The Reduction of Membrane-bound Dopamine β-Monoxygenase in Resealed Chromaffin Granule Ghosts

IS INTRAGranular ASCORBIC ACID A MEDIATOR FOR EXTRAGranular Reducing Equivalents?*

(Received for publication, April 3, 1995, and in revised form, August 30, 1995)

Kandatege Wimalasena† and D. Shyamali Wimalasena
From the Department of Chemistry, Wichita State University, Wichita, Kansas 67260-0051

Dopamine β-monoxygenase (DβM; EC 1.14.17.1) catalyzes the conversion of dopamine (DA) to the neurotransmitter, norepinephrine (NE), within the neurosecretory vesicles of the adrenal medullae and the large dense-cored synaptic vesicles of the sympathetic nervous system. DβM exists in both soluble and membrane-bound forms in these tissues (1–3). The reducing equivalents required for DβM monooxygenation and that internal soluble ascorbate (or dopamine) may not directly reduce or mediate the reduction of membrane-bound DβM in resealed granule ghosts.

The role of internal and external reductants in the dopamine β-monoxygenase (DβM)-catalyzed conversion of dopamine to norepinephrine has been investigated in resealed chromaffin granule ghosts. The rate of norepinephrine production was not affected by the exclusion of internal ascorbate. The omission of ascorbate from the external medium drastically reduced the norepinephrine production without affecting the net rate of dopamine uptake. In the presence of the external reductant, the internal ascorbate levels were constant throughout the incubation period. The rate of norepinephrine production was not affected when ghosts were resealed to contain the DβM reduction site inhibitor, imono-o-glucosaccharide. Ghosts incubated with external imono-o-glucosaccharide reduced the norepinephrine production. The weak DβM reductant, 6-amino-L-ascorbic acid, was found to be a good external reductant for granule ghosts. The outcome of the above experiments was not altered when dopamine was replaced with the reductively inactive DβM substrate, tyramine. These results and the known topology of membrane-bound DβM disfavor the direct reduction of the enzyme by the external reductant. Our observations are consistent with the hypothesis that external ascorbate is the sole source of reducing equivalents for DβM monooxygenation and that internal soluble ascorbate (or dopamine) may not directly reduce or mediate the reduction of membrane-bound DβM in resealed granule ghosts.
ating the electron flux from cytosol to the interior of the granule ghosts through cyt b561 (19–22, 31, 32).

However, in contrast to the above generally accepted mechanism for the reduction of DjM in catecholamine secretory vesicles, several recent reports appear to indicate that the external AscH₂ is the kinetically preferred reductant for the membrane-bound DjM in resealed chromaffin granule ghosts (14, 33). In the present study, we have examined the role of internal and external electron donors in the DjM-catalyzed conversion of DA to NE and the net rate of DA uptake in resealed chromaffin granule ghosts using three new AscH₂ analogs, 6-amino-L-ascorbic acid (6-AAscH₂), 2-amino-L-ascorbic acid (2-AAscH₂), and imino-D-glucosaccharic acid (IGA) as probes (Scheme 1). The results presented in this report suggest that the external reductant, AscH₂, is the sole source of the reducing equivalents for the membrane-bound DjM-catalyzed monooxygenation in resealed chromaffin granule ghosts. More importantly, our results also suggest that internal soluble AscH₂ (or DA) may not directly reduce or mediate the reduction of membrane-bound DjM in chromaffin granule ghosts. The implications of these findings on the mechanism of the reduction of membrane-bound and soluble DjM in relation to in vivo NE biosynthesis is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**

L-Norepinephrine hydrochloride, dl-epinephrine bitartrate, dopamine hydrochloride, Mg-ATP, HEPES, Trizma (Tris base), ascorbate oxidase, and polyoxyethylene 10 tridecyl ether (Emulphogene) were obtained from Sigma. Ascorbic acid (AscH₂) and sodium fumarate were from Aldrich. Ficoll (powder) was obtained from Pharmacia Biotech Inc., and catalase was from Boehringer Mannheim. Protein assay reagents and bovine serum albumin were from Bio-Rad Laboratories. Imidazole, and bovine serum albumin were from Bio-Rad Laboratories. Imidazole hydrochloride, Mg-ATP, HEPES, Trizma (Tris base), ascorbate oxidase (as indicated in the figure legends), and diluted with 0.3 M sucrose, 10 mM HEPES, pH 7.0, and 3.0 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 separate as a band at the 0.4 M sucrose-HEPES/Ficoll interface was drawn out using a disposable syringe, diluted with 5 ml of 0.3 M sucrose, 10 mM HEPES, pH 7.0, and homogenized, and pelleted by the addition of 1/8 volume of a glycerol solution (glycerol, 0.2 M Trisphosphate, pH 7.0, 2 M Tris phosphate, pH 7.0, 3.7). The lysate was added in 1.5-ml aliquots at −78°C. Chromaffin granule ghost membranes were isolated from the stores on the day of each experiment by diluting with water containing 20 mM AscH₂ and 100 μg/ml catalase, leaving for 10 min at 4°C and centrifugation at 36,000 × g for 20 min at 4°C. The pellet was homogenized and resuspended in 2 ml of a solution containing 20 mM Tris phosphate, 100 mM KCl, 150 mM sucrose, 10 mM sodium fumarate, 100 μg/ml catalase, and the desired amounts of AscH₂, IGA, 6-AAscH₂, 2-AAscH₂, or ascorbate oxidase, as indicated in the figure legends, and the pH of the final solution was adjusted to 7.0. The ghost membranes were allowed to resell by incubation for 20 min at room temperature, diluted to 5 ml with the same solution placed inside (but never containing AscH₂, ascorbate derivative, or catalase), and layered over 2.0 ml of 15% Ficoll, 0.3 M sucrose, 10 mM HEPES, pH 7.0, and 3.0 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 separate as a band at the 0.4 M sucrose-HEPES/Ficoll interface was drawn out using a disposable syringe, diluted with 5 ml of 0.3 M sucrose, 10 mM HEPES, pH 7.0, and homogenized, and pelleted by centrifugation at 36,000 × g for 20 min at 4°C. The supernatant was removed, and the pellet was gently coagulated protein, 25 μl of 0.1 M HClO₄ was added, the pellets were washed gently three times with 0.4 M sucrose, 10 mM HEPES, pH 7.0, and the tubes were swabbed dry. Then, 500 μl of 0.1 M HClO₄ was added, the pellets were homogenized, and the extraction was allowed to proceed for 20–25 min at 30°C. After low speed centrifugation to remove coagulated protein, 25 μl of the acidic extracts were analyzed for catecholamines and AscH₂, by reversed phase HPLC-EC. The same protocol was followed for the experiments carried out using tyramine as the substrate, except that tyramine was used at a final concentration of 30 μM, and the aliquots were withdrawn every 4 min for 20 min. The localization of Membrane-bound DjM—Membrane-bound DjM was isolated from chromaffin granule membranes which were stored at −78°C in 20 mM potassium phosphate buffer, pH 7.0, containing 100 μg/ml catalase. The frozen membranes were thawed and centrifuged at 48,500

**Methods**

HPLC-EC Analyses—The catecholamines, NE, E, and DA, and AscH₂ levels of chromaffin granule ghost incubates were quantitated using ion-paired HPLC with electrochemical detection. Perchloric acid extracts of ghosts were separated by a C₁₈ reversed phase column (ESA, HR-80) pre-equilibrated with a mobile phase composed of 50 mM NaH₂PO₄, 0.22 mM sodium EDTA, 1.24 mM octanesulfonic acid, pH 2.6, with 6% CH₃OH at a flow rate of 1 ml/min. All four analytes were detected by electrochemical oxidation at 300 mV. When tyramine was the substrate, octopamine was separated using a mobile phase composed of 75 mM NaH₂PO₄, 10 mV trichloroacetic acid, 0.52 mM sodium dodecyl sulfate, pH 2.8, with 14% CH₃CN. The octopamine levels were detected by electrochemical oxidation at 800 mV. The E and NE levels of these incubates were quantitated under standard conditions by a second injection of the same sample. Sample peak areas were quantitated by comparison to standard curves which were linear over the range of sample sizes encountered.

Protein Assays—Protein contents of the ghost preparations were determined by the method of Bradford (37) using the Bio-Rad protein assay with bovine serum albumin as the standard.

Tissue Preparations—Chromaffin granules were prepared from bovine adrenal medullae obtained within 1 h postmortem from a local abattoir, using a procedure previously described (26, 27) using the original methods of Kirshner (38) as modified by Njus and Radda (39), except that the granules were purified by a discontinuous sucrose density gradient at 58,700 × g for 90 min at 4°C (40). The granules were homogenized in 0.2 mM Tris phospate, pH 7.0, containing 100 μg/ml catalase and lysed by the addition of 1/8 volume of a glycercol solution (glycerol, 0.2 M Tris phosphate, pH 7.0, 3.7). The lysates were stored in 1.5-ml aliquots at −78°C. Chromaffin granule ghost membranes were isolated from the stores on the day of each experiment by diluting with water containing 20 mM AscH₂ and 100 μg/ml catalase, leaving for 10 min at 4°C and centrifugation at 36,000 × g for 20 min at 4°C. The pellet was homogenized and resuspended in 2 ml of a solution containing 20 mM Tris phosphate, 100 mM KCl, 150 mM sucrose, 10 mM sodium fumarate, 100 μg/ml catalase, and the desired amounts of AscH₂, IGA, 6-AAscH₂, 2-AAscH₂, or ascorbate oxidase, as indicated in the figure legends, and the pH of the final solution was adjusted to 7.0. The ghost membranes were allowed to resell by incubation for 20 min at room temperature, diluted to 5 ml with the same solution placed inside (but never containing AscH₂, ascorbate derivative, or catalase), and layered over 2.0 ml of 15% Ficoll, 0.3 M sucrose, 10 mM HEPES, pH 7.0, and 3.0 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 separate as a band at the 0.4 M sucrose-HEPES/Ficoll interface was drawn out using a disposable syringe, diluted with 5 ml of 0.3 M sucrose, 10 mM HEPES, pH 7.0, and homogenized, and pelleted by centrifugation at 36,000 × g for 20 min at 4°C. The supernatant was removed, and the pellet was gently coagulated protein, 25 μl of 0.1 M HClO₄ was added, the pellets were washed gently three times with 0.4 M sucrose, 10 mM HEPES, pH 7.0, and the tubes were swabbed dry. Then, 500 μl of 0.1 M HClO₄ was added, the pellets were homogenized, and the extraction was allowed to proceed for 20–25 min at 30°C. After low speed centrifugation to remove coagulated protein, 25 μl of the acidic extracts were analyzed for catecholamines and AscH₂, by reversed phase HPLC-EC. The same protocol was followed for the experiments carried out using tyramine as the substrate, except that tyramine was used at a final concentration of 30 μM, and the aliquots were withdrawn every 4 min for 20 min. The localization of Membrane-bound DjM—Membrane-bound DjM was isolated from chromaffin granule membranes which were stored at −78°C in 20 mM potassium phosphate buffer, pH 7.0, containing 100 μg/ml catalase. The frozen membranes were thawed and centrifuged at 48,500
The resealed chromaffin granule ghosts prepared to contain 20 mM Asch$_2$, 20 mM Tris phosphate, 100 mM KCl, 150 mM sucrose, 10 mM sodium fumarate, and 100 µg/ml catalase according to the above procedures (standard resealed granule ghosts), actively accumulate DA and convert it to NE in a time-dependent manner in the presence of 5 mM ATP, 5 mM MgSO$_4$, and 20 mM Asch$_2$ in the external incubation medium (standard uptake and conversion incubation conditions). Appropriate control experiments with the standard resealed granule ghosts revealed that the exclusion of ATP or inclusion of 2 µM reserpine in the external incubation medium completely abolishes the DA accumulation and NE production under the above conditions as expected (data not shown).

A series of control experiments were carried out with standard resealed granule ghosts in order to quantify the experimental variability of the rate of DA uptake and its conversion to NE and to examine the viability of the standard ghost preparations during the time course of the experiment under the standard uptake and conversion incubation conditions. Under these conditions, the average internal E levels of the standard resealed granule ghosts varies from 23.0 to 15.7 nmol/mg within a 25-min incubation period (Fig. 1a) probably due to the slow lysis or the leakage of the internal contents of the resealed granule ghosts (see “Discussion”). The data standardized against average constant indigenous E levels show that the average internal Asch$_2$ (reduced) levels were 23.5 nmol/mg and the supernatant was introduced into a Mono Q (HR 10/10) column pre-equilibrated with 20 mM potassium phosphate buffer, pH 7.2, containing 0.25% Emulphogene controlled by a Pharmacia FPLC system. The column was washed with the same buffer until the 280 nm absorbance returned to baseline and then eluted with a KCl gradient of 0–300 mM over a 120-min period using a buffer system containing 20 mM potassium phosphate, pH 7.2, and 0.25% Emulphogene. The fractions with the highest DjM activity were combined and concentrated using an Amicon ultrafiltration device using a YM-30 membrane. The protein content of the concentrated enzyme was determined by the absorbance at 280 nm (ε$^\text{280} = 12.4$ (41)). The specific activity of purified membrane-bound DjM was 3.5 µmol/mg/min.

Membrane-bound DjM from the above preparation was used in the estimation of the inhibition potency of IGA and the substrate activity of 6-AAsch$_2$ using the oxygen monitor assay as described previously (35).

RESULTS

The resealed chromaffin granule ghosts prepared to contain 20 mM Asch$_2$, 20 mM Tris phosphate, 100 mM KCl, 150 mM sucrose, 10 mM sodium fumarate, and 100 µg/ml catalase ac-"
when the granule ghosts were resealed to contain no added AsCH₂ and a high concentration of active ascorbate oxidase (Fig. 2, a and b). The rate of NE production in this preparation was in the normal range in comparison with that of the control standard resealed granule ghosts suggesting a high rate of DA to NE conversion in the absence of internal AsCH₂. On the other hand, when standard resealed granule ghosts were incubated in a medium in the absence of external AsCH₂, but in the presence of a high concentration of external ascorbate oxidase, the rate of DA accumulation was increased by about 3-fold, and NE production was decreased by about 3-fold in comparison with the standard resealed granule ghosts (Fig. 2, a and b). Furthermore, when AsCH₂ was excluded from the interior and the external incubation medium (by the addition of ascorbate oxidase into both media), the rate of NE production was again reduced by about 5-fold without significantly altering the net rate of DA uptake (data not shown).

The effect of external and/or internal IGA, which is a potent inhibitor for the AsCH₂ site of both soluble (Ki = 26.4 ± 5.2 μM (36)) and membrane-bound DjM,² on the rate of DA accumulation and NE production in resealed chromaffin granule ghosts was examined in a series of experiments. When granule ghosts were resealed to contain 10 mM IGA together with 20 mM AsCH₂ and incubated under standard uptake and conversion conditions, the rates of either the DA accumulation or NE production were not significantly altered compared to identical controls without IGA (data not shown). In fact, the rate of NE production under these conditions was in the high normal range in comparison to the control standard resealed granule ghosts. In addition, the rates of both DA accumulation and NE production in ghosts resealed to contain either 1 or 10 mM IGA in the presence (5 mM or 20 mM) or absence of added AsCH₂ were also in the normal range under standard uptake and conversion conditions (Fig. 3, a and b). On the other hand, the rate of NE production was significantly decreased when standard resealed granule ghosts were incubated under uptake and conversion conditions in the presence of 1 mM IGA and 5 mM AsCH₂ with respect to a parallel identical control without IGA. The rate of DA accumulation in the IGA-containing incubates was significantly higher than the rate of the controls suggesting a slow rate of DA to NE conversion without changing the net rate of DA uptake as expected (Fig. 3b). Furthermore, when ghosts were prepared to contain either AsCH₂, IGA, or ascorbate oxidase and incubated in the presence of external IGA (note that IGA is not a substrate or inhibitor for ascorbate oxidase (42)) and ascorbate oxidase, the rate of NE production dropped to the background level while the rate of DA accumulation increased to a very high level confirming that external IGA does not support the DjM monoxygenation reaction in resealed granule ghosts. Control experiments revealed that IGA is not actively or passively accumulated into granule ghosts under the experimental conditions (data not shown).

The specificity of the extragranular reduction site of the chromaffin granule membrane was examined by using the AsCH₂ analog, 6-AAsCH₂, which is a very weak reductant for purified soluble (36) as well as membrane-bound DjM (less than 10% activity at 20 mM concentration in comparison to AsCH₂). The data presented in Fig. 4a clearly demonstrate that in contrast to its inability to reduce soluble or membrane-bound purified DjM efficiently, under standard experimental conditions, external 6-AAsCH₂ supports the NE production in standard resealed granule ghost with the efficiency similar to that of AsCH₂ itself. The data also show that external AsCH₂ supports NE production in 6-AAsCH₂-loaded ghosts with the efficiency similar to that of AsCH₂-loaded ghosts. Furthermore, the rate of DA accumulation is in the normal range for both of these sets of experiments suggesting that external or internal 6-AAsCH₂ has no significant effect on the net rate of DA uptake (Fig. 4, a and b). Although 6-AAsCH₂ has a remarkable structural similarity to DA, control experiments revealed that it is

Fig. 2. The effect of internal and/or external AsCH₂ on the time courses of NE production (a) and DA accumulation (b) in resealed chromaffin granule ghosts. Ghosts were resealed to contain 20 mM Tris phosphate, 100 mM KCl, 150 mM sucrose, 10 mM fumarate, and 100 μg/ml catalase with AsCH₂, without AsCH₂, or with ascorbate oxidase (pH 7.0) and incubated in a medium (2.5 ml total volume) containing 0.3 mM sucrose, 10 mM HEPES, 5 mM Mg-ATP, 5 mM MgSO₄, and 100 μg/ml catalase with AsCH₂, without AsCH₂, or with ascorbate oxidase (pH 7.0) at 30°C for 10 min. At zero time, the uptake and conversion reaction was initiated by adding DA to a final concentration of 200 μM, and, at 5-min time intervals, 400-μl aliquots of the incubate were withdrawn, and HPLC-EC quantitation was performed as noted under “Experimental Procedures.” a, no AsCH₂ inside; b, 20 mM AsCH₂ outside; c, 25 units of ascorbate oxidase inside; d, 20 mM AsCH₂ outside; e, and, 25 mM AsCH₂ inside, 25 units of ascorbate oxidase outside (multiple experiments under the same conditions were carried out in some cases to examine the reproducibility). Note: the experimental variability of the rate of NE production and DA accumulation under standard uptake and conversion incubation conditions (20 mM AsCH₂ outside) with standard resealed ghosts (20 mM AsCH₂ inside) for 5 separate experiments are shown as vertical bars.

In control experiments, 1 mM concentration of IGA was found to inhibit 90% of membrane-bound DjM activity in the presence of 5 mM AsCH₂; a similar inhibition was also observed with lysed membrane fragments under similar conditions.
not accumulated into granule ghosts through the DA uptake mechanism in an ATP- and time-dependent manner, under standard uptake and conversion incubation conditions. Other control experiments indicated that 6-AAA had no significant effect on the viability of resealed ghosts under the experimental conditions.

The data presented in Fig. 5 demonstrate that the chromophoric DβM reductant, 2-AAA (35), also supports the conversion of DA to NE in standard resealed granule ghosts with an efficiency similar to that of AscH₂ (control experiments indicate that 2-AAA does not permeate the granule membrane at a detectable rate although it is somewhat less polar than AscH₂ due to the alteration of the pKₐ of the 3'-OH group). Although quantitative studies have not been completed yet, we have observed the formation of the chromophoric oxidized product of 2-AAA, the red pigment, in a time-dependent manner in these experiments when 2-AAA is the external electron donor (we believe this reaction could be adopted to measure the rate of electron flux into granule ghosts; detailed experiments are in progress). On the other hand, the chromophoric non-

---

**Fig. 3.** The effect of internal and/or external imino-D-glucoascorbic acid (IGA) on the time courses of NE production (a) and DA accumulation (b) in resealed chromaffin granule ghosts. Ghosts were resealed to contain 20 mM Tris phosphate, 100 mM KCl, 150 mM sucrose, 10 mM fumarate, and 100 μM catalase with AscH₂, IGA, ascorbate oxidase, or a combination of the above (pH 7.0) and incubated in a medium (2.5 ml total volume) containing 0.3 mM sucrose, 10 mM HEPES, 5 mM Mg-ATP, 5 mM MgSO₄, and 100 μM catalase with AscH₂, IGA, ascorbate oxidase, or a combination of the above (pH 7.0) at 30 °C for 10 min. At zero time, the uptake and conversion reaction was initiated by adding DA to a final concentration of 200 μM, and, at 5-min time intervals, 400-μl aliquots of the incubate were withdrawn, and HPLC-EC quantitation was performed as noted under "Experimental Procedures." ●, 5 mM AscH₂ inside, 20 mM AscH₂ outside; ■, 5 mM AscH₂ and 1 mM IGA inside, 20 mM AscH₂ outside; □, 20 mM AscH₂ inside, 5 mM AscH₂ and 1 mM IGA outside; ○, 20 mM AscH₂ inside, 5 mM AscH₂ outside; △, 20 mM AscH₂ inside, 20 mM AscH₂ outside; □, 20 mM AscH₂ inside, 5 mM AscH₂ and 1 mM IGA inside, 20 mM AscH₂ outside; ●, no added AscH₂ inside, 20 mM AscH₂ outside. Note: vertical bars are the same as in Fig. 2.

**Fig. 4.** The effect of internal or external 6-aminoascorbic acid (6-AAA) on the time courses of NE production (a) and DA accumulation (b) in resealed chromaffin granule ghosts. Ghosts were resealed to contain 20 mM Tris phosphate, 100 mM KCl, 150 mM sucrose, 10 mM fumarate, and 100 μM catalase with AscH₂ and 6-AAA (pH 7.0) and incubated in a medium (2.5 ml total volume) containing 0.3 mM sucrose, 10 mM HEPES, 5 mM Mg-ATP, 5 mM MgSO₄, and 100 μM catalase (pH 7.0) at 30 °C for 10 min. At zero time, the uptake and conversion reaction was initiated by adding DA to a final concentration of 200 μM, and, at 5-min time intervals, 400-μl aliquots of the incubate were withdrawn, and HPLC-EC quantitation was performed as noted under " Experimental Procedures." ●, 20 mM AscH₂ inside, 20 mM 6-AAA outside; ■, 20 mM 6-AAA inside, 20 mM AscH₂ outside; ◇, 20 mM 6-AAA inside, 5 mM AscH₂ outside; ○, 10 mM IGA and 25 units of ascorbate oxidase inside, 20 mM AscH₂ outside; □, 20 mM AscH₂ inside, 20 mM AscH₂ outside; ●, no added AscH₂ inside, 20 mM AscH₂ outside; X, 10 mM IGA and 25 units of ascorbate oxidase inside, 10 mM IGA outside. Note: vertical bars are the same as in Fig. 2.
ascorbate DβM reductant, N,N-dimethyl-1,4-phenylenedia-
mine (35), does not appear to be an external reductant for
membrane-bound DβM in chromaffin granule ghosts (data not
shown).

The possibility that DA may function as a redox mediator
between the extragranular reduction site and membrane-
bound DβM was investigated by using reductively inactive
tyramine as the DβM substrate. In these experiments, the
rates of intragranular octopamine formation were accurately
quantified by reversed phase HPLC-EC as described under
"Experimental Procedures." The data presented in Fig. 6
displays that standard resealed granule ghosts up-
take tyramine and convert it to the DβM hydroxylation pro-
duct, octopamine, in a time-dependent manner with a rate some-
what slower than that of the DA to NE conversion under
standard uptake and turnover conditions (see Fig. 6, vertical
bars; however, note that the external tyramine concentrations
in analogous experiments were 30 μM, whereas DA concentra-
tions in these experiments were 20 mM). The exclusion of inter-
nal Asch2 by resealing granule ghosts to contain a high
level of highly active ascorbate oxidase3 does not significantly
change the rate of octopamine production in comparison to the
standard controls under identical conditions (Fig. 6). The rate
of octopamine production was not inhibited when ghosts were
resealed to contain 5 mM IGA and no added Asch2 and incu-
bated under standard uptake and turnover conditions (Fig. 6).
In contrast, a slight increase in the rate of octopamine produc-
tion was observed compared to the standard controls when
internal IGA was present, similar to that observed for the DA
to NE conversion. In addition, external 20 mM 6-AAscH2 sup-
ports the tyramine to octopamine conversion in 20 mM
Asch2-loaded granule ghosts with a rate slightly lower than that of
the standard controls.

**DISCUSSION**

The optimum conditions and procedures to examine the DA
uptake and NE production in resealed chromaffin granule
ghosts under various experimental conditions with non-radio-
labeled substrate have been reported previously (26, 27). The
key steps in this procedure were the resealing of the granule
membranes under optimum conditions necessary for maximum
DβM turnover, the termination of the uptake of DA and its
conversion to NE at a given period of time by diluting the
incubates with an ice-cold osmotically balanced solution, the
separation and reisolation of the intact granule ghosts
from the external incubation medium without contamination,
and the quantification of the internal contents of the granule
ghost incubates by HPLC-EC. The ghosts prepared, incubated,
and analyzed according to the above procedures were found to
actively uptake DA and convert it to NE in an ATP- and
time-dependent reserpine-sensitive manner giving consistent
and reproducible results. Therefore, we have used the same
protocols and procedures in the present study without signifi-
cant alterations or modifications.

The resealed granule ghosts prepared according to the above

---

3 In a previous study, Huyghe and Klinman (14) have reported that the rate of conversion of tyramine to octopamine was increased by about 2.7-fold when granule ghosts were resealed to contain a high concen-
tration of Asch2 inside and incubated in an external medium containing
2 mM Asch2. However, we have not observed such a significant change in the
rate of DA to NE or tyramine to octopamine conversion when the internal
Asch2 levels of resealed granule ghosts were changed from none (no added Asch2 and with ascorbate oxidase) to 20 mM Asch2 and
incubated in a medium containing 20 mM Asch2 (Figs. 2, a and b, and
6). We believe this apparent discrepancy may at least be partly due to
the differences in the experimental conditions used in the two studies.
procedures contained relatively constant levels of internal E which were found to slowly decline during the incubation time period (Fig. 1a). Since the depletion of E was observed even when excess AsH₂ was present in both the internal matrix and in the external incubation medium, this could not be due to the auto-oxidation of the catechol moiety of the molecule and must be due to the slow lysis or the leakage of the internal contents of the ghosts during the incubation period or resealing procedure. Therefore, any loss or leakage of internal contents from resealed granule ghosts during the experiments could accurately be corrected (Fig. 1b) by standardizing against the average indigenous E levels since NE is not converted to E within resealed granule ghosts due to the lack of the essential enzyme, phenethylamine N-methyltransferase (5). Furthermore, external contamination or incomplete resealing of ghost preparations prior to the incubation could also be estimated by measuring the DA content at the time 0 point which is usually 0 under the above experimental conditions (Fig. 1, a and b). The normal rates of NE production and DA accumulation in standard resealed granule ghosts (Figs. 1 and 2) were in good agreement with the rates previously reported under similar experimental conditions (26, 27, 43). The observed low ratio of the net rate of DA uptake to NE production (in the range of 1.1–1.3) together with the lack of lag or burst periods in both of these time courses under the standard experimental conditions (Fig. 2, a and b), suggest that both the rate of DA uptake and DβM turnover contribute to the net rate of NE production in resealed granule ghosts which is also consistent with the previous observations (44).

In the experiments where removal of internal AsH₂ contaminants was necessary, ghosts were resealed to contain high concentrations of active ascorbate oxidase. The interior contents of the ghosts reisolated from these preparations, after incubation under standard uptake and conversion conditions, were found to consistently contain an average of 6–7 nmol/mg of AsH₂ regardless of the presence of high levels of intragranular ascorbate oxidase (similar observations have been reported previously (14)). However, these AsH₂ levels were not increased in a time- and/or ATP-dependent manner during the incubation period suggesting that external AsH₂ was not actively taken up into the ghosts (data not shown) and were much less than the external AsH₂ concentration of the incubation medium, ruling out the possibility that external AsH₂ was passively transported and equilibrated during the incubation period. Furthermore, since ascorbate oxidase is a very efficient enzyme (Kcat = 3,000–11,000 s⁻¹ (46)) with very high affinity toward AsH₂ (Km is in the μM range (46)), the observed levels of AsH₂ could not possibly be in the interior soluble matrix of the resealed granule ghosts and not be accessible to ascorbate oxidase. Therefore, a possible simple explanation for the above observation is that AsH₂ nonspecifically and tightly interacts with the external phase of the resealed granule ghost membrane. Since the amino acid sequence and tertiary structure model proposed for cyt b6f1 contains clusters of positively charged amino acid side chains in the cytoplasmic domain of the protein (47), it is possible that negatively charged AsH₂ molecules may specifically and strongly interact with these regions of the protein in order to facilitate the subsequent reaction with the buried heme. Alternatively, it is possible that a small pool of permanently inner or outer membrane-associated externally reducible AsH₂ exists in resealed chromaffin granule ghosts (for example, see Ref. 45) which may not be accessible to the intragranular ascorbate oxidase. However, the present results do not distinguish between these possibilities.

The internal AsH₂ levels in standard resealed granule ghosts remain relatively constant (average of 23.5 nmol/mg of protein of detectable reduced AsH₂ throughout the incubation period (25–30 min) under standard uptake and conversion incubation conditions (Fig. 1, a and b) suggesting that there is no net consumption of internal AsH₂ by the DβM monoxynogenation reaction under these conditions (44). Since it is generally accepted (our results also confirm) that AsH₂ is not actively or passively transported into the interior of the granule ghosts at a detectable rate (11–14), the reducing equivalents essential for the DβM reaction must be provided from the cytosolic pool of the reductant. Alternatively, although the contaminating intragranular catecholamines present in the system could provide necessary reducing equivalents for the DβM-catalyzed DA to NE conversion in theory (for example, see Ref. 45), the rate of depletion of the catecholamine concentration during the incubation period was much slower (about 0.2 to 0.3 nmol/mg min) and was not sufficient (Fig. 1, a and b) to account for the observed rate of NE production. On the other hand, the data presented in Fig. 2a clearly demonstrate that external AsH₂ is essential for the efficient conversion of DA to NE in standard resealed granule ghosts. However, the internal or external AsH₂ has no significant effect on the net rate of DA uptake into resealed granule ghosts (i.e. the combined rates of DA accumulation and NE production; Fig. 2b) under the same experimental conditions. Therefore, it can be concluded that while external AsH₂ is essential for the efficient conversion of DA to NE in resealed chromaffin granule ghosts under standard uptake and conversion incubation conditions, internal AsH₂ has no significant effect.

Internal IGA (an efficient reductant site-competitive inhibitor of DβM) concentrations of up to 10 μM has no significant effect on either the rate of NE production or the rate of DA accumulation under standard uptake and conversion incubation conditions in the presence or even in the absence of externally added AsH₂ in the internal matrix of resealed granule ghosts (Fig. 3, a and b). In fact, close examination of the results indicate that the rate of NE production in the presence of internal IGA was slightly higher in comparison with identical controls without IGA. Therefore, it is clear that although IGA is an excellent inhibitor for both soluble and membrane-bound forms of DβM and effectively interacts with their reduction sites (AsH₂ sites), it does not effectively interact with the reduction site of membrane-bound DβM in resealed granule ghosts from the internal phase of the granule membrane. In addition, the lack of expected competition between internal IGA and AsH₂ for the reduction site of the enzyme strongly suggests that internal AsH₂ also may not directly interact with the reduction site of DβM in the resealed chromaffin granule ghost membrane. On the other hand, externally applied IGA was found to inhibit the intragranular DA to NE conversion efficiently (Fig. 3, a and b) without significantly affecting the net rate of DA uptake (Fig. 3, a and b) suggesting that IGA effectively interacts with the external reduction site of the granule membrane. These results support the proposal that, while intragranular AsH₂ may not directly reduce or mediate the reduction of membrane-bound DβM in resealed chromaffin granule ghosts, the external reductant may be the exclusive reductant for the enzyme under these experimental conditions.
conditions.

In our previous studies (36) we have shown that 6-AAscH₂ was not an efficient reductant for purified soluble DβM. We have now shown that 6-AAscH₂ is a very weak substrate for membrane-bound DβM as well (see “Results”). However, 6-AAscH₂ was found to be a good external reductant for membrane-bound DβM in standard resealed granule ghosts and supports the DA to NE conversion with an efficiency only slightly lower than that of AscH₂ (Fig. 4). In addition, although 6-AAscH₂ is structurally remarkably similar to DA, these results clearly demonstrate that it is not actively or passively taken up into resealed granule ghosts. These results strongly suggest that the specificity of the reduction site of purified DβM must be significantly different from the specificity of the external reduction site of the resealed granule ghost membrane. Therefore, it could be concluded that although membrane-bound DβM in resealed granule ghosts appear to be exclusively reduced by the external reductant, the external reductant may not directly interact with the reduction site of the enzyme from the cytosolic phase of the granule. This conclusion is further substantiated by our preliminary observation that the efficient artificial chromophoric DβM reductant, N,N-dimethyl-1,4-phenylenediamine (35), does not support the DA to NE conversion in standard resealed granule ghosts from the external phase.

Since DA is a known weak reductant for purified DβM (48), it is possible that the high rate of DA to NE conversion in the absence of internal AscH₂ may simply be due to the ability of DA to act as a redox mediator between the external reduction site and membrane-bound DβM. On the other hand, our observation that internal IGA does not competitively inhibit the conversion of DA to NE in resealed granule ghosts in the presence or absence of internal AscH₂ (Fig. 4) appears to rule out this possibility because if DA is an effective redox mediator between the external reduction site and membrane-bound DβM, this process must also be competitively inhibited by IGA, since IGA has been shown to compete with DA for the reduction site of membrane-bound DβM (see “Results”). However, this possibility was further examined by replacing DA in several key experiments with the reductively inactive well characterized alternate DβM substrate, tyramine. The remarkable similar outcome of these key experiments to the corresponding experiments with DA clearly rules out the possibility that under internal AscH₂-depleted conditions DA acts as a redox mediator between the external reduction site and membrane-bound DβM in resealed granule ghosts.

Taken together, the above results clearly demonstrate that the reducing equivalents for the membrane-bound DβM monooxygenation reaction in resealed chromaffin granule ghosts are exclusively provided by the external reductant. These results are in general agreement with the previous proposal of Huyge and Klinman (14). More interestingly, in contrast to the generally accepted notion, the results presented here also strongly suggest that the intravesicular solution AscH₂ (or DA) may not directly reduce or mediate the reduction of membrane-bound DβM in resealed chromaffin granule ghosts. Since the specificity of the reduction site of membrane-bound DβM appears to be different from the specificity of the extragranular reduction site in the membrane and the fact that membrane-bound DβM is anchored to the internal surface of the granule membrane through a short signal sequence (49), it is clear that membrane-bound DβM could not be directly reduced from the external phase. Therefore, we conclude that there must be an electron acceptor site in the external phase of the granule membrane which is capable of accepting and transferring the external reducing equivalents directly or indirectly to the reduction site of the membrane-bound DβM without using intragranular AscH₂ (or DA) as an intermediate.

Numerous previous studies have demonstrated that the transmembrane hemoprotein, cyt b₅₆₃, is capable of transferring external reducing equivalents through the membrane to the interior of the granule (19–22). Furthermore, it has been proposed that the cyt b₅₆₃-mediated reduction of DβM in chromaffin granules requires intragranular AscH₂ as a mediator (50, 51). However, the above results strongly suggest that internal solution AscH₂ (or DA) is not involved in the mediation or the direct reduction of membrane-bound DβM in resealed chromaffin granule ghosts. Therefore, if cyt b₅₆₃ is the external reducing equivalent acceptor, then the reducing equivalents must be transferred to the reduction site of membrane-bound DβM either directly or through an unidentified intermediate electron carrier without using intragranular solution AscH₂ (or DA) as an intermediate. Since this electron transfer process appears to be highly efficient and requires tight functional coupling between cyt b₅₆₃ and the reduction site of membrane-bound DβM, it is very unlikely that the soluble DβM in the granule is also reduced by this same cyt b₅₆₃-mediated electron transport system. Therefore, it is possible that while membrane-bound DβM in chromaffin granules may be reduced through an unidentified external reduction site in the membrane, the soluble enzyme is reduced by intragranular AscH₂ and constant internal AscH₂ levels are maintained through the cyt b₅₆₃-mediated AscH₂ regenerating system as previously proposed (19–22). An attractive alternate possibility would be that the membrane-bound DβM is the only physiologically functional form which is reduced through cyt b₅₆₃ directly or using a mediator other than solution AscH₂ (or DA), and the soluble form is only designated as a disposal form of the enzyme (not recovered and disposed through exocytosis) which might not be functional under physiological conditions. The physiological function of intragranular AscH₂ may be to provide a reducing environment inside the chromaffin granules to protect catecholamines from auto-oxidation. While further experimental evidence is certainly necessary to distinguish between these possibilities, the understanding of the topological arrangement of the various proteins and co-factors in the granule membrane, physiological function(s) of other redox-active proteins or small molecules in the granule matrix, and the role of soluble DβM in intact chromaffin granules are of prime importance.

Acknowledgment—We gratefully thank Dr. Mike Gangel of USDA, Wellington Quality Meats Inc., Wellington, KS, for his aid in obtaining fresh adrenal glands.

REFERENCES
1. Winkler, H., Hortnagl, H., and Smith, A. D. (1970) Biochem. J. 118, 303-310
2. Sabban, E. L., Greene, L. A., and Goldstein, M. (1983) J. Biol. Chem. 258, 7812–7818
3. Slater, E. P., Zaremba, S., and Hogue-Angeletti, R. A. (1981) Arch. Biochem. Biophys. 211, 288–296
4. Ingelsbrensen, O. C., Terland, O., and Flatmark, T. (1980) Biochim. Biophys. Acta 628, 182–189
5. Phillips, J. H. (1982) Neuroscience 7, 1595–1609
6. Diliberto, E. J., Jr., and Allen, P. L. (1960) Mol. Pharmacol. 17, 421–426

Recent experimental findings have demonstrated that the membrane-bound and soluble forms of DβM are derived from one primary translation product. The membrane-bound form of the enzyme is proposed to convert to the soluble form by the proteolytic cleavage of the membrane anchoring signal sequence suggesting that the soluble enzyme is a secondary metabolic product of the membrane-bound enzyme which is disposed of during exocytosis. Therefore, it could be hypothesized that the soluble form of DβM is a disposable form of the enzyme which is not functional under physiological conditions. This hypothesis is further substantiated by the presence of soluble DβM-inhibitory indigene thiols in the chromaffin granule matrix (for example, see Ref. 52).
Reduction of DβM in Chromaffin Granule Ghosts

27524

7. Diliberto, E. J., Jr., and Allen, P. L. (1981) J. Biol. Chem. 256, 3385–3393
8. Skotland, T., and Ljones, T. (1980) Biochim. Biophys. Acta 630, 30–35
9. Foyer, C. H., and Halliwell, B. (1977) Phytochemistry 61, 1347–1350
10. Mapson, L. W., and Moustafa, E. M. (1976) Biochem. J. 62, 248–259
11. Tirrell, J. G., and Westhead, E. W. (1979) Neuroscience 4, 181–186
12. Meniniti, F. S., Knoth, J., and Diliberto, E. J., Jr. (1986) J. Biol. Chem. 261, 16903–16908
13. Levine, M. Marita, K., Heldman, E., and Pollard, H. B. (1985) J. Biol. Chem. 260, 15598–15603
14. Huyghe, B. G., and Klinman, J. P. (1991) J. Biol. Chem. 266, 11544–11550
15. Levine, M., and Pollard, H. B. (1983) FEBS Lett. 158, 134–138
16. Flatmark, T., and Terland, O. (1971) Biochim. Biophys. Acta 253, 487–491
17. Terland, O., Silsan, T., and Flatmark, T. (1974) Biochim. Biophys. Acta 359, 253–256
18. Terland, O., and Flatmark, T. (1980) Biochim. Biophys. Acta 597, 318–330
19. Njus, D., Knoth, J., Cook, C., and Kelley, D. M. (1983) J. Biol. Chem. 258, 27–30
20. Kelley, D. M., and Njus, D. (1986) J. Biol. Chem. 261, 6429–6432
21. Wakefield, L. M., Cass, A. E. G., and Radda, G. K. (1986) J. Biol. Chem. 261, 9739–9745
22. Beers, M. F., Johnson, R. G., and Scarpa, A. (1986) J. Biol. Chem. 261, 2529–2535
23. Levin, M. (1986) J. Biol. Chem. 261, 7347–7356
24. Cororan, J. J., Wilson, S. P., and Kirshner, N. (1984) J. Biol. Chem. 259, 6206–6214
25. J Johnson, R. G., Pfister, D., Carty, S. E., and Scarpa, A. (1979) J. Biol. Chem. 254, 10963–10972
26. Herman, H. H., Wimalasena, K., Fowler, L. C., Beard, C. A., and May, S. W. (1988) J. Biol. Chem. 263, 666–672
27. Wimalasena, K. Herman, H. H., and May, S. W. (1989) J. Biol. Chem. 264, 124–130
28. Johnson, R. G., and Scarpa, A. (1976) J. Biol. Chem. 251, 2189–2191
29. Salama, G., Johnson, R. G., and Scarpa, A. (1980) J. Gen. Physiol. 75, 109–140
30. Mitchell, P. (1979) Science 206, 1148–1159
31. Hannadek, G. J., Ries, E. A., and Njus, D. (1985) Biochemistry 24, 2640–2644
32. Njus, D., Knoth, J., and Zallakian, M. (1981) Curr. Top. Bioenerg. 11, 107–147
33. Ahn, N. G., and Klinman, J. P. (1987) J. Biol. Chem. 262, 1485–1492
34. Irvine, S. (1940) U. S. Patent No. 2,206,374
35. Wimalasena, K., and Wimalasena, D. S. (1991) Anal. Biochem. 197, 353–361
36. Wimalasena, K., Dharmasena, S., and Wimalasena, D. S. (1994) Biochem. Biophys. Res. Commun. 203, 1471–1476
37. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
38. Kirshner, N. (1962) J. Biol. Chem. 237, 231–231
39. Njus, D., and Radda, G. K. (1979) Biochem. J. 180, 579–585
40. Smith, A. D., and Winkler, H. (1976) Biochem. J. 103, 480–482
41. Skotland, T., and Ljones, T. (1977) Int. J. Pept. Protein Res. 10, 311–314
42. Wimalasena, K., and Dharmasena, S. (1994) Biochem. Biophys. Res. Commun. 203, 1471–1476
43. Phillips, J. H. (1974) Biochem. J. 144, 319–325
44. Ahn, N. G., and Klinman, J. P. (1989) J. Biol. Chem. 264, 12259–12265
45. Grouselle, M., and Phillips, J. H. (1983) Biochem. J. 202, 759–770
46. Tollin, G., Meyer, T. E., Cusanovich, M. A., Curir, P., and Marchesini, A. (1993) Biochim. Biophys. Acta 1183, 309–314
47. Perin, M. S., Fried, V. A., Slaughter, C. A., and Sudhof, T. C. (1988) EMBO J. 7, 2697–2703
48. Stewart, L. C., and Klinman, J. P. (1987) Biochemistry 26, 5302–5309
49. Lewis, E. J., and Asnani, L. P. (1992) J. Biol. Chem. 267, 494–500
50. Sirivastava, M., Duong, L. T., and Fleming, P. J. (1984) J. Biol. Chem. 259, 8072–8075
51. Kent, U. M., and Fleming, P. J. (1987) J. Biol. Chem. 262, 8147–8178
52. Rosenberg, R. C., and Lowenberg, W. (1980) Essays in Neurochemistry and Neuropharmacology, Vol. 4, pp. 191–192, John Wiley, New York
The Reduction of Membrane-bound Dopamine \( \beta \)-Monooxygenase in Resealed Chromaffin Granule Ghosts: IS INTRAGranular ASCORBIC ACID A MEDIATOR FOR EXTRAGranular REDUCING EQUIVALENTS?
Kandatege Wimalasena and D. Shyamali Wimalasena

*J. Biol. Chem.* 1995, 270:27516-27524.
doi: 10.1074/jbc.270.46.27516

Access the most updated version of this article at [http://www.jbc.org/content/270/46/27516](http://www.jbc.org/content/270/46/27516)

**Alerts:**
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 51 references, 26 of which can be accessed free at [http://www.jbc.org/content/270/46/27516.full.html#ref-list-1](http://www.jbc.org/content/270/46/27516.full.html#ref-list-1)