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Substrate-induced conformational change of human erythrocyte glucose transporter: inactivation by alkylating reagents

Amrit L. Rampal and Chan Y. Jung

Biophysics Laboratory, Veterans Administration Medical Center, and Department of Biophysical Sciences, State University of New York at Buffalo, Buffalo, NY (U.S.A.)

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The glucose transport carrier in human erythrocyte membranes, when transporting glucose, undergoes a conformation change. In an attempt to delineate the extent of this substrate-induced conformational change, transport inactivation by 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, *N*-ethylmaleimide, iodoacetamide, and 2,4,6-trinitrobenzenesulfonic acid was examined in the presence and in the absence of D-glucose. All these alkylating agents inactivated the carrier. With each of these reagents, with the exception of trinitrobenzenesulfonic acid, D-glucose modified the rate of inactivation as well as the activation enthalpy (ΔH^*) of the inactivation. The inactivation by trinitrobenzenesulfonic acid was not affected by the sugar. Based on these findings, it is suggested that the substrate-induced conformational change mostly occurs within the transmembrane hydrophobic domain while the hydrophilic extramembrane domains are largely outside of this change.

Introduction

The movement of glucose across the plasma membrane of human erythrocytes is a carrier-mediated process (also known as facilitated diffusion [1]), where a transmembrane protein (transporter or carrier) catalyzes translocation of selected sugars across an otherwise almost impermeable membrane diffusion barrier [2]. The transporter protein has been purified [3–5] and its amino acid sequence has been deduced from cDNA sequence [6]. A number of important structural features are now apparent for this transporter molecule. Hydropathy analysis of the amino acid sequence has suggested that the transporter contains twelve transmembrane segments, some of which form a water-filled channel through which the substrate, sugar molecule, may move [6]. Some of these suggested features have been supported by recent circular dichroism and Fourier transform infrared spectroscopic studies [7].

We know, on the other hand, very little about the molecular mechanism by which this transporter functions. Current understanding of the interaction of intrinsic proteins with lipids in membranes together with thermodynamic considerations exclude any form of rotating carrier models in favor of the channel (or gated-pore) model [8]. Furthermore, accumulated knowledge about the structural basis of protein function dictates that conformational dynamics [9] would certainly be an essential feature of the transport function.

Several experimental techniques have been applied to demonstrate the functional importance of conformational transitions in this carrier. These include demonstrations of changes in chemical reactivity [10,11], intrinsic fluorescence [12], and

Correspondence: C.Y. Jung, Veterans Administration Medical Center, 3495 Bailey Avenue ,Buffalo, NY 14215, U.S.A.

hydrogen-tritium exchange kinetics [13] induced by substrates or inhibitors. Of these, the change in chemical reactivity of a protein functional group provided the first clue that a conformational change is involved in the glucose transport function of human erythrocytes. Bowyer and Widdas [14] first demonstrated that the transport function is inactivated by 1-fluoro-2,4-dinitrobenzene, and this inactivation becomes faster in the presence of glucose, implying that a chemical group in the transporter reacting with fluorodinitrobenzene is more exposed or more reactive to the inhibitor during substrate transport. Krupka [15,16] subsequently extended this notion, and showed that only those sugars which are transported induce this conformation of the enhanced reactivity to fluorodinitrobenzene; maltose and phloretin, which are not transported, stabilize the less reactive conformation. Further characterization of the carrier inactivation by fluorodinitrobenzene in our laboratory [17] has provided additional information on the nature of this substrate-induced conformational change: In the absence of the substrate, the inactivation is first order with respect to the fluorodinitrobenzene concentration and shows an activation energy of 32 cal per mol. In the presence of 50 mM D-glucose, the inactivation is nearly second order in terms of fluorodinitrobenzene concentration and shows an activation energy of 17 cal per mol. The pH dependency of the inactivation is also significantly modified by the presence of the sugar substrate. Based on these observations, we have suggested that the transporter may assume at least two readily distinguishable conformational states depending on whether it is free of a substrate (F-conformation) or complexed with a substrate (C-conformation) [17].

A detailed description of this proposed conformational change is not available. In an effort to delineate the extent of this substrate-induced conformational change, the present study examines the reactivities of additional essential functional groups in the carrier as they may be affected by this conformational change. Using human erythrocyte ghosts, inactivation of glucose transport activity by a number of different alkylating reagent was studied both in the presence and in the absence of D-glucose. It was found that in the presence of D-glucose, the carrier inactivations by *N*-ethylmaleimide, chloronitrobenzoxadiazole and iodoacetamide are greatly modified, whereas that by trinitrobenzenesulfonic acid is not. The results indicate that the reactions with the former group of reagents take place within the domain of the substrate-induced conformational change, whereas the reaction of trinitrobenzenesulfonic acid may take place outside this domain. The physicochemical nature of some of the groups reacting with these alkylating reagents is discussed in relation to the conformational change. Valuable insight into the extent of this substrate-induced conformational transition may be obtained if each of these reaction-sites is located as individual amino acid on the amino acid sequence, and eventually at a higher level of the three-dimensional structure. The present study would provide the ground work for such future studies.

Experimental procedure

Materials. Human erythrocytes were obtained from fresh blood drawn from healthy donors, into an ACD bag. Glucose transport-active, resealed ghosts essentially free of hemoglobin were prepared as previously described [17]. D-[^{14}C]Glucose was obtained from Amersham-Searle, Arlington Heights, IL. 7-Chloro-4-nitrobenz-2-oxa-1,3-diazole and 2,4,6-trinitrobenzenesulfonic acid were obtained from Aldrich Chemical Co. D-Glucose, *N*-ethylmaleimide and iodoacetamide were purchased from K & K Laboratories, Plainview, NY.

Treatment of ghosts with alkylating reagents. Ghosts were incubated with a freshly prepared alkylating reagent solution at a specified temperature, in the presence or in the absence of 50 mM D-glucose. When sugar was included, the ghosts were pre-equilibrated with the sugar for 30 min at 37°C prior to the alkylation incubation. Except for the experiments with chloronitrobenzoxadiazole, the incubation was carried out in 1 : 10 isotonic balanced salt solution (BSS), which contained Na^+, 12.5 mM; K^+, 0.5 mM; Ca^{2+}, 0.38 mM; Mg^{2+}, 0.25 mM; all as chlorides, buffered with Tris-HCl (10 to 20 mM) at a specified pH. The incubation with chloronitrobenzoxadiazole was carried out in a 50 mM sodium citrate buffer

at pH 7.0, containing 1 mM EDTA. Whenever the pH changed during the incubation, it was corrected by intermittent titration, which kept pH fluctuation less than 0.3 units. Temperature was kept constant within 0.5 Cdeg. Alkylation reaction was terminated by adding 10–30 volumes of prechilled buffer containing 2–5% (v/v) ethanol. The treated ghosts were recovered by centrifugation at 4°C, and the washing procedure was repeated two or three times more.

Assay for glucose transport activity. The D-glucose carrier activities of ghosts were assayed by measuring rates of the mercuric chloride-sensitive isotopic equilibrium exchange of D-glucose as described elsewhere [18]. All flux measurements were done with a suspension of 10^9 ghosts per ml in 1 : 10 isotonic balanced salt solution at pH 7.4 and 21°C. Ghosts were pre-equilibrated with 10 mM D-glucose for 30 min. After the addition of a tracer amount of D-[^{14}C]glucose at $t = 0$, six aliquots were sampled at 2–3-s intervals, injected into 8 volumes of prechilled, arresting solution containing 2 mM mercuric chloride, and ghosts were recovered by centrifugation (30 000 × g at 4°C). Transport activities were calculated from these isotopic equilibrium time courses in terms of the half-equilibration time ($t_{1/2}$ in seconds) as detailed elsewhere [18].

Spectrophotometric measurements. Absorption data were obtained with a Gilford Spectrophotometer, Model 240. Fluorescence measurements, uncorrected for variations in instrument response, were obtained on an Aminco-Bowman Spectrophotofluorometer equipped with a polarization accessory, according to Jacobson and Wobschall [19].

Results

The glucose carrier activity of human erythrocyte ghosts diminished progressively when the ghosts were incubated with chloronitrobenzoxadiazole (Fig. 1), trinitrobenzenesulfonic acid (Fig. 2), *N*-ethylmaleimide (Fig. 3) and iodoacetamide (Fig. 4). The inactivation was not reversed by repeated washes with buffer free of unreacted reagents. The time courses of the inactivation by each of these reagents appear to fit fairly satisfactorily to a single, pseudo-first-order process (Figs. 1–4). The rates of the inactivation by each of these reagents were increased as the pH was raised. The ranges of pH where the rates were most acutely affected were 7.0 to 7.7 for *N*-ethylmaleimide (with 50 mM glucose), 6.8 to 7.5 for trinitrobenzenesulfonic acid (without glucose), and 7.4 to 8.7 for iodoacetamide (with 50 mM glucose). The rates of transport inactivation by chloronitrobenzoxadiazole, *N*-ethylmaleimide and iodoacetamide, were significantly enhanced in the presence of 50 mM D-glucose (Figs. 1, 3 and 4), whereas that of trinitrobenzenesulfonic acid was neither enhanced nor reduced by the sugar (Fig. 2).

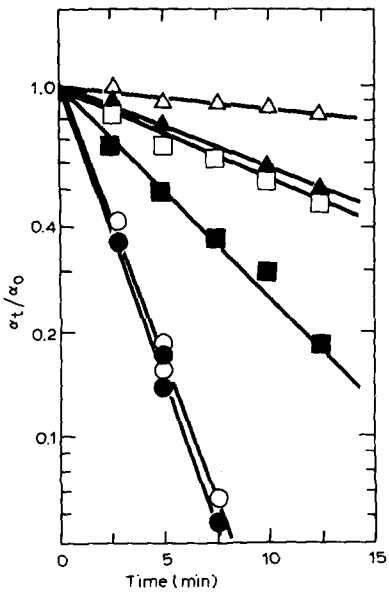

Fig. 1. Time course of the inactivation of the glucose carrier in isolated membranes by chloronitrobenzoxadiazole. Ratio of carrier activities after the treatment (a_t) to that without treatment (a_0) are correlated with the time of the treatment in a semilog plot. Chloronitrobenzoxadiazole, 0.3 mM; ghosts, 1.66 · 10^9 per ml, in 50 mM sodium citrate buffer (pH 7.0), containing 1 mM EDTA. The inactivation experiments were carried out in the absence (open symbols) and in the presence of 50 mM D-glucose (solid symbols) at 37°C (circles), at 22°C (squares) and at 10°C (triangles).

This glucose effect on the inactivation was sensitive to changes in temperature. With chloronitrobenzoxadiazole (Fig. 1), the rate of inactivation was only slightly affected by the sugar at 37°C. However, the sugar effect was much larger at a lower temperature, increasing 2- and 4-fold at

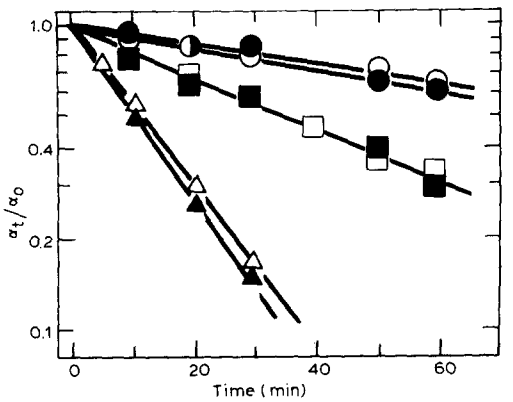

Fig. 2. Time course of the inactivation of the glucose carrier by trinitrobenzenesulfonic acid. Trinitrobenzenesulfonic acid, 3 mM; ghosts, $1.66 \cdot 10^9$ per ml in 1:10 balanced salt solution buffered with 20 mM Tris-HCl at pH 7.9: without glucose (open symbols); with 50 mM glucose (solid symbols); temperature of the inactivation at 37°C (triangles), and at 21°C (circles). Inactivation was also carried out in the presence of excess amounts of $MgCl_2$ (10 mM), at 21°C (squares).

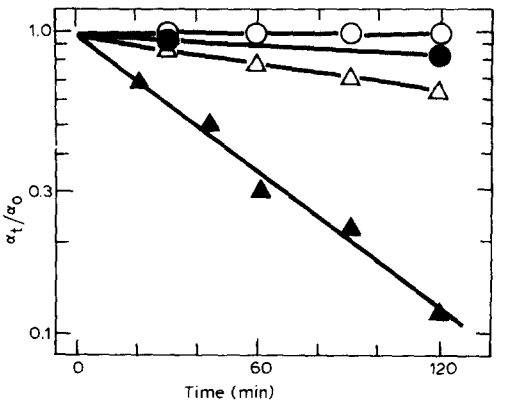

Fig. 4. Time course of the inactivation of the glucose carrier by iodoacetamide. Iodoacetamide, 30 mM; ghosts, $1.66 \cdot 10^9$/ml, in 1:10 balanced salt solution, buffered with 10 mM Tris-HCl at pH 8.7–8.8. Without glucose (open symbols) and with 50 mM glucose (solid symbols). Inactivation at 37°C (triangles) and at 23°C (circles).

22°C and 10°C, respectively. In the case with *N*-ethylmaleimide (Fig. 3), the sugar increased the rate of inactivation approximately 8-fold at 37°C. At 21°C, the rate of inactivation by *N*-ethylmaleimide in the absence of the sugar was so low that accurate measurement was impossible, while the addition of the sugar increased this rate greatly to a readily measurable level. A similar type of glucose effect is observed with iodoacetamide (Fig. 4): At 37°C, treatment of ghosts in the absence of glucose with 30 mM iodoacetamide inactivated the carrier at a rate of 20% per hour, but if 50 mM D-glucose was present, the rate of inactivation was increased by 3.5-fold. At 23°C, the inactivation was undetectable after a 2-h treatment without the sugar, whereas, it became appreciable in the presence of 50 mM glucose. In this case, the sugar enhanced the inactivation more than 5-fold. Carrier inactivation by trinitrobenzenesulfonic acid was unique, showing no glucose effect at all temperatures examined (Fig. 2).

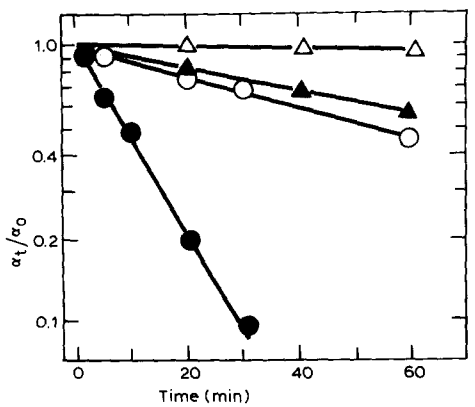

Fig. 3. Time course of the inactivation of the glucose carrier by *N*-ethylmaleimide. *N*-Ethylmaleimide, 4 mM; ghosts, $1.66 \cdot 10^9$/ml, in 1:10 balanced salt solution buffered with Tris-HCl at pH 7.4. Without glucose (triangles) and with 50 mM glucose (circles); at 37°C (solid) and at 21°C (open).

These observations indicated that D-glucose, a typical substrate, affects the activation enthalpy (ΔH^*) of the carrier inactivation by these reagents. Accordingly, a systematic study of the temperature effects on the modulation of transport inactivation by glucose was carried out with chloronitrobenzoxadiazole, trinitrobenzenesulfonic acid, *N*-ethylmaleimide (Figs. 5A, 5B, and 5C, respectively) and iodoacetamide (results not shown). Activation enthalpies calculated from these studies were, without and with 50 mM D-glucose, respectively, 22 and 14 cal/mol (chloronitrobenzoxadiazole), 23 and 19 cal/mol (*N*-ethylmaleimide), 31 and 24 cal/mol (iodoacetamide), and 22 and 22 cal/mol (trinitrobenzenesulfonic acid). Thus, the activation enthalpy of the inactivation by chloronitrobenzoxadiazole, *N*-ethyl-

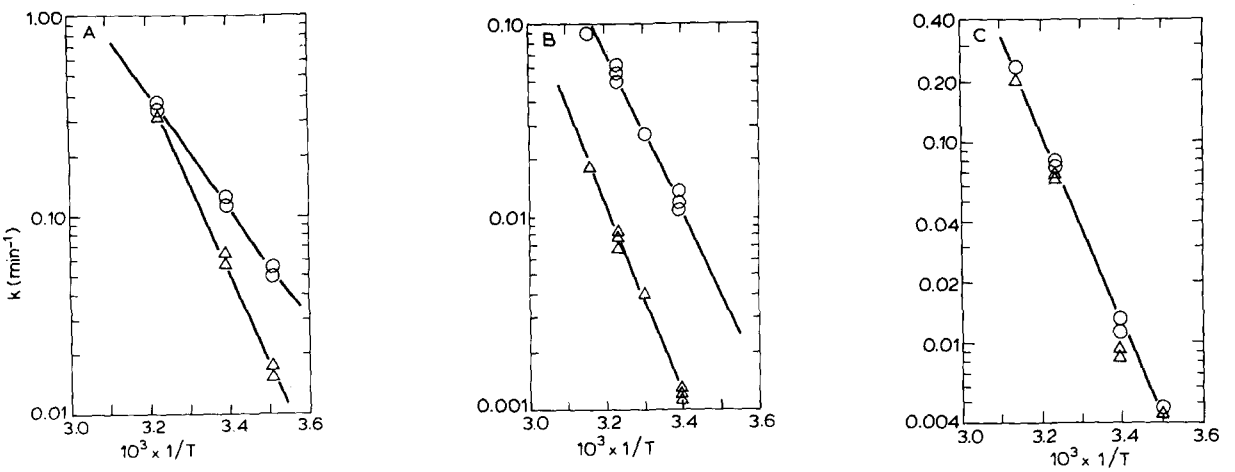

Fig. 5. Temperature effects on the rates of the carrier inactivation by chloronitrobenzoxadiazole (A), *N*-ethylmaleimide (B) and trinitrobenzenesulfonic acid (C), in the presence (○) and in the absence (△) of 50 mM D-glucose. Each point represents results of a single experiment, essentially identical to those for Figs. 1, 2 and 3. Rate constants (k, in min^{-1}) for the inactivation time courses such as in Figs. 1–4 were calculated and correlated with temperature according to the Arrhenius plot.

maleimide or iodoacetamide was significantly reduced in the presence of D-glucose. On the other hand, the activation enthalpy of inactivation by trinitrobenzenesulfonic acid was not affected at all.

Limited attempts were made to define the chemical identity of the functional groups which are responsible for the inactivation by chloronitrobenzoxadiazole. The conditions employed here for the reaction with chloronitrobenzoxadiazole are those which are known to be specific to the reaction with SH groups as demonstrated with several water-soluble enzymes [20]. Spectral studies on the chloronitrobenzoxadiazole-treated ghosts (Table I and Fig. 6) revealed a single absorption maximum at 420 nm without any trace of

TABLE I

SPECTRAL CHARACTERISTICS OF NBD-MODIFIED ERYTHROCYTE GHOSTS AND OTHER NBD DERIVATIVES

Ghosts ($1.66 \cdot 10^9$/ml) were treated with 0.3 mM chloronitrobenzoxadiazole (NBD-Cl) for 20 min at pH 7.0 in 50 mM sodium citrate buffer containing 1 mM EDTA, at 22°C with or without 50 mM D-glucose. They were then washed with 1 : 10 balanced salt solution (pH 7.4) three times and solubilized with 1% SDS. Apo-GAPDH, apoglyceraldehyde-3-phosphate dehydrogenase.

Compound	Absorption maximum (nm)	Excitation maximum (nm)	Emission maximum (nm)	Fluorescence polarization
NBD ghosts				
No glucose	420	437 [a]	508 [b]	0.390 [c]
NBD ghosts				
50 mM glucose	420	434 [a]	508 [b]	0.394 [c]
Apo-GAPDH-NBD [d]	425	425	515	0.38
NBD-phosphorylase [d]	420	430	515	0.41
N-NBD-cyclohexylamide [d]	475	470	545	0.05

[a] 700 V; slits, 1, 1; $\lambda_{em} = 530$ nm.
[b] 700 V; slits, 1, 1; $\lambda_{ex} = 432$ nm.
[c] An average of three measurements using λ_{em} 505 nm, at 23.5°C.
[d] Data from Birkett et al. (1970) [20].

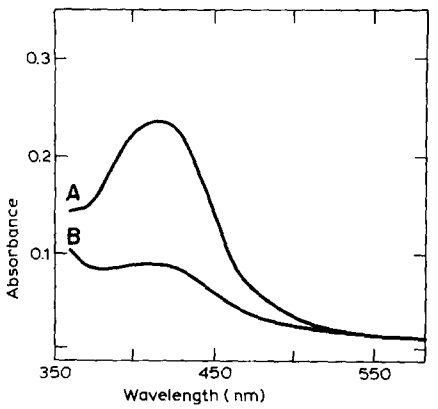

Fig. 6. Absorption spectra of NBD-modified human erythrocyte ghosts. (A) chloronitrobenzoxadiazole (0.3 mM) treated ghosts in 50 mM sodium citrate buffer (pH 7.0) containing 1 mM EDTA for 10 min at 37°C. (B) A treated sample of (A) treated again with 20 mM 2-mercaptoethanol at 20°C for 30 min. In both cases, the samples were solubilized with 1% SDS.

a peak at the 470 nm region, and a fluorescence polarization of 0.39. The absorption at 420 nm was not appreciably affected by the presence of 50 mM D-glucose (data not shown), but drastically reduced in the presence of 2-mercaptoethanol (Fig. 6). These findings are consistent with the conclusion that under the conditions employed here, chloronitrobenzoxadiazole reacts mostly with SH groups of membrane proteins, including the glucose transporter and band 3.

Discussion

We have shown here that chloronitrobenzoxadiazole, trinitrobenzenesulfonic acid, *N*-ethylmaleimide and iodoacetamide, all inactivate the glucose carrier of human erythrocytes, much like fluorodinitrobenzene [17]. There are considerable variations in their potencies; chloronitrobenzoxadiazole > fluorodinitrobenzene > trinitrobenzenesulfonic acid > *N*-ethylmaleimide > iodoacetamide. In one report using a whole cell system [21], iodoacetamide has been shown to be ineffective, and this may have been due to the low concentrations (less than 5 mM) used in the study. With each of these reagents, the time course of the carrier inactivation was essentially a single exponential, indicating that either a single group or several groups with similar reactivities are involved in each inactivation. Participation of some slow reactions by different groups, however, cannot be entirely ruled out.

The present study demonstrates that the carrier inactivation by chloronitrobenzoxadiazole, *N*-ethylmaleimide and iodoacetamide, but not by trinitrobenzenesulfonic acid, was significantly enhanced by the presence of its substrate, D-glucose, while the enthalpy of inactivation was reduced. A similar enhancement of the carrier inactivation has been observed with fluorodinitrobenzene [17]. When more detailed aspects of this substrate effect were considered, some significant differences were noted among the inactivations by these reagents. With *N*-ethylmaleimide, D-glucose greatly enhanced the rate of the inactivation at all temperatures, with only a minimal effect on the enthalpy. With chloronitrobenzoxadiazole, the enthalpy appeared to be more affected than the rate, whereas, with iodoacetamide, as with fluorodinitrobenzene [17], both the rate and the enthalpy were equally greatly affected. In contrast to all of these reagents, the inactivation by trinitrobenzenesulfonic acid was not affected by D-glucose in rate as well as in enthalpy.

It is important to note that the rate of inactivation by each of these alkylating reagents was enhanced, but not inhibited by D-glucose. Since the transport half-saturation constant (K_m) is approx. 25 mM, about 75% of the carriers were bound with D-glucose at the glucose concentration used in this study. This lack of inhibition would then indicate that all of the functional groups concerned here are sterically outside the glucose binding site. Thus, glucose-induced modification of the inactivation with each of these reagents is an allosteric modulation.

The glucose transporter is a transmembrane protein with 50% of the mass being embedded in the membrane lipid bilayer (transmembrane domain) which may accommodate an internal aqueous channel [6]. The unit may contain a substrate recognition site which alternately opens to the inner and outer faces of the membrane [10,12]. Proper interaction of the substrate at its sites and the channel, may induce a series of selective conformational transitions in the carrier protein which is essential for the translocation of the substrate through the channel. This series of substrate-

induced conformational transitions is distinct from those conformational transitions that take place in the absence of substrate.

The results obtained in the present study reveal some additional features of this substrate-induced conformational change. The transporter contains several functional groups, alkylation of which inactivates the carrier presumably by interfering with the conformational transitions needed for substrate translocation. The inactivation-associated alkylation of the functional groups reacting with chloronitrobenzoxadiazole, *N*-ethylmaleimide, iodoacetamide and fluorodinitrobenzene is enhanced by the substrate-induced conformational states, while those reacting with trinitrobenzenesulfonic acid is not affected. This would indicate that the groups reacting with chloronitrobenzoxadiazole, *N*-ethylmaleimide, iodoacetamide, and fluorodinitrobenzene are in the domain that undergoes the substrate-induced conformational change. A group or groups reacting with trinitrobenzenesulfonic acid, on the other hand, appear outside this domain.

There appears to be some correlation between relative hydrophobicity of alkylating reagents and susceptibility of the transport inactivation by these reagents to the substrate-induced conformational transitions. That chloronitrobenzoxadiazole-reacting groups are most likely located in the hydrophobic domain of the carrier is suggested by fluorescence spectrum of chloronitrobenzoxadiazole-treated ghosts. The fluorescence emission spectrum of *N*-acetyl-*S*-NBD-cysteine is sensitive to solvent polarity; in non-polar solvents, the emission maximum is blue shifted from 525 nm in aqueous solution, and the emission intensity is enhanced [22]. The chloronitrobenzoxadiazole-modified, SDS-solubilized ghosts fluorescence at 508 nm (Table I), showing that the emitter groups are mostly located in hydrophobic domains. The transport inactivation by maleimides is known to be potentiated by an increase in hydrophobicity of the molecules [21], suggesting that the SH group (or groups) reacting with *N*-ethylmaleimide for the inactivation may be located in a hydrophobic domain of the transporter. The groups reacting with iodoacetamide and fluorodinitrobenzene, the readily membrane permeating reagents, may also be located in a hydrophobic domain. The groups reacting with trinitrobenzenesulfonic acid, a highly polar, nonpermeating reagent with a unit charge, on the other hand, may be located in hydrophilic domains, most likely at the membrane-aqueous interface [23,24]. Based on these considerations, it may be suggested that the domain of the substrate-induced conformational change encompasses a significant portion of the hydrophobic, transmembrane domain of the transporter, whereas (the extracellular and the two major cytoplasmic) hydrophilic domains [6] are generally excluded. However, the exclusion of hydrophilic domains from this substrate-induced conformational change based on the trinitrobenzenesulfonic acid data alone is less firm, as it is entirely possible that the trinitrobenzenesulfonic acid reacting groups are in this domain and yet may remain unaffected.

To gain insight into the extent of this substrate-induced conformational transition, each of these reaction sites is to be chemically identified and located within the amino acid sequence. None of the reagents studied here is absolutely specific to a particular group. It has been known with water soluble enzymes that chloronitrobenzoxadiazole reacts under certain conditions specifically with SH groups [20], while under other conditions, NH_2 groups also react [25]. Furthermore, the products of the reaction with SH and NH_2 groups are known to show different absorption and fluorescence emission maxima and fluorescence polarizations [20]. The conditions employed here for the carrier inactivation with chloronitrobenzoxadiazole (NBD-Cl) are those known to be specific for the reaction with SH groups of water-soluble enzymes. Spectral studies on the NBD-modified, SDS-solubilized ghosts (Table I) indeed revealed that absorption and fluorescence remission maxima (also see Fig. 6) and fluorescence polarization are those for known S-NBD products (such as NBD-modified apoglyceraldehyde-3-phosphate dehydrogenase and phosphorylase *b*), and are not those for known HN-NBD products (Table I), indicating that SH groups rather than NH_2 groups of the membrane proteins react with chloronitrobenzoxadiazole during the inactivation. However, there are 3–4-times more copies of band 3 than glucose carriers in the ghost and each band 3 molecule has three SH groups compared with six SH groups in glucose

transporter, thus the spectral properties observed here may not be attributed to glucose transporter alone.

Trinitrobenzenesulfonic acid is a reagent which is known to react mainly with NH_2 groups and sometimes with SH groups [26]. An interesting exmaple is that trinitrobenzenesulfonic acid inactivates glutamate dehydrogenase and decreases its allosteric response to the effector, GTP. It was chemically demonstrated that trinitrobenzenesulfonic acid reacted only with NH_2 groups, SH groups in this enzyme being totally excluded from the trinitrophenylation [27]. Further, with human erythrocytes, the reaction of membrane NH_2 groups with trinitrobenzenesulfonic acid is known to be stimulated by Mg^{2+} [24]. A similar stimulatory effect of Mg^{2+} was observed in the carrier inactivation by trinitrobenzenesulfonic acid: An addition of 10 mM Mg^{2+} incresed the carrier inactivation by trinitrobenzenesulfonic acid 2-fold (Fig. 2). It is thus tempting to suggest that a NH_2 group rather than SH is involved in the carrier inactivation by trinitrobenzenesulfonic acid.

The chemical identities of those groups reacting with fluorodinitrobenzene, *N*-ethylmaleimide and iodoacetamide cannot be deduced in the present study alone. In a study with intact erythrocytes, it has been indicated that a NH_2 group is responsible for the carrier inactivation with both fluorodinitrobenzene and *N*-ethylmaleimide, while a SH group may be involved in the inactivation by iodoacetamide [28]. However, Abbott and Schachter [29] have argued in favor of sulfhydryl group involvement in labelling of intact erythrocytes and ghost membranes by *N*-ethylmaleimide derivatives.

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