs present both inside and outside the cell. Likewise the observations on NEM inactivation rates would be difficult to reconcile with any linear mechanism [4, 5].

The two sets of observations are therefore mutually supportive and establish the essential features of the system. A cyclic mechanism involving conformationally distinct inward-facing and outward-facing forms is in accord with moveable carrier models as originally conceived [10, 15] and also with related models involving a gated channel [6, 12–14]. However the occurrence of a conformational change following substrate binding ($q_3 > q_1$) implies a more intimate association between the substrate and the carrier than is absolutely required by these models, which in their simplest forms involve substrate binding at an inert site on the surface of the carrier structure. Intimate association of the substrate with the carrier was also suggested both by the exceptionally strong ionic bond in the carrier-choline complex [7], and by far more restrictive steric requirements for transfer through the membrane than for binding [3]. The latter finding indicates that the substrate molecule may be enclosed by the carrier during transport. Moreover, the striking difference in binding specificity found at the internal and external substrate sites[4] points to a mechanism more complicated than a moveable carrier or gated channel in its simplest form.

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References

1. Devés, R., Krupka, R.M. 1979. A simple experimental approach to the determination of carrier transport parameters for unlabeled substrate analogs. *Biochim. Biophys. Acta* **556**:524–532
2. Devés, R., Krupka, R.M. 1979. A general kinetic analysis of transport: Tests of the carrier model based on predicted relations among experimental parameters. *Biochim. Biophys. Acta* **556**:533–547
3. Devés, R., Krupka, R.M. 1979. The binding and translocation steps in transport as related to substrate structure: A study of the choline carrier of erythrocytes. *Biochim. Biophys. Acta* **557**:469–485
4. Devés, R., Krupka, R.M. 1981. Evidence for a two-state mobile carrier mechanism in erythrocyte choline transport: Effects of substrate analogs on inactivation of the carrier by N-ethylmaleimide. *J. Membrane Biol.* **61**:21–30
5. Edwards, P.A. 1973. Evidence for the carrier model of transport from the inhibition by N-ethylmaleimide of choline transport across the human red cell membrane. *Biochim. Biophys. Acta* **311**:123–140
6. Klingenberg, M., Aquila, H., Kramer, R., Babei, W., Feckl, J. 1977. The ADP, ATP translocation and its catalyst. *In:* FEBS Symposium No. 42. S. Semenza and E. Carafoli, editors. pp. 567–579. Springer-Verlag, New York
7. Krupka, R.M., Devés, R. 1980. The electrostatic contribution to binding in the choline transport system of erythrocytes. *J. Biol. Chem.* **255**:8546–8549
8. Krupka, R.M., Devés, R. 1980. The choline transport system of erythrocytes: Distribution of the free carrier in the membrane. *Biochim. Biophys. Acta* **600**:228–232
9. Krupka, R.M., Devés, R. 1981. An experimental test for cyclic versus linear transport models: The mechanism of glucose and choline transport in erythrocytes. *J. Biol. Chem.* **256**:5410–5416
10. LeFevre, P.G. 1954. The evidence for active transport of monosaccharides across the red-cell membrane. *Symp. Soc. Exp. Biol.* **8**:118–135
11. Martin, K. 1971. Some properties of an SH group essential for choline transport in human erythrocytes. *J. Physiol. (London)* **213**:647–667
12. Patlak, C.S. 1957. Contributions to the theory of active transport: II. The gate type non-carrier mechanism and generalizations concerning tracer flow, efficiency, and measurement of energy expenditure. *Bull. Math. Biophys.* **19**:209–235
13. Singer, S.J. 1974. Lipid-protein interactions in membranes. *In:* Comparative Biochemistry and Physiology of Transport. L. Bolis, K. Bloch, S.E. Luria, and F. Lynen, editors. pp. 95–101. North Holland Publishing Co., Amsterdam
14. Vidaver, G.A. 1966. Inhibition of parallel flux shown by transport models not involving a mobile carrier. *J. Theor. Biol.* **10**:301–306
15. Widdas, W.F. 1952. Inability of diffusion to account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer. *J. Physiol. (London)* **118**:23–39

[4] *See* Footnote 1, p. 100.

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