Kinetic Evidence for Channeling of Dopamine between Monoamine Transporter and Membranous Dopamine-β-monoxygenase in Chromaffin Granule Ghosts*

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The nature of coupling between the uptake and dopamine-β-monoxygenase (DβM) catalyzed hydroxylation of dopamine (DA) was studied in bovine chromaffin granule ghosts. Initial rate and transient kinetics of DA uptake and conversion were determined under a variety of conditions. The uptake kinetics of DA, norepinephrine (NE), and epinephrine under optimal uptake conditions. The transient kinetics of DA accumulation and NE production under both optimal uptake and conversion conditions were zero order with no detectable lag or burst periods. The mathematical analyses of the data show that a normal sequential uptake followed by the conversion process could not explain the observed kinetics, under any condition. On the other hand, all experimental data are in agreement with a mechanism in which DA is efficiently channeled from the vesicular monoamine transporter to membranous DβM for hydroxylation, prior to the release into the bulk medium of the ghost interior. The slow accumulation of DA under optimal conversion conditions appears to be caused by the slow leakage of DA from the channeling pathway to the ghost interior. Because DβM activity in intact granules is equally distributed between soluble and membranous forms of DβM, if an efficient channeling mechanism is operative in vivo, soluble DβM may have access to the substrate, making the catalytic activity of soluble DβM physiologically insignificant, which is consistent with the increasing experimental evidence that membranous DβM may be the physiologically functional form.

Dopamine-β-monoxygenase (DβM); EC 1.14.17.1) catalyzes the conversion of dopamine (DA) to norepinephrine (NE) within the neurosecretory vesicles of the adrenal medulla and the large dense cored synaptic vesicles of the sympathetic nervous system (1–5). Cytosolic DA is actively transported into the storage vesicles through relatively nonspecific vesicular monoamine transporters (VMATs (Refs. 6–9)) prior to the DβM reaction. VMATs are responsible for maintaining a high concentration of catecholamines in storage vesicles. The free energy for this process is provided by pH and electrochemical gradients (10, 11) generated by a transmembrane proton translocating ATPase. Evidence from numerous experiments suggests that both electrochemical (Δφ) and pH gradients (ΔpH) are mandatory for the intragranular accumulation of monoamines (12, 13).

Various mechanistic aspects of DA uptake and DA to NE conversion have been studied using bovine chromaffin granule ghosts as a model for adrenergic neurotransmitter storage vesicles (14–17). Large quantities of purified granule membranes can be easily isolated from bovine adrenals and stored and ressealed to produce fully functional and easily characterizable granule ghosts for detailed mechanistic studies. The coordinated functions of granule membrane proteins with respect to DA uptake and conversion, including membranous DβM (m-DβM), ATPase, cytochrome b561, and VMATs, have been widely studied using chromaffin granule ghosts (14–21). Despite these efforts, some critical aspects of the transport-coupled DβM hydroxylation of DA are not fully understood. For example, numerous studies have shown that DβM activity in granule ghosts, even under optimal conditions, is orders of magnitude less than the total enzyme activity released following the lysis and solubilization (16, 22, 23), suggesting that DA transport may be rate-limiting in granule ghosts. However, isotope effects and other studies appear to indicate that the DβM reaction is rate-limiting in ghosts regardless of the experimental conditions, whereas kinetic studies suggest that the rate of NE production could be much slower than the net uptake of DA, especially under non-optimal conversion conditions (16). Although the DβM activity in granules is distributed between the soluble and membranous forms (23–26), the physiological significance of these two forms of enzyme is not clear, but increasing recent experimental evidence suggests that the catalytic activity of the soluble form of DβM (s-DβM) may not be physiologically significant (15, 23).

To resolve some of these inconsistencies, we examined the nature of the coupling between VMAT-mediated DA transport and the DβM hydroxylation reaction in chromaffin granule ghosts. Initial rate and transient kinetic studies of DA uptake and conversion have been carried out under a variety of conditions, and the experimental data were analyzed and mathematically modeled by assuming a sequential uptake followed by a conversion process. These analyses clearly demonstrate that a sequential uptake and conversion process could not explain the kinetics of DA uptake and conversion under any condition. On the other hand, all the experimental data are in excellent agreement with a channeling mechanism (for recent reviews on substrate channeling in enzymes, see Refs. 27–30) in which DA is efficiently channeled from the VMAT to m-DβM.
for hydroxylation prior to the release of the ghost interior into the bulk medium.

EXPERIMENTAL PROCEDURES

Materials

1-Norepinephrine hydrochloride, n-epinephrine bitartrate, dopamine hydrochloride, MgATP, HEPES, Tris base, and ascorbate oxidase were from Sigma. Ascorbic acid (Asc) and sodium fumarate were from Aldrich. Ficoll was from Amersham Biosciences, and catalase was from Roche Molecular Biochemicals. The protein assay reagent and bovine serum albumin were from Bio-Rad. All other chemicals were of the highest grade obtainable. Membrane pellets were homogenized using glass-Teflon Potter-Elvehjem homogenizers. Centrifugations were performed using Beckman Coulter J-2M and Optima LE-80K refrigerated centrifuges. HPLC-EC analyses were performed using an ESA model 582 solvent delivery module and a Coulouchem-II electrochemical detector with ESA 501 chromatographic software.

Methods

HPLC-EC Analyses—NE, E, and DA contents of granule ghosts under various incubation conditions were quantified using HPLC-EC. Acidic extracts of ghosts were applied to a C18 reversed phase column (ESA, HR-80) equilibrated with a mobile phase composed of 90 mM NaH2PO4, 30 mM citric acid, 0.05 mM Na2EDTA, 1.7 mM octanesulfonic acid sodium salt, pH 3.0, with 10% CH3CN at a flow rate of 1 ml/min as described previously (15). All three analytes were oxidized at 300 mM. Sample peak areas were quantified by comparison to standard curves that were linear over the range of sample sizes encountered.

Preparation of Chromaffin Granular Ghosts—Chromaffin granules were prepared from fresh bovine adrenal medulla using the original methods of Kirshner (33) as modified by Njus and Radda (34) except that the granules were further purified by a discontinuous sucrose density gradient (35) as described previously (14, 15, 32). The granules were homogenized in 0.2 M Tris phosphate, pH 7.0, containing 100 mM Asp and lysed by the addition of 0.14 volume of a glycerol solution (glycerol, 0.2 M Tris phosphate, pH 7.0 (3.7, v/v)). The lysate was stored in 1.5 ml aliquots at –20°C. Granular membranes were isolated from the stores on the day of the experiment by diluting with containing 20 mM Asc and 100 µg/ml catalase, incubating for 10 min at 4°C, and centrifuging at 36,000 × g for 25 min at 4°C. The membrane pellet was homogenized and resuspended in 2 ml of a solution containing 20 mM Tris phosphate, 100 mM KCl, 250 mM sucrose, 10 mM sodium fumarate, 4.0 µM Ca2+, 100 µg/ml catalase, and 20 mM Asc (optional uptake and conversion conditions) or 7.5 units/ml ascorbate oxidase (optimal uptake conditions) adjusted to pH 7.0. The ghost membranes were allowed to reseal by incubation for 20 min at room temperature, diluted to 3.75 ml with the same solution as that placed inside (but without Asc, ascorbate oxidase, or catalase), and layered over 1.5 ml of 15% Ficoll, 0.3 M sucrose, 10 mM HEPES, pH 7.0, and 2.25 ml of 0.4 M sucrose, 10 mM HEPES, pH 7.0, and centrifuged for 30 min at 90,000 × g at 4°C. The resealed ghosts, which separated as a band at the 0.4 M sucrose-HEPES/Ficoll interface, were washed once with 5 ml of 0.3 M sucrose, 10 mM HEPES, pH 7.0, and homogenized and centrifuged at 36,000 × g for 25 min at 4°C. The supernatant was removed, and the pellet was washed with the same buffer and homogenized and resuspended in 1.0 or 2.0 ml of 0.3 M sucrose, 10 mM HEPES, pH 7.0, depending on the experiment, and stored at 0°C. Protein content of ghosts was determined by the Bradford method (31) using the Bio-Rad protein assay with bovine serum albumin as the standard.

DA Uptake Experiments—To aliquots of ghosts prepared under optimal uptake conditions as described above were added 5 mM MgATP, 5 mM MgSO4, 100 µg/ml catalase, 7.5 units/ml ascorbate oxidase. The aliquots were then diluted with 0.3 M sucrose, 10 mM HEPES, pH 7.0, to a final volume of 0.5 ml for initial rate kinetic experiments or 5.0 ml for transient kinetic experiments. This mixture was incubated for 10 min at 30°C, and the reactions were initiated by adding the desired concentration of DA (see corresponding figure legends) and incubated further at 30°C. After the desired time intervals, 400-µl aliquots of the incubate were withdrawn and diluted into 5.0 ml of ice-cold 0.4 M sucrose and 10 mM HEPES, pH 7.0, and stored at 0°C until the incubation was completed. These samples were then centrifuged at 36,000 × g for 25 min at 4°C, the supernatants were removed, the pellets were gently washed three times with 0.4 M sucrose, 10 mM HEPES, pH 7.0, and the tubes were allowed to dry. The pellets were homogenized in 500 µl of 0.1 M HClO4 and allowed to extract for 20 min at room temperature. After low speed centrifugation, 20 µl of the acidic extract was analyzed for catecholamines by HPLC-EC as described above. The raw data were corrected for the loss of internal content of the ghosts by standardizing the DA and NE levels to the average indigenous E levels as reported previously (14–15). Resealed ghosts preparations contained an average of 8–10, 6–9, and 0 nmol/mg of protein of NE, E, and DA, respectively, depending on the preparation.

DA Uptake and Conversion Experiments—These experiments were carried out using identical protocols as described above for uptake experiments, except that the ghosts were prepared under optimal uptake and conversion conditions as described above, and ascorbate oxidase in the external incubation medium was replaced with 20 mM Asc.

RESULTS

The data in Fig. 1 demonstrate that chromaffin granule ghosts prepared according to the above procedures actively accumulated DA, E, and NE in a concentration-dependent manner. The uptake kinetics of all three catecholamines followed simple Michaelis-Menten behavior in a wide concentration range (5–300 µM). The kinetic parameters determined by fitting the initial rate data to the hyperbolic form of the Michaelis-Menten equation show that DA is kinetically a better substrate for the transporter than E and NE (Table I). The depletion of the external substrate during the incubations was estimated to be less than 10% for all the concentrations tested. Therefore, the change in external substrate concentration during the incubation period (6 min) should not have significantly affected the initial rate kinetic parameters determined. In agreement, the control experiments revealed that the corrected uptake rates for all three catecholamines were linear up to 6–8 min of incubation in the entire concentration range.
with a net DA uptake displayed saturable Michaelis-Menten kinetics in good agreement with the initial rate kinetics under similar conditions.

Granule membranes were resealed and ghosts were incubated under optimal uptake and conversion conditions in a total volume of 0.5 ml as detailed under “Experimental Procedures.” The uptake and conversion was initiated by adding desired amounts of DA (0–300 μM final concentration) and, after 6 min time intervals, 400 μl aliquots of the incubate were withdrawn and rates of uptake and conversion were determined as described for Fig. 1. ● DA levels; ○ NE levels; ■ net DA uptake. Dashed lines represent the fit of the experimental data with respect to DA<sub>out</sub> to the hyperbolic form of the Michaelis-Menten equation.

The uptake and conversion of DA inside the granule ghosts as a function of the external DA concentration (DA<sub>out</sub>) under optimal uptake and conversion conditions is shown in Fig. 2. These data show that the maximum rate of NE production was about 60% of the net DA uptake (i.e. internal DA + NE). As expected, net DA uptake displayed saturable Michaelis-Menten kinetics with a K<sub>m</sub> of 33.2 ± 2.4 μM and a V<sub>max</sub> of 1.30 ± 0.03 nmol/mg/min with respect to DA<sub>out</sub>. Interestingly, the initial rates of DA accumulation as well as NE production also showed saturable Michaelis-Menten kinetics with respect to DA<sub>out</sub> with K<sub>m</sub> values of 33.9 ± 3.7 and 32.7 ± 2.0 μM and V<sub>max</sub> values of 0.51 ± 0.02 and 0.79 ± 0.02 nmol/mg/min, respectively (see below).

To determine the effect of intragranular DA concentration (DA<sub>in</sub>) on the rate of NE production, the DA<sub>in</sub> for each DA<sub>out</sub> was estimated based on the average of net DA uptake between 0 and 6 min from the data in Fig. 2. These estimates gave 1.0–3.8 nmol/mg at 10–300 μM DA<sub>out</sub>. However, the rate of NE production was non-saturable and linearly increased with increasing DA<sub>in</sub> in the above concentration range (Fig. 3). Although accurate kinetic parameters could not be obtained, estimates showed that if the enzyme is saturable with respect to DA<sub>in</sub>, K<sub>m</sub> must be >30 nmol/mg (∼15–10 μM), assuming an internal volume of 2–3 μl/mg for granule ghosts (36) and V<sub>max</sub> should be >6.5 nmol/mg/min (Fig. 3).

As shown in Fig. 4A, transient kinetics of DA accumulation and NE production under optimal uptake and conversion conditions were linear in the presence of saturating DA<sub>out</sub> (200 μM). The net rate of DA uptake was 1.3 nmol/mg/min, which is in good agreement with the initial rate kinetics under similar conditions (Fig. 2; V<sub>max</sub> = 1.3 nmol/mg/min), and the rates of DA accumulation and NE production were 0.40 and 0.88 nmol/mg/min, respectively. The data in Fig. 4B show the time courses of net DA uptake and accumulation and NE production under optimal uptake conditions. Again, whereas net uptake and accumulation of DA and NE production were linear, the rate of NE production was significantly reduced, and the rate of DA accumulation was significantly increased, as expected. Under these conditions, the rates of net DA uptake, NE production, and DA accumulation were 1.5, 0.3, and 1.2 nmol/mg/min, respectively, again in agreement with the initial rate kinetics under similar conditions (Table I).

The transient kinetic data presented in Fig. 5A for longer incubation times demonstrate that NE production was linear even up to ~40 min under optimal uptake and conversion conditions in the presence of saturating DA<sub>out</sub> (200 μM). Whereas the rate of DA accumulation was linear only up to about 6–8 min, under these conditions DA<sub>in</sub> reached an apparent steady state with ~7 nmol/mg. However, no lag or burst periods in either DA accumulation or NE production were observed. As shown in Fig. 5B, under optimal uptake conditions, DA accumulation increased by ~50–60% in comparison to the levels determined under optimal uptake and conversion conditions above, whereas the rate of NE production decreased by ~50–60%. Again the DA accumulation reached a plateau (~18 nmol/mg), whereas NE production was linear with no lag or burst periods (Fig 5B). In the presence of limiting DA<sub>out</sub> (25 μM ≡ K<sub>c</sub>; data not shown), both DA accumulation and NE productions were significantly decreased in comparison to the saturating DA<sub>out</sub> experiments under uptake and conversion conditions, as expected. Whereas DA accumulation reached an apparent steady state at ~2 nmol/mg in ~15 min, NE production increased throughout the incubation period, but significantly deviated from the linearity at longer incubation times (likely because of the depletion of DA<sub>out</sub>). Under uptake conditions, the NE production was linear throughout the time course, whereas DA accumulation exceeded the NE production and reached an apparent steady state at ~6 nmol/mg. No lag or burst periods were observed for DA accumulation or NE production under both sets of conditions, similar to that observed under saturating DA<sub>out</sub> conditions (data not shown).

**DISCUSSION**

The procedures that we developed earlier and used in the present study to determine the DA uptake and conversion in granule ghosts give highly reproducible results (15). A key step
in this procedure was the use of cold dilution to separate cleanly the internal contents of the ghost incubates from the external medium and to minimize the back transport of the internal contents. The control experiments have shown that granule ghosts prepared and processed according to these procedures contained relatively constant levels of internal E (6–9 nmol/mg), which slowly declined during longer incubation times, probably because of slow lyses, back transport, nonspecific leakage, etc. Therefore, similar losses of DA and NE in ghost incubates could accurately be corrected by standardizing the DA and NE levels to the average indigenous E levels as reported previously (15), because NE is not converted to E within granule ghosts. We have also shown that ghosts resealed to contain 10–20 mM Asc could be used to examine the DA uptake and conversion in the presence of 10–20 mM extragranular Asc (under optimal uptake and conversion conditions). Ghosts prepared to contain excess ascorbate oxidase with no added Asc could be used to examine essentially the DA uptake in the absence of extragranular Asc (optimal uptake conditions) because intragranular DA to NE conversion is significantly slower under these conditions (15).

The uptake kinetics of DA, NE, and E demonstrate that they all are good substrates for bovine adrenal VMAT and obey Michaelis-Menten kinetics in a wide concentration range under optimal uptake conditions (Fig. 1). The kinetic data in Table I show that DA is a better substrate than NE and E under the same experimental conditions. The \( V_{\text{max}} \) and \( K_m \) parameters determined for DA and E uptake were in good agreement with the previously reported values under similar conditions (14, 32, 38, 39). Although recent studies suggest that bovine adrenal VMAT contains a mixture of VMAT2 (85%) and VMAT1 (15%) (40), our studies could not identify two kinetically distinguishable transport processes for any of the three catecholamines. This may be for two reasons: either the two trans-
porters may be kinetically indistinguishable under the experimental conditions, or the ghost preparation procedures may have enriched a specific population of ghosts with predominantly one of the transporters.

The net DA uptake (DA + NE) into ghosts under optimal uptake and conversion conditions displayed saturable initial rate kinetics with respect to DA_{in} (Fig. 2), and the kinetic parameters determined were similar to previously reported values under comparable experimental conditions (14, 15, 24). The uptake kinetics with respect to estimated DA_{in} (see “Results”) showed that the rate of NE production was apparently non-saturable within the experimental concentration range and linearly increased with increasing DA_{in} (Fig. 3). In addition, kinetic parameters estimated for DA_{in} to NE conversion, assuming saturating kinetics with respect to DA_{in}, are highly inconsistent with the kinetic parameters of the purified m-DβM (15). On the other hand, both NE production and DA accumulation displayed saturable initial rate kinetics with respect to DA_{out} (Fig. 2) with similar \( K_{m} \) parameters that were very comparable with that of net DA uptake, suggesting that DA_{out} could be the kinetically relevant substrate for all three processes (see below).

The transient kinetics of DA uptake, NE production, and DA accumulation were zero-order under optimal uptake and conversion conditions in the presence of saturating DA_{out} (Fig. 4A), and the corresponding rate constants were in good agreement with the initial rate kinetics under similar experimental conditions (see Fig. 2). The apparent zero-order kinetics for DA accumulation and NE production indicate that the rates of back transport of DA_{in} or NE (if any) are insignificant. Therefore, the kinetics of DA uptake followed by m-DβM-catalyzed sequential conversion to NE could be analyzed by a simplified kinetic scheme (Scheme 1) with the following assumptions. (a) DA uptake and conversion are sequential processes. (b) The rate of DA uptake is zero-order (\( k_{in} \)) and irreversible. Because DA_{accum} is saturating (\( -T \times K_{m} \)) and the maximum depletion of DA_{in} because of uptake was estimated to be less than 1–2% under these conditions, the rate of DA uptake should not be dependent on DA_{out}. As mentioned above, the rate of back transport of DA_{in} is insignificant, and DA uptake could be considered irreversible under these conditions. (c) The conversion of DA_{in} to NE is DA_{in}-dependent and first-order (\( k_{ne} \)) with respect to DA_{in} (i.e., all the substrates are saturating or should remain constant) and irreversible. These assumptions are also reasonable, because the extra- and intragranular reductant, Asc, is saturating (20 mM), and the m-DβM reaction is macroscopically irreversible. The above assumption also requires that DA_{in} \( \ll K_{m} \) for DA_{in}, which is also reasonable, because the estimates above indicate \( K_{m} \) for DA_{in} > 30 nmol/mg and the maximum DA_{in} is less than 2 nmol/mg under the same experimental conditions. The above system could be described by the following equations.

\[
\frac{d[DA_{in}]}{dt} = k_{in} - k_{ne}[DA_{in}] 
\]

(Eq. 1)

\[
\frac{d[NE]}{dt} = k_{ne}[DA_{in}] 
\]

(Eq. 2)

\[
[DA_{in}] = k_{d}[Asc][1 - \exp(-k_{ne}t)] 
\]

(Eq. 3)

\[
[NE] = k_{e}[ Asc] [1 - \exp(-k_{ne}t)] 
\]

(Eq. 4)

Although the experimental data in Fig. 4A could not be fitted to equations 3 and 4, the temporal behavior of [DA], [NE], and [DA]+[NE] in the sequential model shown in Scheme 1. \( k_{in} = 0.021 \) nmol/mg/s; \( k_{ne} = 0.015 \) nmol/mg/s; \( k_{2} = 0 \), ---, DA; ----, NE levels; -----, net DA uptake.

The kinetic scheme is first order with respect to DA_{in}, and significant, the kinetics of the system could be analyzed by a simplified scheme (Scheme 2) with the following assumptions. (a) DA uptake and conversion are sequential processes. (b) The rate of DA uptake and conversion is zero-order (maximum depletion of DA_{in} due to uptake is \( -5–6\% \) under these conditions), and re-entry of DA_{in} to the kinetic scheme is first order with respect to DA_{in}. (b) The rate of conversion of DA_{in} to NE is zero-order (\( k_{NE} \)) and independent on DA_{in} under both sets of conditions.\(^2\) (c) The rate of back transport of NE is not significant under the experimental conditions because NE production was linear throughout the time course of the incubation. This system could be described by the following equations:

\[
\frac{d[DA_{in}]}{dt} = k_{in} - k_{2}[DA_{in}] 
\]

(Eq. 5)

\[
[DA_{in}] = k_{d}[Asc][1 - \exp(-k_{ne}t)] 
\]

(Eq. 6)

\[
[NE] = k_{e}[Asc][1 - \exp(-k_{ne}t)] 
\]

(Eq. 7)

The fit of the data in Fig. 5A to equation 6 gave \( k_{1} = 0.52 \pm 0.01 \) nmol/mg/min and \( k_{2} = 0.072 \pm 0.001 \) min\(^{-1} \) under optimal uptake and conversion conditions. Similar fits under optimal

\(^2\) D. S. Wimalasena and K. Wimalasena, unpublished observations.
uptake conditions (Fig. 5B) gave \( k_1 = 1.00 \pm 0.05 \text{ nmol/mg-min} \) and \( k_2 = 0.06 \pm 0.01 \text{ min}^{-1} \). The fit of NE production data to equation 7 resulted in zero-order rate constants \( (k_{\text{NE}}) \) of 0.55 and 0.21 nmol/mg-min, yielding rates of net DA uptake \( (k_u) \) of 1.1 and 1.2 nmol/mg-min under optimal uptake and conversion and uptake conditions (because \( k_2 \) is small \( k_{\text{NE}} \sim (k_{\text{NE}} + k_1) \) at low DA_{in} conditions), respectively. These results are in excellent agreement with the initial rate kinetic data of net DA uptake (see Fig. 2). The steady state concentrations of DA_{in} \( (\text{DA}_{\text{in}} = (k_2/k_1)) \) were determined to be 7.2 and 16.7 nmol/mg-min under optimal uptake and conversion and uptake conditions, respectively, and are also in excellent agreement with the experimental results (Fig. 5, A and B). Furthermore, the similar and relatively small magnitudes of \( k_2 \) determined under both sets of conditions \( (k_{\text{in}} \gg k_2) \) are consistent with the apparent linear transient kinetics observed for net DA uptake and DA accumulation at short incubation times (Fig. 4, A and B). Therefore, all the experimental results are fully consistent with the above model (Scheme 2).

A previous study (16) reported that transient DA accumulation displays an initial burst followed by a sharp decline, whereas NE production displays saturating (or exponential growth) kinetics, using granule ghosts under similar experimental conditions. The authors have used a complex kinetic simulation to explain the results assuming a sequential model. Although we could not reproduce these results, the lack of initial burst or lag periods in either DA accumulation or NE production observed in the present study is consistent with all our previous studies (14, 15, 32). Thus, to explain the observed apparent zero-order transient kinetics of DA uptake and NE production, we propose a novel channeling model (27–30) in which DA is electrochemically channeled from bovine adrenal VMAT to m-DβM for hydroxylation prior to the release into the bulk medium of the ghost interior (Schemes 2 and 3). The slow accumulation of DA, depending on the experimental conditions, must be caused by the leakage of DA from the transporter (or m-DβM) into the interior of the ghosts, and the approach of an apparent steady state of DA_{in} at longer incubation conditions could be caused by the slow concentration-dependent re-entry of DA_{in} into the transport-coupled hydroxylation process from the interior of the granule (Schemes 2 and 3). The rate of NE production is independent of DA_{in} but dependent on DA_{out} and DA_{in} is the kinetically relevant substrate for DA uptake and accumulation and NE production, which is consistent with the similar \( K_u \) parameters determined under initial rate conditions for these three processes with respect to DA_{out}.

The transport-coupled m-DβM activity is usually orders of magnitude less than the total enzyme activity released following the lyses and solubilization of ghosts (16, 22, 23). Because VMAT-mediated transport is directly coupled with the m-DβM activity in ghosts according to the proposed channeling model, m-DβM may not express its optimal activity but efficiently catalyzes the DA to NE conversion at low DA_{out} concentrations. The kinetic isotope effect of the m-DβM reaction in granule ghosts has been determined to be \( -2 \) (similar to the purified enzyme under steady state conditions), suggesting that the m-DβM reaction is rate-limiting relative to the transport under optimal uptake and conversion conditions (16). Although the exclusion of external Asc was expected to reduce the isotope effect caused by the decreased rate of hydroxylation relative to the transport (normally by about 50–60%), the isotope effect was not affected (16). These inconsistencies have been attributed to the non-steady state conditions of the ghost interior. According to the proposed model, removal of external Asc significantly decreases the channeling efficiency because the oxidized enzyme does not interact with the amine substrate efficiently, leading to the increase of DA accumulation through the leakage pathway without affecting the isotope effect. Under both conditions the net uptake of DA remains relatively constant (within 10–20%) regardless of the magnitude of the partition ratio between the DA accumulation and hydroxylation pathways. Therefore, the channeling model provides satisfactory explanations for some of the critical questions regarding the dynamics of DA uptake and conversion in chromaffin granule ghosts.

The concentration of VMAT in chromaffin granule membrane was estimated to be 40–50 pmol/mg (42), and m-DβM was about 7% of the total membrane proteins (37). Assuming a subunit molecular weight of 76,000 for m-DβM (1–3), the molar ratio of m-DβM to VMAT in the membrane is in the range of 1 to 5. Therefore, VMAT molecules in the membrane could theoretically be effectively coupled to m-DβM, providing an efficient channeling probability in vivo (Scheme 3). Because DβM activity in intact granules is equally distributed between s-DβM and m-DβM (23–26), operation of a tightly coupled channeling mechanism in vivo may not give s-DβM access to the substrate, making the catalytic activity of s-DβM to be physiologically insignificant. Interestingly, increasing experimental evidence suggests (15, 23) that m-DβM may be the only functional form and that s-DβM is not functional under physiological conditions and is designated for disposal through exocytosis. We note, however, that the above results do not provide any information about s-DβM in intact granules because s-DβM is lost during the preparation of ghosts. Therefore, a
better understanding of the topological arrangements of VMAT and m-ΔβM and the physiological role of s-ΔβM in intact granules is of prime importance for further confirmation of the proposed channeling mechanism for the transport-coupled hydroxylation of DA in catecholamine storage vesicles.

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