Electron Transfer across the Chromaffin Granule Membrane*

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Membrane vesicles (ghosts) containing ascorbic acid were prepared from bovine chromaffin granules. When ferricyanide or ferricytochrome c were added to the external medium, a membrane potential (internal positive) developed across the ghost membrane. This membrane potential could not be elicited from ascorbate-free ghosts or by ferricyanide added instead of ferricytochrome c. These results indicate that the chromaffin-granule membrane has a transmembrane electron carrier with a midpoint potential between that of ascorbate (+85 mV) and that of cytochrome c (+255 mV). The most likely candidate is cytochrome b-561 (+140 mV).

Catecholamine storage vesicles in adrenergic neurons and in the chromaffin cells of the adrenal medulla must take up reducing equivalents for two purposes. First, the easily oxidized catecholamines must be kept in a reduced state. Second, the synthesis of norepinephrine by the intravesicular enzyme dopamine P-hydroxylase requires a reducing agent as a cofactor. Ascorbic acid provides an intravesicular store of reducing equivalents for two purposes. First, the easily oxidized catecholamines must be kept in a reduced state, dehydroascorbic acid. Ascorbic acid appears to permeate the chromaffin granule membrane only in its fully oxidized form, dehydroascorbate (Tirrell and Weathread, 1979), so reducing equivalents apparently are not taken into the vesicles in the form of ascorbic acid.

We have suggested before that cytochrome b-561 may be a transmembrane electron carrier mediating the uptake of reducing equivalents in the form of electrons (Njus et al., 1981). Cytochrome b-561, also known as chromomembrin B (Apps et al., 1980), is a transmembrane protein (Abbs and Phillips, 1980) and the second most abundant protein in the chromaffin granule membrane. It is also found in sympathetic nerve dense granules (Johnson and Scarpa, 1981). To show that chromaffin granule membranes do have a transmembrane electron carrier, we report here on electron transfer from internal ascorbic acid to an external electron acceptor.

**EXPERIMENTAL PROCEDURES**

Chromaffin granules were prepared from bovine adrenal medulla as described by Kirshner (1962) except that 0.5 M sucrose, 10 mM Hepes, pH 7.0, was used as the isolation medium. Ascorbate-loaded ghosts were prepared by resuspending the granules in 0.1 M ascorbate, 0.15 M Tris/phosphate, pH 7.0, and adding 1/3 volume of 30% glycerol (v/v) in 0.2 M Tris/phosphate, pH 7.0. After incubating for 20 min at 4 °C, the membranes were sedimented by centrifugation at 25,000 × g for 30 min at 4 °C. The membranes were resuspended in 0.1 M ascorbate, 0.15 M Tris/phosphate, pH 7.0, and purified on a discontinuous Ficoll density gradient (Njus and Radda, 1979). All experiments were completed within 18 h of the collection of the ghosts.

Electron transfer from ascorbate trapped within the ghosts to ferricyanide added externally was observed as an increase in the membrane potential (inside positive). To monitor the membrane potential, we used the fluorescent probe ANS. Fluorescence was measured at 480 nm using a Perkin-Elmer Model 204S fluorescence spectrophotometer with the excitation wavelength set at 380 nm. A positive membrane potential promotes ANS binding to the ghost membranes and causes a consequent increase in fluorescence (Njus et al., 1977). Upon addition of KFCN (0.5 mM, final concentration), ANS fluorescence enhancement is observed (Fig. 1A). Absorption by ferricyanide causes a slight instant drop in fluorescence. The subsequent slow increase is the ANS response to the membrane potential. That this indeed reflects a change in the membrane potential is indicated by the fact that the response is abolished by the protonophore FCCP (Fig. 1B). KFCN does not elicit the response (Fig. 1C) proving that the membrane potential change is caused by electron transfer rather than by a K+ diffusion potential. The ferricyanide-dependent fluorescence enhancement is not observed if ghosts are prepared using 0.2 M Tris/phosphate, pH 7.0, instead of the ascorbate-containing buffer. This confirms that the internal electron donor is ascorbate and not residual catecholamine in the ghosts.

Ferricytochrome c can replace ferricyanide as the external electron acceptor. Ferricytochrome c was prepared by adding KFCN (20 mM, final concentration) to 10 mM equine cytochrome c. Ferricyanide was removed by dialyzing the solution against 100 volumes of 0.3 M sucrose, 10 mM Hepes/KOH, pH 7.0, changing the dialysis buffer several times. After a final 5-h dialysis, the concentration of the cytochrome solution was calculated from its absorbance at 410 nm using a value of 106 for the millimolar extinction coefficient (Margo- liash and Schejter, 1966). The dialysate was saved and used as a control for ferricyanide still left in the cytochrome preparation. As shown in Fig. 1D, cytochrome c produces an ANS response. Because the cytochrome solution is strongly absorbing, it causes a large absorbance correction. That the slow subsequent increase is a response to the membrane potential is demonstrated by the fact that it is abolished by FCCP. The dialysate did not elicit a fluorescence response proving that the electron acceptor is cytochrome c rather than residual ferricyanide.

Ferricyanide-dependent fluorescence response is not inhibited by saturating CO, 1 mM KCN, 0.5 mM NaN3, 5 mM antimycin A, 1 mM salicyldihydrassic acid, or 1 mM 1,10-phenanthroline. At 37 °C, the ANS response to ferricyanide decays rather quickly but this dissipation is much reduced at lower temperatures (Fig. 2).

Because the membrane potential does not dissipate at 4 °C, it can be measured by the thioyanate distribution method. To do this, we added KS13CN and H2O to a ghost suspension at 4 °C and then sedimented the membranes by centrifugation at 27,000 × g for 15 min. External and internal SCN- concentrations were calculated from the SCN- activities in the supernatant and pellet, respectively, after making appropriate corrections for external aqueous volume trapped in the pellet (Casey et al., 1977). Membrane potentials, calculated from the SCN- distribution using the Nerst equation (Knoth et al., 1980), confirm the qualitative results obtained using the ANS response (Table I).

Cytochrome c, FCCP, and catalase were purchased from Sigma and ANS (Mg2+ salt) was from Eastman. KS13CN and H2O were

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1The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazinediethanesulfonic acid; A+ ascorbic acid; A- dehydroascorbate; A, dehydroascorbate; ANS, 1-anilinonaphthalene-8-sulfonic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Tris, (tris(hydroxymethyl)methylamine.
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**Fig. 1.** ANS fluorescence enhancement caused by electron transfer. For each trace, 25 μl of ascorbate-loaded ghosts (0.21 mg of protein) were mixed with 2 ml of 0.4 M sucrose, 10 mM Hepes, pH 7.0. At t = 0, 10 μl of 1 mM ANS was added and fluorescence recorded at 7°C as described in the text. Ten-microliter aliquots of 100 mM K₃Fe(CN)₆, 100 mM K₄Fe(CN)₉, 500 μM FCCP, and 2.9 mM ferricytochrome c were added at the times indicated.

**DISCUSSION**

We used two different techniques, the “ANS response” and the thiocyanate distribution method, to measure the membrane potential created by electron transfer. The ANS response is a sensitive and convenient assay and monitors the kinetics of electron transfer. This fluorescence assay, however, is qualitative and can be affected by other environmental changes. The thiocyanate distribution technique, by contrast, provides a quantitative measure of the steady state membrane potential and is less sensitive to other factors.

The membrane potential is a good indicator of transmembrane electron transfer because it requires that the oxidant and reductant be separated by a well sealed membrane. Alternative assays, such as direct measurement of ferricyanide or cytochrome c reduction or ascorbate oxidation, are not definitive. For example, ascorbate-loaded ghosts cause a slow reduction of cytochrome c as measured by the increase in absorbance at 550 nm. However, a similar slow absorbance change is produced by low concentrations of free ascorbate. Thus, using the absorbance assay, it is difficult to tell whether reduction is caused by electron transfer or by a low concentration of ascorbate accumulated in the external space.

The chromaffin granule membrane is quite impermeable to ascorbate. Tirrell and Westhead (1979) reported that [³¹C]ascorbate does not permeate into intact chromaffin granules. We have obtained similar results using chromaffin granule ghosts. The fact that ascorbate oxidation generates a membrane potential also indicates that the chromaffin granule membrane is impermeable to ascorbate. Ghosts retain internal ascorbate for at least 6 h after preparation.

We tested two electron acceptors, ferricyanide and ferricytochrome c. Ferricyanide is the less satisfactory electron acceptor because it is reduced by catecholamines contaminating...
Ten-microliter aliquots of 500 p~ FCCP in ethanol or ethanol alone have also speculated that cytochrome 6-561 may act as a chrome b-561) serves this function. Wakefield Therefore, all of the cytochrome point reduction potential of +380 mV at pH 7.0 (Clark, 1960). Prevailing in our experiments (Clark, 1960). Cytochrome c, by transmembrane electron shuttle.

The midpoint potential of ferricyanide is +360 mV at zero pH (Margoliash and Schejter, 1966) and does not oxidize catecholamines. Therefore, all of the cytochrome c is available to serve as an electron acceptor in the electron transfer reaction.

Since the electron carrier in the chromaffin granule membrane donates electrons to cytochrome c, its midpoint potential should be less than +255 mV. At the pH prevailing inside the chromaffin granule ghosts (pH ~ 6.2), the midpoint potential of ascorbate is +85 mV (Clark, 1960). Consequently, the electron carrier in the chromaffin granule membrane should have a midpoint potential between +85 and +255 mV. This carrier is probably cytochrome b-561. The cytochrome is oxidized by ferricyanide (Flatmark et al., 1971b), is reduced by ascorbate (Flatmark and Terland, 1971), and has a midpoint potential of +140 mV (Flatmark and Terland, 1971).

Chromaffin granules must take up reducing equivalents for the hydroxylation of dopamine. Dopamine β-hydroxylase uses ascorbate as a 1-electron donor and releases semidehydroascorbate as a product (Skotland and Ijones, 1980; Diliberto and Allen, 1981). Diliberto et al. (1982) have shown that the adrenal medulla possesses a semidehydroascorbate reductase activity, but it is located in the outer mitochondrial membrane. Consequently, it will reduce cytotoxic but not intragranular semidehydroascorbate. To complete the electron transfer pathway, an electron carrier in the chromaffin granule membrane is needed to pass electrons from cytotoxic ascorbate to intragranular semidehydroascorbate (Fig. 3). We suggest that the electron transfer activity demonstrated here (cytochrome b-561) serves this function. Wakefield et al. (1982) have also speculated that cytochrome b-561 may act as a transmembrane electron shuttle.

Electron transfer from cytotoxic ascorbate to intragranular semidehydroascorbate will be driven by the membrane potential and pH gradient across the chromaffin granule membrane. AH− oxidizes in two steps to yield A− and then A−: 

\[ \text{AH}^− \rightarrow \text{A}^− + e^- + \text{H}^+ \rightarrow \text{A} + 2e^- + \text{H}^+ \]

Because the oxidation of ascorbate to semidehydroascorbate involves a proton, the midpoint potential should be pH-dependent. Although the midpoint potential of the AH− /A− pair has not been measured as a function of pH, the midpoint potential of the overall reaction (AH− /A−) is about 50 mV higher at pH 5.7 (the intragranular pH) than at pH 7.0 (the cytosolic pH) (Clark, 1960). Consequently, the pH gradient across the chromaffin granule membrane should represent a force of ~50 mV driving electrons from extragranular ascorbate to intragranular semidehydroascorbate. The membrane potential (inside positive), generated by a proton-translocating ATPase in the chromaffin granule membrane (Njus et al., 1981a), will also promote the inward flow of electrons. Because electron transfer is driven by the pH gradient and membrane potential, this mechanism is a novel extension of the chemiosmotic concept of energy coupling.

It is reasonable to suppose that cytochrome b-561, the second most abundant protein in the chromaffin granule membrane, is involved in the granules’ most important electron transfer function which is providing reducing equivalents for dopamine hydroxylation. By acting as a transmembrane electron shuttle, cytochrome b-561 can fill this role in a manner consistent with the electron transfer activity reported here.

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Table I

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