Amine Transport in Chromaffin Granule Ghosts

pH DEPENDENCE IMPLIES CATIONIC FORM IS TRANSLOCATED*

(Received for publication, January 23, 1981, and in revised form, April 10, 1981)

Jill Marie Issacs, Jill Marie Issacs, and David Njus
From the Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202

Chromaffin granules have a translocator-mediated uptake system for the monoamines dopamine, serotonin, norepinephrine, and epinephrine. These substrates are predominantly cationic at physiological pH but they also exist in neutral, zwitterionic, and anionic forms. The cationic fraction is nearly pH-independent between pH 6.8 and pH 7.6. Over the same pH range, the neutral and zwitterionic fractions increase by a factor of 6.3 and the anionic fraction increases by a factor of 40. In chromaffin granule ghosts, the apparent $K_m$ values for dopamine and serotonin transport are independent of pH between 6.8 and 7.6. Consequently, the translocator probably binds the cationic form of the substrate. $V_{max}$ values for dopamine and serotonin uptake increase by a factor of 2 between pH 6.8 and pH 7.6.

In biological membranes, unmediated permeation of weak acids and bases may occur because the minor neutral species crosses the membrane. Likewise, mediated transport of ionizable molecules could occur via translocators which accept a minor ionic form. For proton-linked systems in particular, transport of a minor species could simplify the exchange or co-transport mechanism. It has been suggested, for example, that catecholamines might be transported into adrenal medullary chromaffin granules in the neutral form rather than in the predominant cationic form (Johnson and Scarpa, 1979). Chromaffin granules take up catecholamines via an $H^+$ antiport or exchange diffusion mechanism with a stoichiometry of 2 $H^+$ per catecholamine cation (Knoth et al., 1980; Phillips and Apps, 1980). The monoamine translocator could catalyze this exchange directly or it could instead exchange one neutral or zwitterionic catecholamine for one proton. The latter exchange is mechanistically simpler, but it would require the translocator to have a higher affinity for its substrate.

The form of the amine that binds to the translocator can be identified by examining the kinetic parameters governing uptake. Substrates of the transport system (epinephrine, norepinephrine, dopamine, and serotonin) all have two ionizable groups (an amino and a hydroxyl) and can exist in cationic, zwitterionic, neutral, or anionic forms. The relative concentrations of the various amine species have different pH dependences. Because the ionizable groups have pH, values above 8.5, the cationic fraction predominates in the physiological pH range and is relatively pH-independent. A 1-unit increase in pH, however, will increase the neutral and zwitterionic fractions by a factor of 10; the anionic fraction will increase by a factor of 100. Because the kinetic parameter $K_m$ is the substrate concentration at half-saturation, the apparent $K_m$ should reflect the concentration of the species that binds to the translocator. The observed $K_m$ should be proportional to the $H^+$ activity if the neutral species is translocated, whereas it should be relatively pH-independent if the cationic form is translocated. We report here on the pH dependences of $K_m$ and $V_{max}$ for dopamine and serotonin transport in chromaffin granule membrane vesicles ( ghosts).

**EXPERIMENTAL PROCEDURES**

Chromaffin granule ghost membranes were prepared from bovine adrenal medulla as previously described (Knoth et al., 1980; Njus and Radda, 1979). All experiments were completed within 1 h of slaughter. Protein concentrations were determined using the biuret assay (Casey et al., 1976).

To measure uptake of $[^3H]$dopamine or $[^3H]$serotonin, a measured amount of ghosts (0.4 to 0.9 mg of protein in 50 or 100 pl) was added to 0.9 ml of 400 mM sucrose, 40 mM Hepes buffer at the pH indicated (6.8, 7.2, or 7.6). Twenty-five pl of 100 mM ATP, 100 mM $MgSO_4$, pH 7, was added with the appropriate amount of $[^3H]$dopamine (10 mCi/mmol) or $[^3H]$serotonin (25 mCi/mmol). Samples were incubated for 10 min at 30 °C, collected under gentle suction on cellulose acetate filters (25-mm diameter, 0.45-μm pore size), and washed with about 1 ml of 400 mM sucrose, 40 mM Hepes buffer. Filters were placed in vials with 10 ml of scintillation fluid and $^3H$ activity was counted on a Beckman LS 100C liquid scintillation counter. A known amount of $^3H$-labeled monoamine was also counted, and this value was used to convert counts per min to nanomoles. $[^3H]$Dopamine (21.5 Ci/mmol) and $[^3]H$serotonin (28.9 Ci/mmol), purchased from New England Nuclear, were mixed with the unlabeled amine (Sigma Chemical Co.) to give the specific activities cited above.

The kinetics of dopamine and serotonin uptake were investigated using Lineweaver-Burk analysis. Typical plots obtained at pH 6.8, 7.2, and 7.6 are shown in Fig. 1. For each plot, substrate concentrations were varied from 0 μM to 50 μM (dopamine) or from 2 μM to 20 μM (serotonin). Uptake was assayed two or three times at each concentration and replicate values were averaged. A straight line, fit to the average values by the least squares method, gave $-1/K_m$ from the x-intercept and $1/V_{max}$ from the y-intercept. Background uptake (no added ATP or no incubation) was negligible and was not subtracted. Several Lineweaver-Burk plots were obtained with each substrate at each pH. Values of $1/K_m$ and $1/V_{max}$ from replicate experiments were averaged (Table I). For both dopamine and serotonin, the apparent $K_m$ values remain relatively constant between pH 6.8 and pH 7.6. However, the $V_{max}$ values increase with increasing alkalinity of the medium.

**RESULTS AND DISCUSSION**

Chromaffin granules, the storage vesicles of the adrenal medulla, use a coupled transport system to maintain high internal catecholamine concentrations (Njus and Radda, 1978; Njus et al., 1981). A proton-translocating adenosine triphosphate pumps protons into the granule interior establishing a transmembrane gradient in $H^+$ electrochemical potential. Catecholamines are then taken up in exchange for these protons. This exchange is apparently mediated by a mem-

* This investigation was supported by the National Science Foundation under Grant BNS-7904752. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Monoamine uptake into chromaffin granule ghosts is a slow process requiring approximately 2 h to reach steady state (Phillips and Apps, 1980). Both dopamine and serotonin uptake (Phillips, 1978) are linear for about 30 min, irrespective of the external pH. Consequently, 10 min is an appropriate duration for obtaining initial rates for kinetic studies. The rate of transport depends on the size of the proton gradient (∆pH and ∆p) rather than on the adenosine triphosphatase activity itself. The force driving transport is (4.6 RT/F)ApH + ∆p (Phillips and Apps, 1980). The magnitude of this force is apparently independent of the external pH since dopamine uptake approaches the same steady state level whether the pH is 6.8, 7.4, or 8.0.

We have investigated the kinetics of monoamine transport as a function of pH (Table I). The apparent Km is about 20 μM for serotonin and about 30 μM for dopamine. Both values are independent of pH. For both serotonin and dopamine, Vmax increases from about 0.7 nmol/min/mg of protein at pH 6.8 to about 1.5 nmol/min/mg of protein at pH 7.6. The kinetics of monoamine transport has been studied by others. Phillips (1974) found the apparent Km of serotonin uptake to be 9 μM and the Vmax to be 7 nmol/min/mg of protein at pH 7.0. Kanner et al. (1979) also obtained a low Km for serotonin transport (4.3 μM). Both Phillips and Kanner et al. measured uptake at 37°C as opposed to the 30°C used in our experiments. The difference in temperature and in ghost preparations could account for the difference in Km and Vmax values.

Phillips (1974) noted that the results differed from one ghost preparation to another, but neither he nor Kanner et al. (1979) gave values for experimental variability. We found that the error in 1/Km was ~30%, while the error in 1/Vmax was ~65% (Table I). While the variability in 1/Km could obscure a slight pH dependence, this would not conceal a difference in the apparent Km of more than a factor of 2. The higher variability in Vmax is probably a consequence of variability in the force driving transport. Differences in proton permeability of the ghost membranes will lead to differences in Vmax.

Taungner (1972) investigated the dependence of transport on pH and obtained results comparable to our measurements of Vmax. She found that the rate of epinephrine uptake increased about 2-fold (from 0.4 to 0.9 nmol/min/mg of protein) between pH 6.8 and 7.6. Greater activity at higher pH is probably a property of the translocator since, as noted before, the force driving transport does not seem to be dependent on the external pH. This is not surprising because deprotonation of the translocator and the probability that a monoamine replaces the H+ both should increase with increasing pH (Fig. 2A).

The amines dopamine and serotonin have two ionizable groups, the amine and the hydroxyl. Because the pKα values of these groups are all greater than 8.5 (Dawson et al., 1969; Martin, 1971), the majority of the molecules are in the cationic form below pH 8.0. Nonetheless, the neutral, zwitterionic, and anionic forms do exist. In theory, the translocator could accept any one of these forms. The possible translocator-mediated exchanges consistent with an overall stoichiometry of 2 H+/catecholamine cation (Knott et al., 1980; Phillips and Apps, 1980) are shown in Fig. 2.

Because the relative concentrations of the various species exhibit different pH dependences, we can distinguish the

---

**Table I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>1/Km (μM⁻¹)</th>
<th>1/Vmax (μmol/min/mg)</th>
<th>Km (μM)</th>
<th>Vmax (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>6.8</td>
<td>0.031 ± 0.010</td>
<td>1.5 ± 1.2</td>
<td>32</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>0.039 ± 0.011</td>
<td>1.3 ± 0.9</td>
<td>25</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>7.6</td>
<td>0.035 ± 0.002</td>
<td>0.7 ± 0.2</td>
<td>29</td>
<td>1.4</td>
</tr>
<tr>
<td>Serotonin</td>
<td>6.8</td>
<td>0.056 ± 0.040</td>
<td>1.4 ± 1.2</td>
<td>18</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>0.033 ± 0.011</td>
<td>0.6 ± 0.4</td>
<td>19</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>7.6</td>
<td>0.047 ± 0.012</td>
<td>0.7 ± 0.4</td>
<td>21</td>
<td>1.4</td>
</tr>
</tbody>
</table>
species that actually binds to the translocator. The fraction in the cationic form remains relatively constant over the pH range from 6.8 to 7.6. The relative concentrations of the neutral, zwitterionic, and anionic forms, however, increase with increasing pH. The neutral and zwitterionic fractions increase by a factor of 6.3 with a 0.8-unit increase in pH; the anionic fraction increases by a factor of 40. If these latter species are translocated, the total substrate concentration at half-saturation (apparent $K_m$) should be strongly pH-dependent. Because the relative concentrations of the neutral and zwitterionic species increase 6.3 times between pH 6.8 and 7.6, the measured $K_m$ should decrease by the same factor. Since the apparent $K_m$ values for dopamine and serotonin remain constant over the pH range between 6.8 and 7.6, the cationic species is most likely translocated.

It is, of course, possible that the affinity of the translocator for the substrate might itself be pH-dependent. Thus, if the translocator affinity were 6.3 times higher at pH 6.8 than at pH 7.6, this would exactly balance the decrease in the relative concentration of the neutral or zwitterionic species. However, besides being an unlikely coincidence, this would require the translocator to have a much stronger affinity for the substrate. The pKₐ values for dopamine are approximately 8.9 (hydroxyl) and 10.6 (amino), while those for serotonin are about 11.1 (hydroxyl) and 9.8 (amino) (Dawson et al., 1969). These pKₐ values are approximate because the two ionizations are competitive as well as dependent on temperature and ionic strength (Martin, 1971). Nevertheless, it is possible to make some qualitative observations. The zwitterion is the second largest fraction of dopamine but the third largest fraction of serotonin. If the zwitterions are transported, the $K_m$ for the zwitterionic dopamine species would be on the order of 1 μm and the $K_m$ value for the zwitterionic serotonin species would be about 1 nm. The neutral fraction, on the other hand, is the second largest fraction of serotonin but the third largest fraction of dopamine. Thus, if the neutral forms are transported, the $K_m$ for the neutral serotonin species would be on the order of 100 nm while that for dopamine would be around 10 nm. Transport of either the neutral or zwitterionic species would require an improbably low $K_m$ for either dopamine or serotonin. Only transport of the cation is consistent with a reasonable $K_m$ for both amines.

We have shown that the translocator most likely binds the monoamine cation. It is possible that a proton is released subsequent to binding but prior to translocation. If the released proton is derived from the amine, one could argue that the neutral species is actually translocated. Even if this were the case, however, the translocation process would be more complex than a simple exchange of one neutral amine for one proton.

The actual mechanism by which the exchange shown in Fig. 2A occurs is unknown. One could imagine, however, two possible mechanisms. Translocation of the amine into the granules might occur simultaneously with the export of the protons (two-site model). On the other hand, the translocator could alternately accept monoamine from one side and H⁺ from the other (one-site model). This could be achieved, for example, by a mobile carrier moving the amine across the membrane and then returning with two protons. These possible mechanisms are currently the focus of studies of the erythrocyte anion transporter (Rothstein et al., 1976) and the mitochondrial phosphate transporter (Lavat et al., 1979). Having identified the cation as the translocated species, we can now seek the mechanism of monoamine/H⁺ exchange in chromaffin granules.

Acknowledgment—We thank Dr. Hans Winkler for stimulating suggestions.

REFERENCES
Maron, R., Fishkes, H., Kanner, B. I., and Schuldiner, S. (1979) Biochemistry 18, 4781-4785
Taugner, G. (1972) Naunyn-Schmiedeberg’s Arch. Pharmacol. 274, 298-314