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Dithiols and monothiols are linked with GABA transport in membrane vesicles of rat brain synaptosomes

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The properties of γ-aminobutyric acid (GABA) transport into membrane vesicles derived from synaptosomes of rat brain have been studied using membrane-permeable and -impermeable sulfhydryl reagents, dithiol-specific reagents and oxidizing reagents. GABA transport is inhibited, reversibly, by very low concentrations of the membrane-permeable trivalent arsenical, phenylarsine oxide. Preincubation with this reagent only partially protects GABA transport from inactivation by *N*-ethylmaleimide (NEM). Thorin, a negatively charged trivalent arsenical, has no influence on GABA transport at concentrations 100-fold higher than that of the inhibitory phenylarsine oxide. The impermeant oxidizing agent, potassium ferricyanide, did not inhibit transport whereas the permeant reagent, diamide, was inhibitory. These data indicate that the GABA transporter possesses an activity-linked dithiol in a hydrophobic region of the carrier not accessible to charged, polar reagents. *p*-Chloromercuribenzenesulfonate (PCMBS) also inhibits but does not protect against NEM inactivation, suggesting the occurrence of an activity-linked monothiol in a polar region of the carrier.

Phenylarsine oxide; *N*-Ethylmaleimide; Sulfhydryl reagent; Neurotransmitter; γ-Aminobutyric acid

1. INTRODUCTION

High-affinity transport systems are assumed to be responsible for terminating the action of some neurotransmitters on postsynaptic receptors and for maintaining constant levels of neurotransmitter in the neuron [1,2]. The carriers have been identified in membranes of brain preparations including synaptosomes and membrane vesicles derived from synaptosomes. Kanner and Erecinska and their co-workers have characterized a high-affinity γ-aminobutyric acid (GABA) transport system in membranes from rat brain [3–9]. The system has a K_m of 2–10 μM, has an absolute requirement for Na^+ and Cl^-, is dependent on the membrane potential, and is sensitive to sulfhydryl reagents. We have investigated a number of bacterial transport systems known to be sensitive to sulfhydryl reagents and found that the sensitivity is due not to a monothiol but to a dithiol which can be protected against irreversible modification by prior oxidation to a disulfide [10–13]. Although the role of the thiol groups in transport proteins has not yet been defined, we have recently shown that the dithiol, in the fructose phosphotransferase carrier, can go through a redox cycle during turnover of the carrier [14]. This suggests that there may be a mechanistic role for the dithiols in

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Abbreviations: GABA, γ-aminobutyric acid; NEM, *N*-ethylmaleimide; PCMBS, *p*-chloromercuribenzenesulfonate; Thorin, 4-(2-arsonophenyl)azo-3-hydroxy-2,7-naphthalenedisulfonic acid

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these systems. Kanner [9] used triphenyltin chloride as a probe of the Cl requirement of the GABA transporter. This compound is capable of collapsing a Cl^- gradient by exchanging Cl^- for OH^- [18]. Inhibition of GABA uptake and efflux was observed after treatment of the energized vesicles with triphenyltin chloride. However, DTT, which destroys the compound, partially reversed the inhibition suggesting that a direct modification of the carrier by triphenyltin chloride also occurred [6,9]. We have observed that micromolar concentrations of triphenyltin chloride could inactivate dithiol-containing carriers which were not driven by Cl^- concentration gradients. DTT reversed the inactivation. In addition, triphenyltin chloride protected against irreversible inactivation by sulfhydryl alkylating reagents. The inactivation and protection are attributable to triphenyl tin-dependent oxidation of dithiols to disulfides. The triphenyl tin and sulfhydryl reagent sensitivity of the GABA carrier led us to investigate the occurrence of an activity-linked dithiol in this transporter as well.

2. MATERIALS AND METHODS

Membrane vesicles were prepared from the brain of adult male or female rats as described by Kanner [9]. Transport assays were performed essentially according to Kanner [9] and Rudnick [15]. Vesicles were rapidly thawed at 37°C and incubated in 0.1 M potassium phosphate (pH 6.7) and 1 mM $MgSO_4$ for 5 min in a shaking water bath. They were centrifuged for 10 min at 40000 × *g* and 4°C. The pellet was resuspended in the same solution at a final concentration of 1 mg protein/ml and stored at 4°C until use. The external solution during transport measurements (uptake buffer) consisted of 1 mM $MgSO_4$, 0.1 M NaCl and 0.14 μM [2,3-^{3}H]GABA (spec. act. 30 Ci/mmol). At the specified times 200 μl of the uptake suspension was pipetted onto Millipore HAWP02500 filters (0.45 μm pore size) under vacuum and washed twice, immediately, with 2 ml ice-cold 0.15 M NaCl. The filters were dried at 50°C and counted by liquid scintillation. The zero time measurement consisted of pipetting a concentrated vesicle suspension directly onto the filter simultaneously with the proper amount of GABA-containing buffer and washing as described above.

Protein concentrations were determined by the method of Lowry et al. [16].

[2,3-^{3}H]GABA was obtained from New England Nuclear, NEM, phenylarsine oxide and dithiothreitol from Aldrich and Thorin (4-(2-arsonophenyl)azo-3-hydroxy-2,7-naphthalenedisulfonic acid) from Ventron. All other chemicals were of reagent grade.

3. RESULTS AND DISCUSSION

3.1. *Occurrence of activity-linked sulfhydryls*

Fig.1 shows the effect of NEM and PCMBS on the accumulation of GABA by membrane vesicles derived from brain synaptosomes. Membrane vesicles were incubated with 500 μM NEM for 15 min, and subsequently with 10 mM DTT to quench the alkylation reaction. This protocol results in 70% inactivation. Exposure to 500 μM PCMBS results in 100% inactivation; DTT (10 mM) is capable of returning the activity to 40% of the control value depending on the length of the incubation period with DTT. These data are in agreement with those of Kanner [9] and Troeger et al. [4] on the sensitivity of the GABA transport process to sulfhydryl reagents in vesicles and synaptosomes, respectively. If PCMBS and NEM react at the same site, prior exposure of the carrier to PCMBS should protect against the irreversible inactivation caused by alkylation with NEM. The data in fig.1 show that this is not the case. Prior exposure to 500 μM PCMBS, followed by 500 μM NEM and then 10 mM DTT to quench the NEM reaction and reverse the PCMBS inhibition results in the same residual activity as that found without PCMBS pretreatment. Apparently, there is a site in a hydrophobic region accessible to NEM but not to PCMBS.

3.2. *Occurrence of activity-linked dithiols*

The occurrence of activity-linked dithiols was examined using trivalent arsenicals. Phenylarsine oxide, an apolar arsenical, is a potent inhibitor of GABA accumulation even at 40 μM (fig.2). Its effect is reversed by DTT as expected. Protection experiments were again carried out to determine whether phenylarsine oxide and NEM were reac-

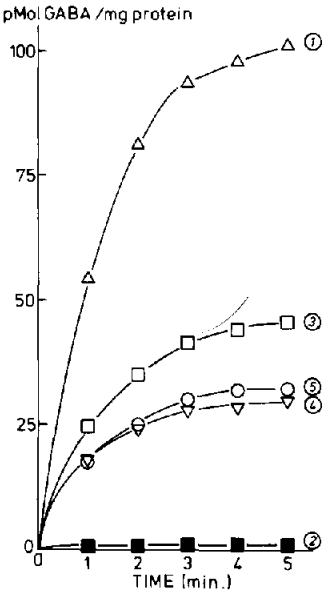


Fig.1. Influence of PCMBS, NEM and a combination of these reagents on the accumulation of GABA in membrane vesicles derived from synaptosomes. Vesicle suspension, washed and taken up in potassium phosphate, $MgSO_4$-containing buffer as stated in section 2, was used for each curve at a protein concentration of 0.7 mg/ml. PCMBS, NEM and DTT were added to the suspension at the stated times. After incubation with these reagents at room temperature, the vesicle suspension was diluted 10-fold with uptake buffer and GABA accumulation was measured as stated in section 2. Curves: 1, 25 min incubation at room temperature followed by a 30 min incubation in the presence of 10 mM DTT; 2, 10 min incubation with 500 μM PCMBS followed directly by assaying; 3, 25 min incubation with 500 μM PCMBS followed by a 30 min incubation with 10 mM DTT; 4, 10 min incubation with 500 μM PCMBS followed by a 15 min incubation with 500 μM NEM then by a 30 min incubation with 10 mM DTT; 5, 15 min incubation with 500 μM NEM followed by a 30 min incubation with 10 mM DTT.

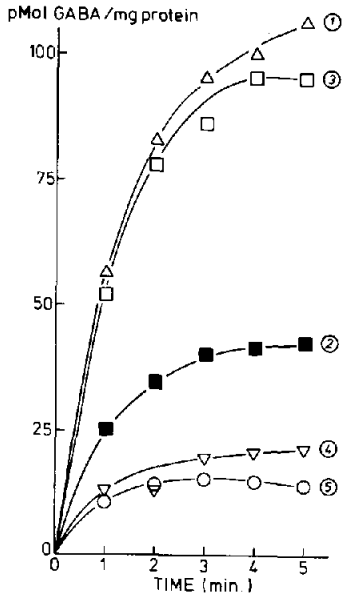


Fig.2. Influence of phenylarsine oxide as a reversible inhibitor of GABA accumulation in membrane vesicles derived from synaptosomes and as a protector against irreversible inhibition by NEM. Vesicle suspensions prepared as described above (final protein concentration, 1 mg/ml) were incubated at 25°C. Curves: 2, with 40 μM phenylarsine oxide in ethanol for 10 min; 1, with an equal amount of ethanol alone for 10 min followed by a 30 min incubation with 10 mM DTT; 3, with 40 μM phenylarsine oxide in ethanol for 25 min followed by a 30 min incubation with 10 mM DTT; 4, with 40 μM phenylarsine oxide in ethanol for 10 min followed by a 15 min incubation with 500 μM NEM, then by a 30 min incubation with 10 mM DTT; 5, same procedure as for curve 4 but using ethanol in place of phenylarsine oxide in the first incubation.

ting at a single unique activity-linked sulfhydryl site. Prior incubation with phenylarsine oxide should protect against irreversible NEM inactivation if the two compounds react at the same site. Preincubation with 40 μM phenylarsine oxide, followed by 500 μM NEM and then 10 mM DTT resulted in an activity which was only slightly higher than when NEM was used without protection by phenylarsine oxide. The activity was much lower than that observed with phenylarsine oxide and DTT. The lack of protection is evidence for the occurrence of an activity-linked monothiol which does not form a stable adduct with phenylarsine oxide, possibly the PCMBS site.

Thorin is a disulfonic acid-containing arsenical which is capable of complexing dithiols in the same manner as phenylarsine oxide [17]. It does not inhibit GABA transport even at a concentration up to 1 mM after a 60 min incubation period (not shown). Apparently, the activity-linked dithiol is located in a hydrophobic region not accessible to bulky, charged reagents.

The existence of the dithiol was confirmed using

oxidizing reagents. Diamide, an uncharged, sulfhydryl-specific oxidizing agent completely inhibits GABA accumulation (fig.3). Its effect can be reversed by subsequent treatment with DTT. As in the case of phenylarsine oxide, diamide provided only partial protection against NEM inactivation. In contrast to diamide, potassium ferricyanide did not inhibit at all up to concentrations of 10 mM. These data are also consistent with the existence of an activity-linked dithiol located in a hydrophobic region of the carrier.

3.3. *GABA binding does not influence the accessibility of the dithiol*

GABA has been included during incubations with phenylarsine oxide or NEM to test whether the substrate is capable of protecting the activity-linked sulfhydryls. Vesicle suspensions were prepared in 0.1 M potassium phosphate buffer (pH 6.7) and 1 mM $MgSO_4$ using the same procedure as stated in section 2. They were then incubated with a final concentration of 0.13 or 65 μM GABA for 10 min at 25°C followed by 60 μM phenylarsine oxide or 500 μM NEM for 20 min at the same temperature. The vesicle suspensions were then centrifuged at 40000 × g for 10 min, washed once with the above buffer and resuspended to the original volume with the same buffer. GABA transport was measured directly after resuspension as described in section 2. Neither concentration of GABA was able to influence the inhibition caused by phenylarsine oxide or NEM. The levels of GABA accumulation were the same in vesicles containing GABA as those lacking GABA during exposure to the sulfhydryl reagents.

In summary, the current data show that the GABA transporter possesses different classes of activity-linked sulfhydryl groups, a monothiol in a hydrophilic region and a dithiol in a hydrophobic region of the molecule. There may also be monothiols in the hydrophobic region, however reaction at these sites could not be distinguished from reaction at the monothiols in the hydrophilic region of the carrier. The role of dithiols in transport proteins has yet to be defined. We have provided evidence that, in the phospho-*enol*pyruvate-dependent bacterial sugar transport enzymes, the activity-linked dithiol is an intersubunit dithiol involving the same cysteine residue

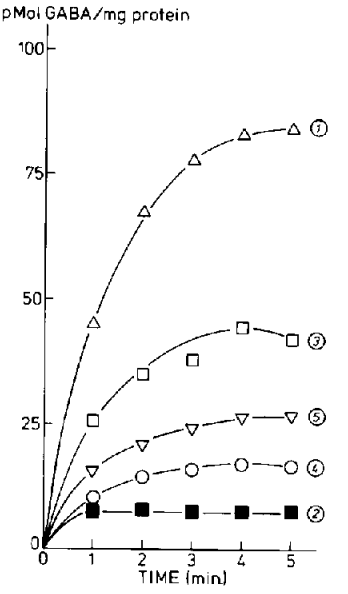


Fig.3. Influence of diamide as a reversible oxidant on GABA accumulation in membrane vesicles derived from synaptosomes and as a protector against irreversible inhibition by NEM. Vesicle suspensions, prepared as described in the legend to fig.2, were incubated at 25°C. Curves: 1, with 20 mM DTT for 40 min, then diluted directly into uptake buffer and measured for GABA accumulation activity; 2, same as curve 1 but replacing the DTT incubation by a 30 min incubation with 10 mM diamide; 3, same as curve 2 but including a 40 min incubation with 20 mM DTT after the diamide incubation; 4, same as curve 3 but using 500 μM NEM in place of diamide; 5, same as curve 2 but the diamide incubation was followed by a 30 min incubation with 500 μM NEM and then a 40 min incubation with 20 mM DTT.

from each subunit. The dithiol appears to be situated in a transmembrane channel formed by the enzyme subunits. We have also shown that the accessibility of the dithiol can change during turnover of the carrier. These characteristics suggests a possible mechanistic role for the dithiols in the transport event. Whether this is the case for the activity-linked dithiol of the GABA carrier remains to be determined.

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