Electron Transfer across Posterior Pituitary Neurosecretory Vesicle Membranes*

James T. Russell†, Mark Levine§, and David Njus¶

From the †Laboratory of Neurochemistry and Neuroimmunology, National Institute of Child Health and Human Development and the Laboratory of Cell Biology, National Institute of Mental Health and the ¶Laboratory of Cell Biology and Genetics, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892 and the §Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202

Secretory vesicles from the neurohypophysis have a transmembrane electron carrier very similar to that found in adrenal medullary chromaffin granules. Two different tests show that ascorbic acid contained in the vesicles will reduce an external electron acceptor. First, reduction of cytochrome c or ferricyanide in the medium by a neurosecretory vesicle suspension can be followed spectrophotometrically. Second, the membrane potential (inside positive) generated by electron transfer can be monitored using the membrane potential-sensitive optical probe Oxonol VI. As in chromaffin granules, this electron transfer is probably mediated by cytochrome b₅₆₁. It may function to regenerate internal ascorbic acid and to provide reducing equivalents needed by the intravesicular amidating enzyme.

The neurosecretory vesicles in the nerve endings of the posterior pituitary are responsible for the storage and secretion mainly of vasopressin and oxytocin. In recent years, it has become apparent that these vesicles are metabolically quite active. Like many secretory vesicles, the vesicle membrane possesses an inwardly directed H⁺-translocating ATPase (1, 2). The membranes also contain a cytochrome b₅₆₁ spectrophotometrically and immunologically similar to the one found in adrenal medullary chromaffin vesicles (3). In addition, the neurosecretory vesicles contain an amidating enzyme which converts the COOH- terminus of vasopressin and oxytocin into an amide group during processing of the prohormone (4-6), a modification which is required for the biological activity of the peptides (8). The amidating enzyme contains copper and uses ascorbic acid as an electron donor (4-7).

Some insight into the functions of the ATPase and cytochrome b₅₆₁ may be obtained by comparing the neurosecretory vesicle with the chromaffin vesicle, the catecholamine storage vesicle of the adrenal medulla. Chromaffin vesicles also have a copper-containing enzyme, dopamine β-hydroxylase, which uses ascorbic acid as an electron donor (9, 10). Eipper et al. (5, 6) have stressed the parallels between the amidating enzyme found in the pituitary and dopamine β-hydroxylase found in the adrenal (see also Ref. 3). In the chromaffin vesicle, cytochrome b₅₆₁ is thought to be involved in transfer-

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1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DCIP, 2,6-dichloroindophenol; Pipes, piperazine-N,N′-bis(2-ethanesulfonic acid); HPLC, high-performance liquid chromatography.
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Assay of Ascorbic Acid—HPLC analysis of ascorbic acid was performed exactly as described previously (15, 16). Ascorbic acid was separated using a 5-μm radial compression C₁₈ column and quantified using an ultraviolet detector set at 254 nm (Waters Associates). The mobile phase was 0.2% dicyclohexylamine phosphate run at 3.0 ml/min and 1500 p.s.i. Ascorbic acid eluted at 5.6 min.

Materials—Oxonol VI (bis(3-propyl-5-oxoisoxazol-4-yl)penta-methine oxonol) was obtained from Molecular Probes, Junction City, OR and ascorbate oxidase was from Boehringer Mannheim. S-15 (N-3-tert-butyl-5-chlorosalicyl)-2-chloro-4-nitroanilide) was a gift from Dr. P. G. Heytler, E. I. du Pont de Nemours & Co., Wilmington, DE. Cytochrome c (Type VI) was from Sigma. Protein was assayed using fluorescamine (17).

RESULTS

Reduction of External Electron Acceptor—Cytochrome c is slowly reduced by suspensions of intact neurosecretory vesicles as indicated by the increase in absorbance at 550 nm (Fig. 1). The rate of reduction depends upon the composition of the medium: in 140 mM potassium gluconate, between 1.1 and 1.6 neq of cytochrome c are reduced per min per mg of vesicle protein. In 130 mM K₂SO₄, the rate is only about 0.5 neq/min/mg. In these experiments, KCN was added to prevent reduced cytochrome c from being reoxidized by cytochrome oxidase which might be present in mitochondrial membranes contaminating the neurosecretory vesicle preparation. Ascorbate oxidase was also added 5 min prior to the measurement to ensure that cytochrome c was not being reduced by ascorbic acid external to the vesicles. That cytochrome c is being reduced indirectly by an intravesicular electron donor is shown by the difference in reduction caused by intact and lysed vesicles. The rate of cytochrome c reduction does not depend on the pH of the medium when the vesicles are intact (Fig. 2C). When the vesicles are lysed with Triton X-100, however, the rate of reduction is markedly slowed at pH 5.5 (Fig. 2D). This is expected if ascorbic acid is the electron donor because the reduction potential of the electron couple ascorbate:semidehydroascorbate is shifted toward higher values at more acidic pH (18, 19).

To show that the vesicles contain ascorbic acid and that cytochrome c is being reduced by this intravesicular ascorbic acid, we examined cytochrome c reduction by lysed vesicles (Fig. 2A). Whereas ascorbate oxidase does not affect reduction of cytochrome c by intact vesicles (Fig. 1), it abolishes over 80% of the reducing capacity of vesicles lysed by treatment with 0.2% Triton X-100. This suggests that ascorbic acid sequestered inside the vesicles is responsible for most and perhaps all of the cytochrome c reduction.

Ferricyanide is also reduced by neurosecretory vesicles (Fig. 3) but at a much faster rate (about 100 neq/min/mg of protein). Moreover, the rate of ferricyanide reduction in sul-

FIG. 1. Reduction of cytochrome c by neurosecretory vesicles. Vesicles (430 μg of protein) were suspended in 1 ml of 140 mM potassium gluconate (a) or 130 mM K₂SO₄ (b), in both cases buffered with 10 mM Pipes/Tris, pH 6.5, containing 1 mM EDTA, 0.6 mM KCN, and 10 μg of ascorbate oxidase. Cytochrome c reduction was initiated by adding 25 nmol of cytochrome c at t = 0 (arrow).

FIG. 2. Reduction of cytochrome c by intact and lysed neurosecretory vesicles and ascorbic acid. In panel A, vesicles (430 μg of protein) were suspended in 1 ml of medium buffered with 10 mM Pipes/Tris, pH 6.5, containing 1 mM EDTA, 0.6 mM KCN, and 0.2% Triton X-100. In panel B, ascorbate (30 nmol) was added instead of vesicles. In both panels, cytochrome c reduction was initiated by adding 25 nmol of cytochrome c at t = 0 (arrow). Media were 140 mM potassium gluconate (a), 130 mM K₂SO₄ (b), and 140 mM potassium gluconate, 10 μg/ml ascorbate oxidase (c). In panels C and D, neurosecretory vesicles (592 μg of protein) were suspended in 1 ml of 140 mM K gluconate, 1 mM EDTA, 0.6 mM KCN, 10 mM Pipes/Tris at the indicated pH. In panel D, vesicles were lysed by including 0.2% Triton X-100 in the medium. In both panels, 25 nmol of cytochrome c were added at the arrow.
Ferricyanide/mg of vesicle protein. Vesicles lysed by treatment with 0.2% Triton X-100 reduce a comparable amount of ferricyanide (Fig. 4). Moreover, ascorbate oxidase destroys most of the ferricyanide-reducing capacity of the lysed but not of the intact vesicles, indicating that the reduction is attributable to intravesicular ascorbic acid.

**Ascorbic Acid Content of Neurosecretory Vesicles**—The ascorbic acid content of the neurosecretory vesicles was estimated by four different methods: 1) the rate at which lysed vesicles reduce cytochrome c, 2) the capacity of lysed vesicles to reduce ferricyanide, 3) the capacity of intact vesicles to reduce DCIP, and 4) direct measurement of extracted ascorbic acid by high-performance liquid chromatography. We compared the rates of cytochrome c reduction caused by lysed neurosecretory vesicles and by known amounts of ascorbic acid (Fig. 2, A and B). Since the rate of cytochrome c reduction varies with ascorbate concentration (data not shown), the rate can be used to estimate the amount of ascorbate present in the vesicle lysate. In Fig. 2, lysed vesicles (430 μg of protein) caused a rate of reduction comparable to 30 nmol of ascorbic acid. This equivalence is found in both gluconate and sulfate media. Therefore, the rate of cytochrome c reduction indicates an ascorbate content of 70 nmol/mg of protein. In two experiments of this type, the average ascorbate content of the vesicle lysate was 48 ± 22 neq/mg of protein. The traces in Fig. 3 show that intact vesicles reduce 150 nmol of ferricyanide/mg of vesicle protein. Lysed vesicles reduced 115 ± 9 nmol/mg as shown by the slope in Fig. 4. These values correspond to ascorbate contents of 75 and 58 nmol/mg, respectively. The extent of DCIP reduction was measured by adding neurosecretory vesicles (364 μg of protein) to 25 nmol of DCIP and measuring the decrease in absorbance at 600 nm. Intact vesicles reduce 55 nmol of DCIP/mg of vesicle protein, suggesting that the vesicles contain an equivalent amount of ascorbate. In four preparations of neurosecretory vesicles, the ascorbic acid content was 19.4 ± 6.0 nmol/mg of protein (average ± S.D.) as assayed by high-performance liquid chromatography. The different techniques summarized in Table I all demonstrate the presence of ascorbic acid within the neurosecretory vesicles. The value obtained by HPLC is considerably lower than those estimated by cytochrome c, ferricyanide, or DCIP reduction. This discrepancy may be caused by incomplete extraction of ascorbic acid or by losses occurring during the extraction and assay procedure. In any case, the ascorbate content is between 20 and 70 nmol/mg of protein. Using the value of 3.02 μl/mg of protein for the intravesicular volume (1, 2), this translates into an internal ascorbic acid concentration between 7 and 20 mM.

Membrane Potential Associated with Electron Transfer—Reduction of external electron acceptor by internal ascorbic acid

**Table I**

| Method of measurement | Reducing capacity (neq/mg protein) |
|-----------------------|----------------------------------|
| Ascorbate content     | 39                               |
| DCIP reduction        | 110                              |
| Ferricyanide reduction| 115                              |
| Cytochrome c reduction| 97                               |
acid must involve electron transfer across the vesicle membrane. This charge transfer should depend on the membrane potential. By fueling the inwardly directed $H^+$-translocating ATPase, MgATP creates a membrane potential (inside positive) which should oppose the flow of electrons from internal ascorbate to external ferricyanide under our experimental conditions. In accordance with this, MgATP slows the rate of ferricyanide reduction in intact vesicles. The evidence that the reducing agent is internal ascorbic acid is as follows. First, cytochrome c and ferricyanide are reduced by intact vesicles treated with ascorbate oxidase and by the vesicle lysate in the absence of the enzyme, but not by the vesicle lysate treated with ascorbate oxidase (Figs. 2 and 4). Second, the amount of ferricyanide reduced by both intact and lysed neurosecretory vesicles correlates roughly with the ascorbic acid content of the vesicles (Fig. 3 and Table I). Third, the rate of cytochrome $c$ reduction by lysed vesicles is consistent with the ascorbate content of the vesicles (Table I), and the pH dependence of that rate in the vesicle lysate is consistent with the pH dependence of the midpoint reduction potential of ascorbic acid (Fig. 2). Fourth, intact vesicles oxidized with DCIP will not reduce cytochrome $c$, and the
Adding the electron acceptor ferricyanide elicits a membrane potential by the fact that the change is abolished by the uncoupler 2,4-dinitrophenol (DCIP). Ferricyanide is an effective electron acceptor, but it cannot reduce cytochrome c. Thus, the question remains as to whether ascorbic acid is present at high concentrations within neurosecretory vesicles. The amount of ascorbate in the vesicles estimated from the reducing capacity (cytochrome c, ferricyanide, and DCIP) is between 45 and 60 nmol/mg of vesicle protein (Table I). Quantitation of ascorbic acid using an HPLC method yields a value of 19.4 nmol/mg of vesicle protein, which is significantly lower than that estimated by the other techniques. This is probably caused by incomplete extraction of ascorbic acid from the vesicles or by oxidation occurring during the extraction procedure. The possibility that the vesicles contain a significant concentration of another reducing agent is unlikely because more than 80% of the reducing power of the vesicle lysate was abolished by treatment with ascorbate oxidase, and only one peak (ascorbic acid) was seen when the vesicle lysate was subjected to HPLC with electrochemical detection. However, the presence of an intravesicular reducing system that is responsible for cycling ascorbic acid cannot be discounted. Ascorbic acid is not taken up by neurosecretory vesicles. Thus, the question remains as to whether ascorbic acid is packaged in nascent vesicles or is accumulated in a permeable form (perhaps dehydroascorbic acid) and later reduced within the vesicle by electron transfer.

Several factors affect the rate of electron transfer across the neurosecretory vesicle membrane. First, the composition of the medium seems to have an important effect on the rate of cytochrome c reduction. This is probably attributable to anion binding by cytochrome c (20), since the medium affects the rate, whether the electrons are contributed by intact or membrane c. The membrane potential also influences the rate of electron transfer (Fig. 5). Because electron transfer from internal ascorbate to external cytochrome c will generate a membrane potential (inside positive), this membrane potential will inhibit electron transfer. Dissipating the membrane potential by adding the uncoupler S-13 relaxes this constraint on electron transfer and accelerates the rate. MgATP increases the membrane potential (inside positive) and hence slows electron transfer.

Since the membrane potential affects the rate of electron transfer, electron efflux must be electrogenic and should itself create a membrane potential (inside positive). The membrane potential probe Oxonol VI confirms this expectation (Fig. 6). Adding the electron acceptor ferricyanide elicits a membrane potential in neurosecretory vesicles. The electron donor ferrocyanide does not. That the absorbance change reflects an increase in internal positive membrane potential is indicated by the fact that the change is abolished by the uncoupler S-13. Finally, that this response requires an internal electron donor is indicated by the fact that it cannot be elicited from DCIP-treated vesicles.

We have postulated that cytochrome b561 is responsible for the electron transfer activity. The reasons for this are several. First, this cytochrome in chromaffin vesicles has a midpoint potential of +100 to +140 mV (21, 22). This is the appropriate potential for an electron carrier mediating electron flow between ascorbic acid (+110 mV at the intravesicular pH of 5.5) and cytochrome c (+240 mV). Second, both chromaffin vesicles and neurosecretory vesicles have cytochrome b561 (3), and both vesicles catalyze electron transfer. Finally, although the rates of electron transfer in the two membranes have been measured under different conditions, the rates correlate with the relative amounts of cytochrome b561 in the membranes. Chromaffin vesicle membranes contain 7 nmol of cytochrome b561/mg of membrane protein, while neurosecretory vesicle membranes contain about 1 nmol/mg (3). Chromaffin vesicle ghosts containing 30 nm ascorbic acid reduce 19 μM cytochrome c at a rate of about 20 nmol/min/mg of membrane protein. Neurosecretory vesicles catalyze electron transfer at about one-seventh this rate (Fig. 1) given that only 40% of the vesicle protein is in the membrane.

Neurosecretory vesicles offer some advantages over chromaffin vesicles as a system in which to investigate electron transfer. The catecholamines, stored at massive concentrations in chromaffin vesicles, complicate studies of electron transfer. It is particularly difficult to observe ferricyanide reduction by chromaffin vesicles because catecholamines leaking out of the vesicles reduce ferricyanide directly. Studies of electron transfer in neurosecretory vesicles, however, are free of complications caused by this additional reductant.

The role of the electron transfer system in vivo may be to provide reducing equivalents for the intravesicular aming enzyme. This enzyme uses ascorbic acid as an electron donor, so electrons must be imported from a cytosolic reductant to regenerate the intravesicular ascorbate. This inward electron transfer will be promoted by the membrane potential (inside positive) established by the H+-translocating ATPase. Moreover, because the midpoint reduction potential of ascorbic acid is pH-dependent, ascorbic acid will be more stable at the lower pH prevailing in the vesicle interior so that it will, in fact, take electrons from cytosolic ascorbic acid. Since many biologically important peptide hormones are carboxyl-terminally amidated, this system may also be found in other peptidergic vesicles. Indeed, cytochrome b561 has been identified in pituitary intermediate lobe secretory vesicles as well as in some regions of the brain (3). Thus, this system appears to be common to both peptidergic and aminergic vesicles and perhaps should be sought in other types of vesicles as well.

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