Rate of Electron Transfer between Cytochrome b_{561} and Extravesicular Ascorbic Acid*

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Cytochrome b_{561} transfers electrons across secretory vesicle membranes in order to regenerate intravesicular ascorbic acid. To show that cytosolic ascorbic acid is kinetically competent to function as the external electron donor for this process, electron transfer rates between cytochrome b_{561} in adrenal medullary chromaffin vesicle membranes and external ascorbate/semidehydroascorbate were measured. The reduction of cytochrome b_{561} by external ascorbate may be measured by a stopped-flow method. The rate constant is 1.2 (± 0.5) × 10^{13} M^{-1} s^{-1} at pH 7.0 and decreases slightly with pH. The rate of oxidation of cytochrome b_{561} by external semidehydroascorbate may be deduced from rates of steady-state electron flow. The rate constant is 1.2 (± 0.5) × 10^{13} M^{-1} s^{-1} at pH 7.0 and decreases strongly with pH. The ratio of the rate constants is consistent with the relative midpoint reduction potentials of cytochrome b_{561} and ascorbate/semidehydroascorbate. These results suggest that cytosolic ascorbate will reduce cytochrome b_{561} rapidly enough to keep the cytochrome in a mostly reduced state and maintain the necessary electron flux into vesicles. This supports the concept that cytochrome b_{561} shuttles electrons from cytosolic ascorbate to intravesicular semidehydroascorbate, thereby ensuring a constant source of reducing equivalents for intravesicular monoxygenases.

In secretory vesicles, such as adrenal medullary chromaffin vesicles, ascorbic acid functions as the electron donor for at least two intravesicular monoxygenases: dopamine β-monooxygenase and peptidyl-glycine a-amidating monooxygenase. The intravesicular store of ascorbate is maintained by ascorbate and cytochrome b_{561}; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; 1, liter.

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EXPERIMENTAL PROCEDURES

Results and Discussion

Reduction of Cytochrome b_{561} by External Ascorbic Acid—The rate of reduction of cytochrome b_{561} by external ascorbic acid may be observed directly using the stopped-flow method (Fig. 1A). In this experiment, ascorbate-free ghosts are employed so the cytochrome is obtained in a completely oxidized state. Upon mixing with ascorbate (AH^{-}), the cytochrome becomes reduced (Fig. 2); the initial rate of cytochrome b_{561} reduction is proportional to the ascorbate concentration at least at low [AH^{-}] (Fig. 3).

The simplest kinetic analysis assumes that this reaction is characterized by a rate constant that is first-order in each substrate.

\[
AH^{-}_{ext} + B_{red} \rightarrow AH_{red} + B_{ox} + H^{+} \quad (I)
\]

Then, the rate constant k_{1} is equal to the initial rate divided by the ascorbate and cytochrome concentrations (Equation 4, Supplementary Material) and is given by the slope of the plots shown in Fig. 3. Values for k_{1} determined in this way increase slightly with pH (Table I).

Although determined from initial rates of cytochrome reduction, k_{1} should also account for the subsequent time course of the stopped-flow experiment (Fig. 2). Since the back reaction (Reaction II) may become important at later times, it must also be considered.

\[
AH_{red} + B_{ox} + H^{+} \rightarrow AH_{ox} + B_{red} \quad (II)
\]

Portions of this paper (including "Experimental Procedures" and Equations 3–15) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

The abbreviations used are: AH^{-}, ascorbate; A_{ox}, semidehydroascorbate; A_{red}, dehydroascorbate; B_{ox}, ferrocyanochrome b_{561}; B_{red}, ferrocyanochrome b_{561}; E_{ox}, midpoint reduction potential of ascorbate/semidehydroascorbate; E_{red}, midpoint reduction potential of ferrocyanochrome b_{561}/ferrocyanochrome b_{561}; Mes, 2-(N-morpholino)ethanesulfonic acid; 1, liter.

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FIG. 1. A, reaction scheme for measuring the rate constant $k_{11}^+$ by the stopped-flow method. B, reaction scheme for measuring $k_{11}^-\,\text{using the steady-state experiment.}$

The rate constant for the back reaction ($k_{11}^-$) may be calculated from the measured value of $k_{11}^+$, because their ratio is equal to the equilibrium constant, which can be calculated from the midpoint reduction potentials of the two redox pairs.

$$\frac{k_{11}^-}{k_{11}^+} = K_{eq} = \exp[(E_{aex} - E_{aex}')(F/RT)] \quad (1)$$

Also important is the disproportionation of the ascorbate free radical which is characterized by a second-order rate constant $k_{disr}$.

$$A^+ + A^- + H^+ \xrightarrow{k_{disr}} AH^- + A \quad (III)$$

When the rate equations for these reactions are numerically integrated, it is apparent that a good fit is obtained at earlier times and divergence occurs only later as accumulated error becomes large (Fig. 2). This shows that the back reaction accounts at least in part for the nonexponential shape of the time course; semidehydroascorbate, generated as cytochrome $b_{553}$ is reduced, slows the reduction rate at later times.

Oxidation of Cytochrome $b_{553}$ by External Semidehydroascorbate—The rate of oxidation of cytochrome $b_{553}$ by external semidehydroascorbate may be determined by a steady-state method (Fig. 1B). In this experiment, ascorbate-loaded ghosts are employed so the cytochrome is obtained in a mostly reduced state. When these ghosts are suspended in an ascorbate-containing medium and ascorbate oxidase is added, cytochrome $b_{553}$ becomes transiently oxidized (Fig. 4B). Ascorbate oxidase oxidizes ascorbate to semidehydroascorbate (Yamazaki and Piette, 1961) and semidehydroascorbate in turn oxidizes cytochrome $b_{553}$ (Kelley and Njus, 1986). The oxidation state of the cytochrome, therefore, reflects steady-state electron flow from internal ascorbate to external ascorbate free radical. The rate constant for electron transfer from internal ascorbate to cytochrome $b_{553}$ is known (Kelley and

Fig. 2. Reduction of cytochrome $b_{553}$ by external ascorbic acid. A, ghosts (final concentration 0.602 mg protein/ml) were mixed by stopped flow with 32 μM ascorbate (upper trace) or 3.2 μM ascorbate (lower trace) at pH 6.0. B, Ghosts (final concentration 0.372 mg protein/ml) were mixed by stopped flow with 14 μM ascorbate (upper trace) or 1.4 μM ascorbate (lower trace) at pH 8.0. In all cases, absorbance was monitored at 561–569 nm and converted to units of μmol/g protein by dividing by the molar extinction coefficient and protein concentration. Broken lines are simulations of these traces calculated by numerical integration of Equations 3, 5, and 6 using applicable values for initial ascorbate and protein concentration.

Fig. 3. Dependence of the initial rate of cytochrome $b_{553}$ reduction on ascorbate concentration. Initial rates of cytochrome $b_{553}$ reduction were determined from traces of the kind shown in Fig. 2. These are plotted as a function of ascorbate concentration for two experiments, one done at pH 6.0 (O) and one done at pH 8.0 (○). The straight lines, fit to the data points by linear regression, have slopes of $4.04 \times 10^{-7}$ l/g-min and $11.2 \times 10^{-7}$ l/g-min, respectively. These slopes are measures of the rate constant $k_{11}^-\,\text{.}$

Ascorbate (μM)
TABLE I
Rate constants for electron transfer reactions

| pH | $k_{-1A}$ (stopped flow exp) | $k_{-1A}$ (steady state exp) | $E^*_m$ (predicted) | $E^*_m$ (pre-dieted) |
|----|-------------------------------|------------------------------|---------------------|---------------------|
| 6.0 | (4.3 ± 0.2) x 10^-7 (3) | 270 ± 100 (3) | 390 | 168 |
| 7.0 | (6.2 ± 2.6) x 10^-7 (4) | 170 ± 70 (4) | 330 | 129 |
| 8.0 | (7.8 ± 2.6) x 10^-7 (3) | 24 ± 3 (3) | 270 | 124 |

Fig. 4. Semidehydroascorbate concentration and cytochrome $b_{562}$ oxidation produced by ascorbate/ascorbate oxidase. A, Semidehydroascorbate concentration was monitored following the addition of ascorbate oxidase (at $t = 0$) to medium containing 600 µM ascorbate at pH 6.0 (●), 7.0 (○) or 8.0 (▲). The absorbance difference (600-420 nm) was recorded and converted to semidehydroascorbate concentration by dividing by the extinction coefficient. Points are averages (± S.D.) for three (● and ▲) or five (○) replicate traces. Lines are fifth-order polynomials fit to the data points. B, cytochrome $b_{562}$ oxidation was monitored following the addition of ascorbate oxidase (at $t = 0$) to ascorbate-loaded ghosts suspended in medium containing 600 µM ascorbate at pH 6.0 (●), 7.0 (○), or 8.0 (▲). The absorbance difference (561-569 nm) was recorded and converted to fractional oxidation ($B_{562}$/B). Points are averages (± S.D.) for three (● and ▲) or five (○) replicate traces. Lines are fifth-order polynomials fit to the data points.

Fig. 5. Values of $k_{-1A}$ calculated by numerical integration of the rate equations. Polynomials describing the time courses of $[B_{562}]$ and $[A^*_m]$, obtained from the data shown in Fig. 4, were used to numerically integrate the time course of the steady-state experiment (see Supplementary Material). A value for $k_{-1A}$ was calculated at each time point using Equation 9.

Njus, 1988, as is the internal ascorbate concentration. Consequently, if the external semidehydroascorbate concentration can be determined, the rate constant for electron transfer from cytochrome $b_{562}$ to external semidehydroascorbate may be calculated.

The semidehydroascorbate concentration may be measured under the same conditions but in the absence of ghosts (Fig. 4A). It is evident that the oxidation of the cytochrome parallels the presence of semidehydroascorbate. The low semidehydroascorbate concentration at pH 6.0 is consistent with rapid disproportionation at low pH (Bielski et al., 1981). The data in Fig. 4A may be used to calculate the disproportionation constant of semidehydroascorbate. Disproportionation constants determined in this way are pH-dependent and in good agreement with those given in the literature (Table II).

To analyze the steady-state experiment, electron transfer reactions on the internal side of the membrane (Reactions IV and V) must be considered along with Reactions I, II, and III.

$$\text{AH}_{\text{m}} + B_{\text{m}} \rightarrow A^*_m + B_{\text{red}} + H^+ \quad \text{(IV)}$$

$$\text{A}^*_m + B_{\text{red}} + H^+ \rightarrow \text{AH}_{\text{m}} + B_{\text{m}} \quad \text{(V)}$$

Then, a value for $k_{-1A}$ may be computed for any time in the steady-state experiment using Equation 9 (see Supplementary Material). Values are reasonably time-independent until later times when accumulated error becomes large (Fig. 5). Table I summarizes values for $k_{-1A}$ determined in this way.

Implications of the Rate Constants—The rate constants $k_{-1A}$ and $k_{1A}$ have been measured by independent methods but

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their ratio should be consistent with Equation 1. From this ratio and the known midpoint potential of the ascorbate/semidehydroascorbate pair (Iyanagi et al., 1984), the midpoint potential for cytochrome b561 can be calculated. As shown in Table I, this agrees well with the measured value of +0.14 V (Flatmark and Terland, 1981; Apps et al., 1984). This consistency is strong evidence that the two methods both give accurate estimates of the rate constants.

The rate constant for the reduction of cytochrome b561 by external ascorbic acid is $4.3 \times 10^{-2}$ l/g.min at pH 6.0 (Table II). The rate constant for the reduction of cytochrome b561 by internal ascorbic acid, measured previously (Kelley and Njus, 1988), is $6.2 \times 10^{-3}$ l/g.min. This indicates that the cytochrome reacts considerably faster with external than with internal ascorbate. Since the cytochrome is designed to transfer electrons from external ascorbate to internal semidehydroascorbate, reduction by external ascorbate may be kinetically favored over reduction by internal ascorbate.

Finally, the redox state of cytochrome b561 in vivo may be considered. To maintain steady state electron flow across the membrane, external ascorbate must reduce cytochrome b561 at the same rate as the cytochrome is oxidized by internal semidehydroascorbate.

$$k_{\text{red}}[A]_e/[B]_i = k_{\text{ox}}[A]_i/[B]_i$$

(2)

The intravesicular semidehydroascorbate concentration is probably about $2 \times 10^{-7}$ M (Diliberto and Allen, 1981). External ascorbate is about 5 mM (Ingebretsen et al., 1980). Using values of 150 l/g.min for $k_{\text{red}}$ (Kelley and Njus, 1988) and 6.2 x $10^{-3}$ l/g.min for $k_{\text{ox}}$, (Table I) implies that $[B]_i/[B]_m$ is about 10. Thus, cytochrome b561 will be maintained in a mostly reduced state, poised to reduce intravesicular semidehydroascorbate as soon as it is formed by the intravesicular monooxygenases. This kinetic analysis, therefore, shows that cytochrome b561 is capable of very efficient regeneration of intravesicular ascorbate. This further substantiates the role of the cytochrome in electron transfer across secretory vesicle membranes and the role of cytosolic ascorbic acid as the external electron donor.

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Supplementary Material for:

RATE OF ELECTRON TRANSFER BETWEEN CYTOCHROME b561 AND EXTRAVESICULAR ASCORBIC ACID

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EXPERIMENTAL PROCEDURES

Chromaffin vesicles were isolated from bovine adrenal medulla as described by Kirshner (1962) except that 0.3 M sucrose, 10 mM Hepes(NaOH), pH 7.0, was used in the isolation medium. Chromaffin vesicle membrane vesicles (ghosts) were prepared by lysing chromaffin vesicles and removing the membranes as described previously (Njus and Radda, 1977). Kelley and Njus, 1985. The lysates contained either 0.2 M Tris(HCl), pH 7.5 (ascorbate-free ghosts) or 0.1 M ascorbate, 0.15 M Tris(HCl), pH 7.0 (ascorbate-loaded ghosts). After rescaling, the ghosts were dialyzed against the lysing medium for 40-48 hr (Kelley and Njus, 1980), purified on a Ficollurex density gradient (Njus and Radda, 1976), and used immediately.

For steady-state flow experiments (Figure 4), cytochrome b561 reduction was followed spectrophotometrically using an Aminco-2icus stopped-flow apparatus equipped with an Aminco DMS spectrophotometer operating in the dual-wavelength mode. One channel of the stopped-flow apparatus contained ascrobate-free ghosts (0.17.8 mM), ascorbate (1.15.8 mM) in 0.5 M sucrose, 250 mM KCN, 10 mM buffer. The other channel contained 0.4 M sucrose, 250 mM KCN, 10 mM buffer and a sto concentration of ascorbate. The buffer was Mes(HCl) at pH 6.0 and Hepes(NaOH) at pH 7.0 and 8.0. Ghosts and ascorbate were mixed by injection, and the absorbance difference (561 nm - 549 nm) was recorded. The amounts of cytochrome reduction were calculated from the absorbance difference using a molar extinction coefficient of 17,730 (Silsand and Flatmark, 1974). This was normalized by dividing by the protein concentration.

For steady-state experiments (Figure a), ascorbic acid (25 µM) was suspended in 0.5 µM potassium fluoride, 250 mM KCN, 10 mM ascorbate, and 10 mM buffer. The buffer was Mes(HCl) at pH 6.0 and Hepes(NaOH) at pH 7.0 and 8.0. Ascorbate was added externally to a concentration of 0.1 µM and the absorbance difference (561 nm - 549 nm) was monitored using an Aminco DMS spectrophotometer. At 1 > 0, ascorbate had a (0.4 units) was added. Finally, ferricyanide (1-3 M final concentration) was added. The fraction of cytochrome in the oxidized state (B) was determined by assuming the initial absorbance to represent 100% reduction (B = 0) and the absorbance following ferrocyanide addition to represent 100% oxidation (B = 1). At least three replicate traces were obtained and corresponding points at 20-second intervals were averaged. A fifth-order polynomial was then fit to these data points. In parallel experiments, the semidehydroascorbate concentration was determined in the absence of ghosts by adding the same concentration of ascorbate to 0.2 M potassium fluoride, 250 mM KCN, 10 mM ascorbate, and 10 mM buffer. The absorbance difference (561 nm - 549 nm) was recorded and recorded in semidehydroascorbate concentration using an extinction coefficient of 1320 M⁻¹ cm⁻¹ (Schuler, 1977). At least three replicate traces were obtained and corresponding points at 10-second intervals were averaged. A fifth-order polynomial was then fit to these data points.

Concentrations of intravesicular and extravesicular ascorbate during the steady-state experiment were calculated by numerical integration of equations 12 and 13 using a time interval of 0.0 sec. Initial concentrations of external and internal ascorbate were taken as
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600 μM and 50 mM, respectively. The latter value approximates the ascorbate concentration actually present in the ghosts at the time of the experiment (Yamagami et al., 1986). The rate constant $k_{A}^{b}$ (Cavanagh et al., 1986) was taken to be 0.102 μmol$^{-1}$ g$^{-1}$ (pH 8) and $k_{A}^{d}$ was computed from equation 1a as described below. $E_{t}^{0}$ (Yamagami et al., 1986) was assigned the appropriate value from Table I. Other parameters were $P_{12} = 0.14$ (V), and $P_{13} = 0.10$ (V) (Mandel and Terlindale, 1974; Apps et al., 1984). $T = 0.033 s$ is the protein (Koch et al., 1981) and $T = 209 s$. The oxidation state of cytochrome b$_{551}$ during the stopped-flow experiment (Figure 1) was computed by numerical integration of equations 3, 5, and 6 using a time interval of 0.05 sec. Values used for $k_{A}^{d}$ (Dellaporta et al., 1981) were 2.112566 x 10$^{-6}$ g$^{-1}$ μmol$^{-1}$ g$^{-1}$ and $0.113 x 10^{-4}$ (pH 7), or $0.179 x 10^{-4}$ (pH 6) s$^{-1}$. Other parameters were evaluated as above. Numerical integrations were performed using a DEC computer program run on a National 8085 microcomputer.

Protein concentrations were determined using the BCA assay (Pierce Chemical Co.). Ascorbate oxidase (2000 units) was prepared as a 20% sonicated solution. Ascorbate oxidase, potassium glutamate, Hepes, Mes, and Tris were obtained from Sigma Chemical Co.

**ANALYSIS OF THE STOPPED-FLOW EXPERIMENT**

When ascorbate is mixed rapidly with ascorbate-free chemiluminescent ghosts (Figure 1A), the internal reactions (IV and V) may be ignored. The rate at which reduced cytochrome b$_{551}$ is reduced is then

$$d [B]/dt = k_{A}^{d} ([A]_B - [B])$$

At $t = 0$, $[A]_B = 0$ and $[B] = B$, where $B$ is the total concentration of cytochrome b$_{551}$. Therefore,

$$k_{A}^{d} = (d [B]/dt)_0$$

Equation 4 implies that the rate constant $k_{A}^{d}$ is equal to the slope of the plot shown in Figure 2.

The rate equations may be integrated to predict the time course of the stopped-flow experiment. The rate at which the semidehydroascorbate concentration changes in this experiment is

$$d [A]_D / dt = k_{A}^{d} ([B]_B - [A]_D)$$

The rate equation for ascorbate, including its disproportionation, is

$$d [A]_B / dt = - k_{A}^{d} ([B]_B - [A]_D) + k_{A}^{d} ([A]_D - [A]_B) + k_{A}^{d} [A]_D$$

Equation 5 implies that the rate constant $k_{A}^{d}$ is equal to the slope of the plot shown in Figure 3.

The rate equations may be integrated to predict the time course of the stopped-flow experiment.

**ANALYSIS OF THE STEADY-STATE EXPERIMENT**

In the steady-state experiment (Figure 1B), both internal reactions (I and II) and internal reactions (IV and V) must be considered. If we assume that electron transfer quickly reaches steady-state, then reactions (I and IV) must prevail at the same rates as reactions (II and V).

$$d [B]/dt = k_{A}^{d} ([A]_B - [B])$$

The cytochrome concentrations when related to the internal volume are much higher than when related to the external volume (ID$_{ex} >>$ ID$_{int}$). To avoid potential confusion, it is convenient to divide equation 7 by $B$, the total cytochrome concentration, thus eliminating the cytochrome concentration to dimensionless fractions. The value of $k_{A}^{d}$ may be calculated from $k_{A}^{d}$ using equation 1, and $k_{A}^{d}$ may be estimated from $k_{A}^{d}$ using a similar relation:

$$k_{A}^{d} = k_{A}^{d} \exp (E_{t}^{0} - E_{t}^{0}(FERT))$$

After making these substitutions, equation 7 may be solved for $k_{A}^{d}$.

$$k_{A}^{d} = \frac{k_{A}^{d} E_{t}^{0}([A]_D - [B])}{[A]_D - [B]}$$

Consequently, $k_{A}^{d}$ may be calculated from the values of $[B]_B$, $[A]_D$, $[A]_D$, and $[A]_D$ existing at any given time during the steady-state experiment. The time course of $[A]_D$ was measured (Figure 4B). The time courses of the other concentrations may be determined as follows: The internal free-radical concentration $[A]_D$ may be deduced from integrating it at steady state.

$$d [A]_D / dt = - k_{A}^{d} [A]_D ([B]_B - [A]_D) + k_{A}^{d} [A]_D$$

This quadratic equation may be solved to give $[A]_D$ as a function of $[B]_B$ and $k_{A}^{d}$.

$$[A]_D = \frac{k_{A}^{d} [B]_B - k_{A}^{d} [A]_D}{k_{A}^{d}}$$

In this and the previous equation, $T$ is the specific volume of the ghosts, and $B_{i}$ is the fractional volume contained within the ghosts. These terms correct for the volume difference between internal and external spaces.

Equation 11 may be solved to give $[A]_D$ in terms of $[A]_D$ and the measured concentration of $[A]_D$ (Figure 4A).

The concentrations of $[A]_D$ and $[A]_D$ may be determined from their known initial values by numerical integration of the respective rate equations:

$$d [A]_D / dt = - k_{A}^{d} [A]_D [B]_B + k_{A}^{d} [A]_D [A]_D$$

$$d [A]_D / dt = - k_{A}^{d} [A]_D [B]_B + k_{A}^{d} [A]_D$$

The latter equation is true because $[A]_D$ is at steady-state. Therefore, the rate at which external ascorbate disappears is equal to the rate at which external dehydroascorbate appears, and the latter forms only by disproportionation. equations 10 and 11 may be numerically integrated using measured values of $[B]_B$ and $[A]_D$ and values of $[A]_D$ and $[A]_D$ calculated from equations 10 and 11 respectively. Then $k_{A}^{d}$ may be computed using equation 9.

The disproportionation constant of semidehydroascorbate was calculated from the data in Figure 4A as follows: To reduce one molecule of O$_2$ to H$_2$O$_2$, ascorbate oxidase oxidizes 4 molecules of ascorbate (AH$_{-}$) to semidehydroascorbate (A). Following disproportionation (reaction III), the net yield is 2 dehydroascorbate (A) per O$_2$. In these experiments, the semidehydroascorbate concentration decays to zero because the solution becomes anaerobic; ascorbate (600 μM) is present in excess over dissolved O$_2$ (assumed to be 250 μM). Therefore, the amount of dehydroascorbate formed by disproportionation must equal twice the O$_2$ consumed:

$$2 \times [A]_D = \int \frac{d [A]_D}{dt}$$

The time at which $[A]_D$ decays to zero. Consequently,

$$k_{A}^{d} = 2 \times [A]_D$$

From the polynomial fit to the time course of $[A]_D$, $k_{A}^{d}$ may be calculated as the first positive root. The polynomial may be squared, integrated, and divided into $2 \times [A]_D$ to obtain $k_{A}^{d}$.

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