Modification of Arginyl or Histidyl Groups Affects the Energy Coupling of the Amine Transporter†

Raul Suchi, Yael Stern-Bach, and Shimon Schuldiner*

Institute of Life Sciences, Hebrew University of Jerusalem, Givat Ram, 91904 Jerusalem, Israel

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ABSTRACT: We have characterized the effects of phenylglyoxal and diethyl pyrocarbonate (DEPC) on the catalytic cycle of the amine transporter in chromaffin granule membrane vesicles. Both reagents inhibited transport in a dose-dependent reaction (with IC_{50} values of 8 and 1 mM, respectively). The inhibition by DEPC was specific for histidyl groups since transport could be restored by treatment with hydroxylamine. Neither phenylglyoxal nor DEPC inhibited binding of either R1- or R2-type ligands, indicating that the inhibition of transport is not due to a direct interaction with either of the known binding sites. Interestingly, however, the acceleration of reserpine binding (an R1 ligand) by a transmembrane H^+ gradient is inhibited by both reagents at concentrations identical to those which inhibit transport. As previously demonstrated, transport of one proton across the transporter is required for this acceleration to take place [Rudnick, G., Steiner-Mordoch, S., Fishkes, H., Stern-Bach, Y., & Schuldiner, S. (1990) *Biochemistry 29*, 603–608]. Therefore, we suggest that either proton transport or a conformational change induced by proton transport is inhibited by both types of reagents.

The biogenic amine transporter is responsible for accumulation of serotonin, dopamine, norepinephrine, epinephrine, and histamine within secretory vesicles in a variety of cells (Kanner & Schuldiner, 1987; Njus et al., 1986).

Where it has been studied, the mechanism of amine accumulation into isolated storage organelles, or membrane vesicles derived therefrom, always involves exchange of extravesicular amine with intravesicular H^+ catalyzed by a reserpine-sensitive transporter (Kanner & Schuldiner, 1987; Winkler, 1976; Njus et al., 1981, 1986). An H^+-pumping ATPase in the organelle membrane generates an electrochemical gradient of H^+ (acid and positive inside) which provides the driving force for amine–H^+ exchange (Njus et al., 1986; Rudnick, 1986). All the organelles studied display the same inhibitor sensitivity, irrespective of the amine normally stored within. These findings have led to the proposal that a closely similar or identical protein catalyzes amine–H^+ exchange in all biogenic amine storage organelles (Njus et al., 1986; Rudnick et al., 1980). Similar mechanisms, as far as coupling to H^+ ion gradients, have also been suggested for storage of neurotransmitters other than biogenic amines: glutamate, γ-aminobutyric acid (GABA), glycine, and acetylcholine (Naito & Ueda, 1985; Kish et al., 1989; Anderson et al., 1982).

Two distinct types of binding sites have been characterized: (a) Reserpine binds to the R1-type site, and its binding is inhibited by the transport substrates (Deupree & Weaver, 1984; Scherman & Henry, 1984). (b) Tetrabenazine and ketanserin bind to the R2 sites which have low affinity for the transport substrates (Scherman & Henry, 1983; Darchen et al., 1988).

Binding of [^{3}H]reserpine is accelerated upon imposition of a proton electrochemical gradient across the membrane (Scherman & Henry, 1984). Once bound, [^{3}H]reserpine dissociates very slowly, if at all, from the transporter (Rudnick et al., 1990). Binding is very stable, and reserpine remains bound even after solubilization, a finding which has proved very useful in the purification of the transporter (Stern-Bach et al., 1990).

The role of functional groups has been studied. The amine transporter is not inactivated by sulfhydryl reagents (Kanner & Schuldiner, 1987). It has a carboxyl residue sensitive to modification by *N,N'*-dicyclohexylcarbodiimide (DCC)[1] (Schuldiner et al., 1978; Gasnier et al., 1985) whose availability is influenced by the occupancy of the R2 site (Suchi et al., 1991). DEPC has been shown to inhibit the overall transport activity (Isambert & Henry, 1981), but no support was presented that indeed histidine residues are involved in the inhibition. Moreover, there is no information whether binding of substrates or other specific steps are the target of the inhibition.

In this work, we characterized the mode of action of DEPC and phenylglyoxal. We demonstrated that DEPC at low concentrations acts specifically on a histidyl moiety on the protein, since treatment with hydroxylamine, which displaces the ethoxycarbonyl moiety from the imidazole nitrogen of histidine, restores transport activity. In addition, we have analyzed in detail the effect of both reagents on binding of ligands to two different sites on the protein and on reactions which require H^+ transport. We show that both reagents inhibit only those reactions in which transport of hydrogen ions is involved. We propose that histidine and arginine residues are involved in the coupling of the amine transport to the proton electrochemical gradient.

EXPERIMENTAL PROCEDURES

Preparation of Membranes. Chromaffin granule membrane vesicles were prepared as described previously (Schuldiner et al., 1978) by repeated osmotic lysis of chromaffin granules isolated by differential sedimentation.

Loading of Membrane Vesicles with K^+. Membranes (1 mL of a 5 mg of protein/mL suspension) were diluted into

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* Correspondence should be addressed to this author. Phone: 972-2-585992. FAX: 972-2-666804. Bitnet: SHIMONS@HUJIVMS.

[1] Abbreviations: KN, 10 mM K-Hepes, pH 7.4, 125 mM KCl, and 25 mM KSCN; DCC, *N,N'*-dicyclohexylcarbodiimide; DEPC, diethyl pyrocarbonate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; SH, 0.3 M sucrose/10 mM K-Hepes, pH 7.4.

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40 mL of a prewarmed (37 °C) solution containing 10 mM K-Hepes (pH 7.4). After 5-min incubation, 25 mM KSCN and 125 mM KCl were added and incubated for an additional 5 min at 37 °C. They were then centrifuged for 30 min at 40000*g* and resuspended in KN (pH 7.4) to a final protein concentration of 1 mg/mL.

Assay of ΔpH-Dependent Transport. ΔpH-dependent transport was assayed at 37 °C in 300 μL of a medium containing 0.3 M sucrose, 10 mM Tris/Hepes (pH 8.5), 2.5 mM $MgSO_4$, 5 μM nigericin, and 0.4 μM [^{3}H]serotonin (12.6 Ci/mmol). The reaction was initiated by a 1:100 dilution of membranes loaded with K^+ as described above (15–20 μg of protein). At a given time, uptake was terminated by rapid addition of 2 mL of a cold solution of the same buffer and immediate filtration through membrane filters (Schleicher & Schuell, 0.45-μm pore size). The filters were then washed with another 2 mL of the latter solution, removed from the suction apparatus, dried, and assayed for radioactivity by liquid scintillation spectrometry.

Reserpine Binding in the Absence of $\Delta\tilde{\mu}_{H^+}$. Membranes were diluted to a protein concentration of approximately 0.2 mg of protein/mL in KN, pH 7.4. [^{3}H]Reserpine (20.1 Ci/mmol) was added to a final concentration of 3 nM, and the mixture was incubated for 4 h at 37 °C. At this time, a 300-μL sample of the suspension was applied to a column of 2 mL of Sephadex, G-50 fine, which had been packed in a 2-mL disposable syringe and precentrifuged at 100*g* for 10s. The column with the applied sample was centrifuged once more for 1 min at 225*g* and the effluent counted in 4 mL of 40% Lumax (Lumac, Landgraaf, Netherlands) in toluene. In parallel, reaction mixtures containing 2 μM reserpine were used to substract nonspecific binding.

Reserpine Binding in the Presence of $\Delta\tilde{\mu}_{H^+}$. Membranes loaded with K^+ were diluted to a protein concentration of approximately 0.2 mg/mL in a medium containing 0.3 M sucrose, 10 mM Tris/Hepes (pH 8.5), 2.5 mM $MgSO_4$, and 5 μM nigericin, and [^{3}H]reserpine (20.1 Ci/nmol) was added to a final concentration of 3 nM. The mixture was incubated for 20 min at 37 °C and then assayed for reserpine binding as described previously for reserpine binding in the absence of $\Delta\tilde{\mu}_{H^+}$.

Ketanserin Binding. Membranes were diluted to a protein concentration of approximately 0.25 mg/mL in 0.3 M sucrose containing 20 mM K-Hepes, pH 8.5, and 0.5 nM [^{3}H]-ketanserin. The mixture was incubated for 5 min at 4 °C. Specific binding was measured essentially as described (Darchen et al., 1988): At the indicated times, the reaction mixture was diluted with 2 mL of ice-cold SH, filtered on GF/C Whatman filters, and washed with an additional 2-mL aliquot. Parallel reaction mixtures containing 3 μM TBZ were used to subtract nonspecific binding. The blank values were about 20% of the control.

Reconstitution. Membranes (5 mg of protein/mL in 0.3 M sucrose and 10 mM K-Hepes, pH 7.4) were solubilized by adding sodium cholate to a final concentration of 1%. After incubation at 4 °C for 10 min, the suspension was centrifuged at 100000*g* for 60 min, and the supernatant was collected.

A mixture of 5 mg of bovine brain lipids and 0.5 mg of asolectin was dried under N_2. The solubilized protein was added to the dry lipids and sonicated for a few seconds in a bath-type sonicator. The suspension was dialyzed overnight against 300 volumes of 150 mM NH_4Cl, 10 mM K-Hepes, pH 7.4, and 1 mM $MgSO_4$ and for 2 h against the same buffer at pH 8.5. The proteoliposome preparation was concentrated by centrifugation (100000*g*, 105 min) to a minimal volume (200 μL) to measure serotonin transport.

The ΔpH-driven transport in the proteoliposomes was assayed after a 1:100 dilution of the NH_4^+-loaded proteoliposomes into a medium devoid of NH_4^+, conditions at which a pH gradient is generated across the membrane due to the higher permeability of the NH_3 species relative to the charged NH_4^+ ion. The reaction was carried out at 27 °C in 0.3 mL of medium containing 150 mM sodium isethionate, 10 mM Tris/Hepes, pH 8.5, 1 mM $MgSO_4$, and 0.4 μM [^{3}H]serotonin (12.6 Ci/mmol). The reaction was initiated by addition of 3 μL of proteoliposomes. The reaction was stopped by dilution with 2 mL of the above ice-cold solution, filtered on 0.22-μm pore size Millipore filters, and washed with an additional 2 mL. The filters were dried and assayed for radioactivity. The data presented represent the specific fraction of transport. The nonspecific values, which were subtracted from serotonin transported, were never higher than 40%. These values were obtained by adding various transport inhibitors and were essentially identical whether tetrabenazine, reserpine, or unlabeled serotonin were added at a range between 5 and 50 μM (Stern-Bach et al., 1990).

Materials. Cholic acid (Sigma) was recrystallized 3 times from ethanol and neutralized with NaOH to pH 7.0. [^{3}H]-Reserpine was obtained from the Nuclear Research Centre (Negev, Israel). [^{3}H]Serotonin was purchased from Amersham. [^{3}H]Ketanserin was from New England Nuclear; soybean phospholipids (asolectin) were partially purified according to Kagawa and Racker (1971). Ketanserin was a gift from Jansenn Pharmaceutica (Belgium). Crude bovine brain lipids were extracted according to Folch et al. (1957). All other reagents were of the highest quality commercially available.

RESULTS AND DISCUSSION

DEPC Inhibits the $\Delta\tilde{\mu}_{H^+}$*-Dependent Acceleration of Reserpine Binding, but Not Binding Itself.* Present knowledge on the vesicular amine transporter suggests the presence of three sites which interact with each other in various ways: (1) the substrate binding site, generally believed to be identical to the reserpine binding site (also called the R1 site); (2) the tetrabenazine binding site, occupation of which results in transport inhibition; transport substrates or inhibitors such as reserpine interact only very weakly with it; (3) the proton binding site; two protons are believed to be necessary for transport. The first one is necessary to recruit the binding site, as measured by acceleration of reserpine binding; the second one may be necessary for release of the substrate in the interior of the vesicle or for its protonation (Rudnick et al., 1990). We know how to measure binding to the R1 and R2 sites and how to estimate the effect of H^+ binding and translocation on reserpine binding and transport. We therefore analyze the effect of transport inhibitors, DEPC and phenylglyoxal, on the partial reactions catalyzed by the transporter.

The results in Figure 1 show the effect of DEPC treatment of chromaffin granule vesicles on their ability to transport serotonin and bind reserpine and ketanserin. As previously reported (Isambert & Hentry, 1981), DEPC inhibits serotonin transport in a dose-dependent manner. Thus, membrane vesicles treated at a DEPC concentration of 3 mM transport at a rate of only 20% that of the control (□). Binding of reserpine (○) accelerated by the imposition of an artificially imposed $\Delta\tilde{\mu}_{H^+}$ is similarly inhibited by DEPC.

In contrast, binding of reserpine to nonenergized membranes (●), a slower process which displays, however, very similar properties (k_D, B_{max}, and inhibition by other substrates), is not inhibited at all by 3 mM DEPC.

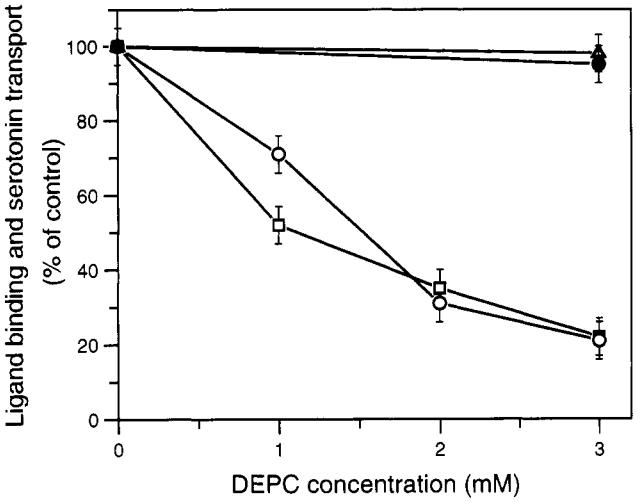

FIGURE 1: Effect of DEPC treatment of the amine transporter on binding and transport. Membranes loaded with K^+ as described under Experimental Procedures were suspended in a buffer containing 0.3 M sucrose and 0.01 M K-MES, pH 6.0, to a protein concentration of 1 mg/mL. After 10-min exposure at room temperature to the indicated DEPC concentrations, they were diluted with either KN, pH 7.4 (□, ○), or SH whose pH was titrated to 8.5 (●, △). The membrane suspensions were centrifuged, resuspended in the appropriate buffers, and assayed as described under Experimental Procedures for either ΔpH-driven transport of serotonin (□), reserpine binding in the presence (○) or absence (●) of a pH gradient, or ketanserin binding (△). Control values were 400 pmol mg^{-1} min^{-1}, 3.2 pmol/mg, 2.5 pmol/mg, and 0.35 pmol/mg, respectively.

Ketanserin and tetrabenazine bind to R2 sites on the transporter in a process independent of $\Delta\tilde{\mu}_{H^+}$. Binding of ketanserin (△) is not inhibited by DEPC.

Inhibition by DEPC Is on the Transporter. As explained above, the difference between the four reactions tested lies in their energy requirement. Thus, while both transport and $\Delta\tilde{\mu}_{H^+}$-accelerated reserpine binding require the generation of a transmembrane proton electrochemical gradient, neither ketanserin nor slow reserpine binding do. Therefore, a possible explanation for the difference in sensitivity could lie in the indirect effect of DEPC on the H^+ ion gradient rather than in a direct effect on the transporter. This explanation seems unlikely in view of the fact that the dependence of both reactions on $\Delta\tilde{\mu}_{H^+}$ is dramatically different (Rudnick et al., 1990) while the inhibition by DEPC is essentially at the same concentrations (Figure 1). In order to rule out such an indirect effect, we solubilized and reconstituted the transporter into proteoliposomes. In this experiment, treated and untreated membranes were solubilized in 1% sodium cholate. After centrifugation, the solubilized proteins were reconstituted as described immediately or after partial purification by DEAE chromatography (Stern-Bach et al., 1990). In both cases, the proteoliposomes in which the DEPC-treated protein was incorporated displayed only 16–20% of the control activity, a level of inhibition almost identical to the one detected in the native membranes. Control values in these experiments were 43 ± 4 pmol of serotonin (mg of protein)$^{-1}$ min^{-1} for the crude transporter and 449 ± 45 pmol (mg of protein)$^{-1}$ min^{-1} for the purified; the inhibited values were 8 ± 2 and 76 ± 8 pmol (mg of protein)$^{-1}$ min^{-1}, respectively.

Inhibition Is Due to Modification of a Histidyl Moiety. The specificity of the DEPC reaction is not absolute even under the most stringent conditions (pH 6.0). Thus, DEPC can react not only with histidine but also with tyrosine, cysteine, and primary amines (Miles, 1977). Hydroxylamine can attack the *N*-carbethoxyhistidyl compound and regenerate a free histidine. Therefore, treatment with hydroxylamine should restore the activity if the reaction was with a histidine residue (Grillo & Aronson, 1986; Padan et al., 1979; Sokol et al., 1988).

Table I: Restoration of Activity of DEPC-Treated Membranes with Hydroxylamine[a]

[DEPC] (mM)	time of DEPC treatment (min)	serotonin transport (% of act.) hydroxylamine		$\Delta\mu_{H^+}$-accelerated reserpine binding (% of act.) hydroxylamine	
		−	+	−	+
0		100 ± 7	85 ± 6 (100)	100 ± 6	90 ± 5 (100)
3	10	40 ± 3	65 ± 3 (75)	30 ± 3	72 ± 5 (80)
3	60	5 ± 0.9	40 ± 2 (50)	nd	nd
6	10	25 ± 3	40 ± 3 (50)	nd	nd
6	60	5 ± 0.8	5 ● 1 (7)	nd	nd

[a] Membranes (1 mg of protein/mL) loaded with K^+ were incubated in a buffer containing 0.3 M sucrose and 0.01 M K-MES, pH 6.0. After exposure to the indicated DEPC concentration for either 10 or 60 min, hydroxylamine was added to 150 mM, and incubation was continued for an additional 60 min. ΔpH-dependent serotonin transport was assayed after centrifugation (40000g, 30 min) and resuspension in KN. Control values were 350 pmol mg^{-1} min^{-1} for serotonin transport and 2.5 pmol/mg for reserpine binding. The values in parentheses indicate the percentage within each experimental group.

In order to check this point, we tested the effect of hydroxylamine treatment on transport by membranes treated with two DEPC concentrations for two different times. The results, shown in Table I, demonstrate that at 3 mM DEPC, a considerable restoration of the transport capability can be observed; after 10-min DEPC treatment, the activity rises upon exposure to hydroxylamine from 40% of the control to 75% of the hydroxylamine-treated control; after 60 min, the increase in activity is 10-fold from 5% to 50%, respectively. When the DEPC concentration is increased to 6 mM, hydroxylamine has only a slight effect at the short time period and none at the long one. Similarly, the $\Delta\mu_{H^+}$-accelerated binding of [^{3}H]reserpine is restored by hydroxylamine treatment of membranes treated with DEPC (Table I).

We conclude that under appropriate conditions DEPC inhibits transport through modification of a histidyl moiety on the transporter. At higher DEPC concentrations, however, hydroxylamine cannot restore transport. This suggests either that at higher concentrations DEPC reacts with residues other than histidine or with a histidine which is not available to hydroxylamine or that an additional DEPC reacts with the second imidazole nitrogen (Miles, 1977). Hydroxylamine cannot restore a free histidine from the latter compound.

Effect of Phenylglyoxal, an Arginine Modifier, Is Identical to That of DEPC. Phenylglyoxal is considered to be a specific arginine modifier at pH 7.4 (Riordan et al., 1977; Takahashi, 1977). Membrane vesicles exposed to phenylglyoxal lose 50% of their transport capacity at 7 mM reagent and more than 90% at 50 mM (Figure 2).

As described above for DEPC, phenylglyoxal at a 10 mM concentration inhibits 75% of reserpine binding in the presence of $\Delta\tilde{\mu}_{H^+}$ but has no effect on the binding in the absence of $\Delta\tilde{\mu}_{H^+}$ (Table II). Again, in order to test whether the effect on the transporter is a direct effect on the protein, we solubilized and reconstituted it after treatment with 10 mM phenylglyoxal. The inhibition detected after reconstitution (53%) is essentially identical to that observed in the native membrane (data not shown).

Conclusions and Significance of the Findings. The findings presented here suggest that histidine and arginine residues in the amine transporter play an important role in the mechanism of proton/amine exchange. Exposure of vesicles to DEPC or phenylglyoxal compounds known to modify histidine and

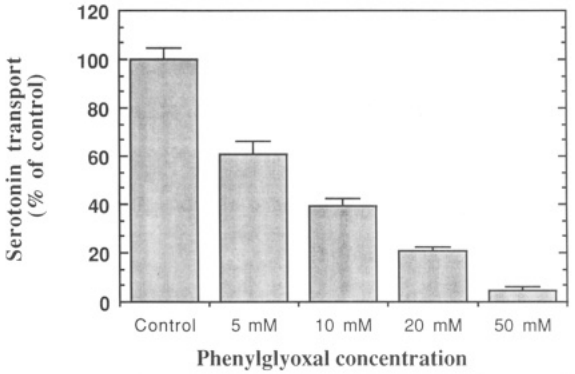

FIGURE 2: Inhibition of ΔpH-driven transport of serotonin by phenylglyoxal. Membranes loaded with K^+ as described under Experimental Procedures were suspended in buffer KN to 1 mg of protein/mL and incubated at room temperature with the indicated concentration of phenylglyoxal. After 10 min, they were centrifuged (40000*g*, 30 min), resuspended in KN, and assayed for transport as described under Experimental Procedures.

Table II: Effect of Phenylglyoxal on Reserpine Binding[a]

conditions	reserpine binding (pmol/mg of protein)	
	−phenylglyoxal	+phenylglyoxal
no gradient	1.25 ± 0.14	1.3 ± 0.13
+pH gradient	1.95 ± 0.17	0.4 ± 0.05

[a] Membranes (1 mg of protein/mL) loaded with K^+ were incubated in KN buffer at room temperature for 10 min in the presence or absence of 10 mM phenylglyoxal. Thereafter they were collected by centrifugation (40000*g*, 30 min), resuspended, and assayed for reserpine binding in the presence or absence of a pH gradient. The former was assayed for 20 min, the latter for 4 h.

arginine residues, respectively, lead to inactivation of the amine transporter. Loss of activity occurs when the function is monitored by ΔpH-driven transport or by ΔpH acceleration of reserpine binding. Importantly, however, neither reagent interferes with the ability of the protein to bind either [^{3}H]-reserpine or [^{3}H]ketanserin.

Although we have not demonstrated that the effects of DEPC and phenylglyoxal are due specifically to modification of histidyl and arginyl groups, a number of lines of evidence support this argument. First, both DEPC and phenylglyoxal have been shown to specifically modify these residues in a number of soluble enzymes (Lundblad & Noyes, 1984a,b). Second, hydroxylamine displaces the ethoxycarbonyl moiety from the imidazole nitrogen of histidine (Heinrikson & Kramer, 1974) and regenerates transport activity in DEPC-treated vesicles.

Several other transport systems have been shown to be sensitive to DEPC (Grillo & Aronson, 1986; Sokol et al., 1988; Padan et al., 1979) and phenylglyoxal (Sokol et al., 1988; Tse et al., 1984; Stipani et al., 1986). In the case of the *lac* carrier, both chemical modification studies and the results of site-directed mutagenesis of histidine and arginine residues have supported a specific role of the above in H^+ translocation or in the coupling of H^+ and lactose translocations. Our results are consistent with such a role of histidyl and arginyl residues also in the catalytic cycle of the amine transporter.

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Registry No. H^+, 12408-02-5; serotonin, 50-67-9; reserpine, 50-55-5; ketanserin, 74050-98-9; histidine, 71-00-1; arginine, 74-79-3.