Reaction of Ascorbic Acid with Cytochrome b_{561}

CONCERTED ELECTRON AND PROTON TRANSFER

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Rate constants for reduction of cytochrome b_{561} by internal ascorbate (k_{r1}) and oxidation by external ferricyanide (k_{r1}') were determined as a function of pH from rates of steady-state electron transfer across chromaffin-vesicle membranes. The pH dependence of electron transfer from cytochrome b_{561} to ferricyanide (k_{r1}') may be attributed to the pH dependence of the membrane surface potential. The rate constant for reduction by internal ascorbate (k_{r1}), like the previously measured rate constant for reduction by external ascorbate (k_{r1}), is not very pH-dependent and is not consistent with reduction of cytochrome b_{561} by the ascorbate dianion. The rate at which ascorbate reduces cytochrome b_{561} is orders of magnitude faster than the rate at which it reduces cytochrome c, despite the fact that midpoint reduction potentials favor reduction of cytochrome c. Moreover, the rate constant for oxidation of cytochrome b_{561} by ferricyanide (k_{r1}') is smaller than the previously measured rate constant for oxidation by semidehydroascorbate, despite the fact that ferricyanide has a higher midpoint reduction potential. These results may be reconciled by a mechanism in which electron transfer between cytochrome b_{561} and ascorbate/semidehydroascorbate is accelerated by concerted transfer of a proton. This may be a general property of biologically significant electron transfer reactions of ascorbic acid.

Cytochrome b_{561}, a protein found in the membranes of secretory vesicles, functions to import electrons into the vesicles for the purpose of maintaining intravesicular ascorbic acid (vitamin C). At least two secretory vesicle enzymes require ascorbic acid as an electron donor. These enzymes, dopamine \( \beta \)-monooxygenase and peptidylglycine \( \alpha \)-amidating monoxygenase, probably use ascorbic acid as a one-electron donor and convert it to the ascorbate free radical, semidehydroascorbate. At its internal surface, cytochrome b_{561} donates an electron to the free radical, reducing it back to ascorbate. The cytochrome then takes an electron at its external surface from ascorbic acid present in the cytosol. Thus, the cytochrome serves as an electron shuttle, maintaining ascorbate inside the vesicles at the expense of ascorbate on the outside (Njus et al., 1983, 1986).

Because cytochrome b_{561} has no other catalytic function, it is an ideal model for studying electron transfer reactions of ascorbic acid. In an earlier study of cytochrome b_{561} in chromaffin-vesicle ghosts, we (Kelley et al., 1990) measured rate constants for reduction by external ascorbate and for oxidation by external semidehydroascorbate. Ascorbate reduces cytochrome b_{561} more quickly than it reduces cytochrome c despite the fact that the midpoint reduction potential of cytochrome b_{561} is 120 mV lower than that of cytochrome c. Moreover, while reduction of cytochrome c is strongly pH-dependent, consistent with reduction by the ascorbate dianion, reduction of cytochrome b_{561} is not. This suggests that ascorbate reduces cytochromes b_{561} and c by different mechanisms.

To elucidate the mechanism of electron transfer between ascorbate and cytochrome b_{561}, we have determined the effect of pH on the reduction of the cytochrome by external ascorbate. Reduction by internal ascorbate, like that by external ascorbate, is rapid and pH-independent. A pH dependence of these reactions could be concealed by compensatory pH-dependent changes in cytochrome b_{561} or membrane surface potential. These possibilities were tested and excluded by examining the effect of pH on the rate of cytochrome b_{561} oxidation by ferricyanide and on binding of the fluorescent surface-potential probe TNS.

MATERIALS AND METHODS

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 as the isolation medium. The vesicles (20-ml suspension) were purified further by layering over 14 ml of 1.6 M sucrose, 10 mM Hepes-pH 7.0, and centrifuging at 90,000 x g for 36 min at 4 °C in an SW 28 rotor. The pellet was resuspended in a lysis medium consisting of 0.1 M ascorbate, 0.15 M Tris adjusted to pH with NaOH (pH 8.0), malonic acid (pH 6.0), or phosphoric acid (pH 7.0). The vesicles were lysed by adding 0.12 volume of 30% glycerol, 70% lysis medium. After 20 min at 4 °C, the membranes were pelleted by centrifugation for 20 min at 36,000 x g and resuspended in the lysis medium. The ascorbate-loaded chromaffin-vesicle ghosts were then dialyzed against the lysis medium for 48 h (Kelley and Njus, 1986), purified on a Ficoll/sucrose density gradient (Njus and Radla, 1979) and used immediately.

To measure the rate of ferricyanide reduction as a function of external ferricyanide concentration (Fig. 1A), chromaffin-vesicle ghosts were suspended in assay medium (100-320 µg of protein/ml) at pH 6.0, 7.0, or 8.0 and incubated at room temperature with ascorbate oxidase to scavenge external ascorbate. Ferricyanide was added to the desired final concentration and the absorbance difference (418 - 480 nm) was monitored using an Aminco DW-2 spectrophotometer operated in the dual wavelength mode. The initial rate of absorbance change was measured, converted to a rate of ferricyanide reduction using a molar extinction coefficient of 1023, and normalized by dividing by the membrane protein concentration.

To measure the rate of ferricyanide reduction as a function of

1 The abbreviations used are: TNS, 2-toluidinyl-6-naphthalene sulfonic acid; AH\(^+-\), ascorbate monoanion; A\(^+\), ascorbate dianion; A\(^2-\), semidehydroascorbate anion; A\(^-\), protonated semidehydroascorbate; D, dehydroascorbate; bC, cytochrome b_{561}; FeCy, ferri/ferricyanide; Hepes, N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; ox, oxidized; red, reduced.
internal ascorbate concentration (Fig. 1B), chromaffin-vesicle ghosts were incubated with ascorbate oxidase at room temperature in assay medium at pH 6.0, 7.0, or 8.0. After ~15 min, a desired amount of ferricyanide was added to oxidize some of the internal ascorbate via cytochrome b_{561}. Then, the vesicle suspension (60–240 µg of protein/ml) was placed in the spectrophotometer to monitor the absorbance difference at 520 nm and a test concentration of ferricyanide was added. Again, the initial rate of absorbance change was measured and converted to a normalized rate by dividing by the molar extinction coefficient and the membrane protein concentration.

To measure TNS fluorescence (Table II), chromaffin-vesicle ghosts (160–210 µg of protein) were suspended in 2.56 ml of assay medium at pH 6.0, 7.0, or 8.0. TNS was added to a final concentration of 3.9 or 7.8 µM, and the fluorescence was measured at 440 nm (325 nm excitation) using a Perkin-Elmer Model 204S spectrophotometer. Fluorescence at each TNS concentration was normalized to the value observed at pH 6.0.

The assay medium consisted of 0.2 M potassium gluconate, 10 mM methymaline·HCl, 250 µM KCN, and 10 mM Mes (pH 6.0) or 10 mM Hepes (pH 7.0 or 8.0). Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce Chemical Co.). Curve fitting was done by regression analysis using a BASIC program run on a Microtosh SE/30 microcomputer. k_{ro} was assigned a value of 1.275 × 10^{7} liter/min-mol, 1.924 × 10^{7}, and 2.992 × 10^{5} m^{-1} min^{-1} at pH 6, 7, and 8 respectively (Bielski et al., 1981). K_{a} was assigned values of 18.674 (pH 6), 1.763 (pH 7), or 166 (pH 8) based on midpoint reduction potentials of +0.14 V for cytochrome b_{561} (Flatmark and Terland, 1971; Appuzo et al., 1984) and +0.39 V (pH 6), +0.33 V (pH 7), or +0.27 V (pH 8) for ascorbate/semidehydroascorbate (Iyaniyi et al., 1984). Ascorbate oxidase, Hepes, Mes, Tris, gluconate, and TNS were obtained from Sigma.

RESULTS

In a previous study (Kelley and Njus, 1988), we showed that steady-state electron transfer from ascorbate trapped within chromaffin-vesicle ghosts to external ferricyanide may be used to determine rate constants for electron transfer from internal ascorbate to cytochrome b_{561} (k_{ro}) and from cytochrome b_{561} to external ferricyanide (k_{ro}'). In the earlier study, rate constants were measured only at pH 7. Moreover, because the assay medium had a low ionic strength, the membrane surface potential probably caused the rate constant for oxidation by external ferricyanide (k_{ro}') to be underestimated greatly. In this study, the effect of surface potential was minimized by using an assay medium with a higher ionic strength. In addition, 10 mM methylamine was included to equilibrate internal and external pH so the pH dependence of the internal reaction could be assessed accurately.

Rates of steady-state electron flow across the chromaffin-vesicle membrane were measured in two different experiments. In one (Fig. 1A), the concentration of external ferricyanide was varied. In the other (Fig. 1B), the internal ascorbate concentration was varied by preincubating the vesicles with varying amounts of ferricyanide. After thus oxidizing a portion of the internal ascorbate, the rate of electron transfer was measured by adding a constant test concentration of ferricyanide. In the earlier study (Kelley and Njus, 1988), these two experiments were done separately using different ghost preparations. k_{ro} and k_{ro}' were determined from the first experiment assuming a value for the internal ascorbate concentration ([AH+]_ro). k_{ro} and [AH+]_ro were determined from the second experiment assuming a value for k_{ro}'. Here, the two experiments have been done sequentially using the same ghost preparation. The first experiment was used to determine k_{ro}' and [AH+]_ro, and the second experiment was used to calculate k_{ro} and [AH+]_ro. This procedure permits the trapped ascorbate concentration to be determined for each experiment rather than be assigned the same average value for all ghost preparations.

Steady-state electron transfer from internal ascorbate to external ferricyanide involves the following reactions (Fig. 2).

\[
B_{ro} + \text{AH}_{ro} \rightarrow B_{red} + A_{ro} + H^{+} \quad (I)
\]

\[
B_{red} + \text{FeCy}_{red} \rightarrow B_{ro} + \text{FeCy}_{red} \quad (II)
\]

In addition, the oxidation of cytochrome b_{561} by internal semidehydroascorbate (A') and the disproportionation of the free radical must be considered.

\[
B_{red} + A_{ro} + H^{+} \rightarrow B_{ro} + \text{AH}_{ro} \quad (III)
\]

\[
A_{ro} + A_{ro} + H^{+} \rightarrow \text{AH}_{ro} + A_{ro} \quad (IV)
\]

The disproportionation of semidehydroascorbate has been measured as a second-order reaction, and the rate constant k_{dis} has been measured as a function of pH (Bielski et al., 1981). Assuming that the other reactions are also second-order, we may write the following rate equation (Kelley and Njus, 1988).
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\[
\frac{V}{B} = \frac{k_F k_0 [\text{FeCy}]}{k_F [\text{FeCy}]_{\text{out}} + k_0 [\text{AH}]_{\text{in}} + k_\text{red} [\text{A}^-]_{\text{in}}} \tag{1}
\]

\[
V/B = \frac{k_F [\text{FeCy}]_{\text{out}}}{1 + k_F [\text{FeCy}]_{\text{out}} + k_0 [\text{AH}]_{\text{in}} + k_\text{red} [\text{A}^-]_{\text{in}}} \tag{2}
\]

When ascorbate-loaded ghosts are preincubated with a given amount of ferricyanide (F\text{red}), the internal ascorbate concentration is reduced from its initial value (A\text{in}) by an amount equal to 167 F\text{red} (Kelley and Njus, 1988). Equation 1 may then be written as

\[
\frac{V}{B} = \frac{k_F [\text{FeCy}]_{\text{out}}}{1 + k_F [\text{FeCy}]_{\text{out}} + k_0 [\text{AH}]_{\text{in}} + k_\text{red} [\text{A}^-]_{\text{in}} + k_F [\text{FeCy}]_{\text{out}}} \tag{3}
\]

To determine the rate constants, therefore, initial rates of electron transfer using a constant, high ferricyanide concentration ([F\text{red}]). These were then tested for rate of electron transfer using a constant, high ferricyanide concentration ([F\text{Cy}]). These rates (Fig. 1A) were fit to Equation 2 to obtain values for \(k_F^\text{red}\) and [AH]\text{in} assuming a value for \(k_0^\text{red}\). At the same time, ghosts were preincubated with varying amounts of ferricyanide (F\text{red}). These were then tested for rate of electron transfer using a constant, high ferricyanide concentration ([F\text{Cy}]). These rates (Fig. 1B) were fit to Equation 3 to obtain values for \(k_0^\text{red}\) and \(k_F^\text{red}\) assuming a value for \(k_F^\text{red}\). By iteration, optimum values for \(k_F^\text{red}\) and \(k_0^\text{red}\) were determined. Table I summarizes values determined from several experiments at pH 6.0, 7.0, and 8.0.

The pH dependence of observed rates of electron transfer may reflect pH-dependent changes in the membrane surface potential. To assess this effect, the anionic fluorescent probe TNS was used to evaluate the pH dependence of the surface potential \(\Psi\). At low concentrations, TNS binds to biological membranes in proportion to its aqueous concentration at the surface of the membrane. The latter is related to the TNS concentration far from the membrane ([TNS]\text{out}) by the Boltzmann equation (McLaughlin and Harary, 1976).

\[
[TNS]_{\text{bound}} = \text{constant} \times [\text{TNS}]_{\text{out}} \exp(\Psi/RT) \tag{4}
\]

Because TNS fluoresces when bound in a hydrophobic environment but not when free in aqueous solution, fluorescence is a convenient measure of TNS binding. Consequently, if chromaffin-vesicle ghosts are mixed with a fixed concentration of TNS in media at different pH, the relative fluorescence is a measure of the change in membrane surface potential.

\[
\Psi = \frac{(RT/3F) \ln [k_F^\text{red}/k_0^\text{red}]}{-TNS} \tag{5}
\]

Table II

<table>
<thead>
<tr>
<th>pH</th>
<th>TNS fluorescence</th>
<th>(\Delta \Psi) from TNS</th>
<th>(\Delta \Psi) from (k_F^\text{red})</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.0</td>
<td>67.5 ± 4.8</td>
<td>-10</td>
<td>+1</td>
</tr>
<tr>
<td>8.0</td>
<td>48.6 ± 3.8</td>
<td>-18</td>
<td>-21</td>
</tr>
</tbody>
</table>

**DISCUSSION**

We have analyzed steady-state electron transfer from internal ascorbate to external ferricyanide and calculated rate constants for reduction of cytochrome b561 by internal ascorbate (\(k_0\)) and oxidation of the cytochrome by external ferricyanide (\(k_F\)). Comparison of these rate constants with those measured previously at pH 7.0 (Table I) show that values of \(k_0\) are comparable. Values found for \(k_F\) are considerably higher, probably because a negative membrane surface potential at low ionic strength caused the earlier values to be underestimated greatly. The surface concentration of ferricyanide, a trivalent anion, should be especially sensitive to membrane surface potential.

The rate constant for reduction of ferricyanide by cytochrome b561 appears to be markedly pH-dependent, but this may be an artifact caused by the pH dependence of the membrane surface potential. The rate of electron transfer will be proportional to the ferricyanide concentration at the membrane surface, and that will be related to the ferricyanide concentration far from the membrane according to the Boltzmann equation. Consequently, the observed rate constant will be equal to the actual rate constant multiplied by exp (3F\Psi/RT). If the actual rate constant is pH-independent, then the relative surface potential \(\Delta \Psi\), can be calculated from the ratio of observed rate constants for ferricyanide reduction (\(k_F\)).

Values for \(\Delta \Psi\), calculated from rate constants agree fairly well with values determined using TNS (Table II). Because ascorbate and semidehydroascorbate have only a single negative charge, their rate constants are not as sensitive to surface potential as are those for ferricyanide. Over the pH range from 6 to 8, \(k_0\) and \(k_F\) would not be expected to change by more than a factor of 2.5 because of surface potential. The absolute magnitude of the surface potential should be considered also. Matthews et al. (1972) measured the surface potential of chromaffin vesicles suspended in 0.16 M KCl and found it to be about -15 mV between pH 6 and 8. The surface potential, therefore, may cause the rate constants for ascorbate and semidehydroascorbate to be underestimated slightly, especially at higher pH. These effects, however, should not be large enough to alter the conclusions drawn below.

The rate constant for oxidation by external ferricyanide (\(k_F\)) clarifies the paradoxical observation that external fer-
Cytochrome constant for electron transfer from species 1 to species 2 be expected for reduction by the dianion (Table 111). More-

E"" pH unit. This is consistent with reduction of cytochrome potential than cytochrome contrast, the rate of cytochrome b561 reduction by either internal ratem constants were calculated using Equation 9. Values for

rocyanide stimulates the rate of β-hydroxylation catalyzed by dopamine β-monooxygenase in chromaffin-vesicle ghosts. Grouselle and Phillips (1982) reported that 250 µM external ferrocyanide stimulates the rate of intravesicular tyramine hydroxylation by ~3 nmol/min·mg of ghost protein. Ahn and Klinman (1987) recently reported that external ferrocyanide (0.2–2 mM) also stimulates the rate of intravesicular dopamine hydroxylation by about 4 nmol/min·mg. Because ferrocyanide does not reduce cytochrome b561, both groups concluded that there must be another pathway for electron flow from external ferrocyanide to internal dopamine β-monooxygenase.

The fact that ferrocyanide does not cause net reduction of cytochrome b561 does not mean, however, that the cytochrome is incapable of mediating electron flow from ferrocyanide. The rate constant for reduction of cytochrome b561 by ferrocyanide (k-1) may be calculated from the rate constant for oxidation of cytochrome b561 by ferricyanide (k+1).

\[
k_{+1} \cdot k_{-1} = \exp(\Delta G^\circ/(RT))
\]

The rate constant for oxidation of cytochrome b561 by ferrocyanide (k+1) is ~90 liters/g·min (Table I). This and the midpoint reduction potentials of cytochrome b561 (E° = +0.14 V) and ferri/ferrocyanide (E° = +0.42 V) imply that the rate constant for the reduction of the cytochrome by ferrocyanide is 2 × 10⁻¹⁰ liters/g·min. Consequently, if dopamine β-monooxygenase turnover keeps cytochrome b561, in a mostly oxidized state, the rate of electron flow from 1 mM ferrocyanide to cytochrome b561 will be ~2 nmol/min·mg. This would account for much of the observed stimulation of dopamine β-monooxygenase activity. Thus, stimulation of β-hydroxylation by external ferrocyanide may be attributable to electron flow through cytochrome b561.

Cytochrome c is reduced by ascorbate at a relatively slow rate at physiological pH (Table III). Moreover, this rate is highly pH-dependent, increasing by a factor of ~10 with every pH unit. This is consistent with reduction of cytochrome c by the ascorbate dianion (Al-Ayash and Wilson, 1979). By contrast, the rate of cytochrome b561 reduction by either internal or external ascorbate is not nearly as pH-dependent as would be expected for reduction by the dianion (Table III). Moreover, cytochrome b561, which has a lower midpoint reduction potential than cytochrome c, should be reduced by ascorbate more slowly. We find, however, that ascorbate, either internally or externally, reduces cytochrome b561 much more rapidly than cytochrome c.

The rate constants may be compared quantitatively using the Marcus theory for electron transfer reactions in solution (Marcus and Sutin, 1985). The cross-relation gives the rate constant for electron transfer from species 1 to species 2 (k12) in terms of the self-exchange rate constants for each species (k+1 and k-1) and the equilibrium constant for the reaction (K12).

\[
k_{12} = \sqrt{(k_{+1}k_{-1})}K_{12}^{1/2}
\]

The cellision factor f12 is usually close to unity and may be omitted. Consequently, the rate constants for reduction of cytochrome b561 and cytochrome c by ascorbate should be related by Equation 9.

\[
k_{12}/k_{AC} = \sqrt{(k_{+1}k_{-1})}\exp(\Delta G^\circ/(RT))
\]

If we assume that the self-exchange rate constants for cytochromes b561 and c are comparable (k+1 = k-1) and use the appropriate midpoint potentials (E° for cytochrome c and E° for cytochrome b561) to calculate the equilibrium constants, the cross-relation may be used to predict the rate constant for reduction of cytochrome b561 by ascorbate (k12) given the rate constant for reduction of cytochrome c (kAC). It is evident that electron transfer between ascorbate and cytochrome b561 occurs more than 2 orders of magnitude faster than expected (Table III). The assumption that k12 = kAC may be questioned, but it seems most unlikely that the discrepancy can be explained by a higher value of k12. Cytochrome c is small (M, 12,384) and has an exposed heme edge. Cytochrome b561 is larger (M, 30,061) and is a transmembrane protein. Thus, it is difficult to imagine how the heme in cytochrome b561 could be more accessible (from both sides of the membrane) than the heme in cytochrome c.

Rates of oxidation of cytochrome b561 by semidehydroascorbate and ferricyanide may also be compared. The rate of oxidation by semidehydroascorbate is also faster than expected when compared with the rate of oxidation of cytochrome b561 by ferricyanide (Table IV) even though the midpoint reduction potential of semidehydroascorbate (+0.076 V for A+/A-) is lower than that for ferri/ferrocyanide (+0.42 V). Again, rate constants for oxidation by semidehydroascorbate (k+1) may be predicted from rates of oxidation by ferricyanide (k+1) using the Marcus theory.

\[
k_{+1}/k_{+1} = \sqrt{(k_{+1}k_{-1})}\exp(\Delta G^\circ/(RT))
\]

The self-exchange rate constants for semidehydroascorbate (k12) and ferricyanide (k12) are 1 × 10⁻⁵ M⁻¹·s⁻¹ (Williams and Yandell, 1982) and 5 × 10⁻⁵ M⁻¹·s⁻¹ (Butler et al., 1981), respectively. This calculation does not require assumptions about the self-exchange rate constant of cytochrome b561, and it again suggests that the reaction between cytochrome b561 and semidehydroascorbate occurs too quickly to be attributed to outer sphere electron transfer (Table IV).

The value for the midpoint reduction potential of the A+/A- pair (E° = +0.076 V) was calculated from the pK values of ascorbate (pK1 = 4.04; pK2 = 11.23; Taqui Khan and Martell, 1969), from the pK of semidehydroascorbate (pK2 = 0.45; Laroff et al., 1972) and from the midpoint reduction potential at pH 7 (E° = +0.330 V; Iyanagi et al., 1984) using Equation 11.

\[
E^\circ = \frac{E^\circ_{2}}{(RT)\ln[(1 + [H^+]_c/K_c) + ([H^+]^2/K_c)](1 + [H^+]_c/K_c)}
\]

Values of E° given by others (+0.019 V, Williams and Yandell, 1982; +0.05 V, Creutz, 1981) are even lower and would suggest an even more unfavorable equilibrium for oxidation of cytochrome b561 by semidehydroascorbate.

Because ascorbate and semidehydroascorbate are both univalent anions at physiological pH, the oxidation of ascorbate involves loss of both an electron and a proton (Fig. 3). The speed and pH independence of cytochrome b561 reduction by

<table>
<thead>
<tr>
<th>TABLE III</th>
<th>Rate constants for reduction by ascorbate</th>
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<tbody>
<tr>
<td>pH 6.0</td>
<td>pH 7.0</td>
</tr>
<tr>
<td>Cytochrome c (E° = +0.260 V)</td>
<td>0.83</td>
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<tr>
<td>Al-Ayash and Wilson, 1979</td>
<td>2.5</td>
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<tr>
<td>Yamazaki, 1962</td>
<td>35 ± 12</td>
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<tr>
<td>Cytochrome b561 (E° = +0.140 V)</td>
<td>310 ± 10</td>
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<tr>
<td>k+1 (inside)</td>
<td>0.1</td>
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<tr>
<td>k-1 (outside)</td>
<td>1</td>
</tr>
</tbody>
</table>
Reaction of Ascorbic Acid with Cytochrome b₅₆₁

<table>
<thead>
<tr>
<th>pH 6.0</th>
<th>pH 7.0</th>
<th>pH 8.0</th>
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<tbody>
<tr>
<td>Ferricyanide (E⁺⁺⁺ = +0.420 V)</td>
<td>Outside (kₓ)</td>
<td>(6.2 ± 2.7) x 10⁸</td>
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<tr>
<td>Semidehydroascorbate (E⁺⁺⁺⁺ = +0.076 V)</td>
<td>Outside (kₓ)</td>
<td>(2.0 ± 0.7) x 10⁶</td>
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<tr>
<td>Predicted from ferricyanide</td>
<td>1 x 10⁶</td>
<td>1 x 10⁴</td>
</tr>
</tbody>
</table>

**Fig. 3. Alternative mechanisms of ascorbate oxidation.** Cytochrome c and ferricyanide oxidize the ascorbate dianion by an "outer sphere" mechanism. Cytochrome b₅₆₁ may oxidize the ascorbate monoonion directly by concerted electron and proton transfer.

asmorcate are not compatible with separate electron transfer and deprotonation steps. Electron transfer followed by deprotonation would form a high energy intermediate, the protonated ascorbate radical (AH⁻). At ~+0.7 V (Creutz, 1981; Williams and Yandell, 1982), the midpoint potential of the AH⁻/AH⁻ pair is too high for reduction to occur via this pathway. Deprotonation followed by electron transfer, in addition to being pH-dependent, should be limited kinetically by the low concentration of the ascorbate dianion. This argues that electron and proton transfer occur by a concerted mechanism; cytochrome b₅₆₁ binds the ascorbate monoonion and releases the semidehydroascorbate anion.

Similar logic argues that oxidation of cytochrome b₅₆₁ by semidehydroascorbate does not occur via separate electron transfer and protonation steps. The reaction is too fast and too weakly pH-dependent to be compatible with protonation of A⁺ to AH⁻ followed by electron transfer. Electron transfer to form A⁺ followed by protonation is also unlikely since the A⁺/A⁻ pair has a midpoint potential (+0.076 V) that is too low to oxidize cytochrome b₅₆₁ at the observed rate. Again, concerted proton/electron transfer is a plausible alternative; semidehydroascorbate is converted directly to the ascorbate monoonion instead of the energetically unfavorable ascorbate dianion.

Cytochrome b₅₆₁ is adapted specifically to react with ascorbate/semidehydroascorbate whereas cytochrome c is not. Consequently, it would not be surprising to find that cytochrome b₅₆₁ reacts by a more efficient process than the outer sphere mechanism which characterizes the reaction of cytochrome c with ascorbate. Concerted electron and proton transfer provides such a mechanism; it permits ascorbate and semidehydroascorbate to interconvert without passing through either of the energetically unfavorable intermediates: the ascorbate dianion (A⁺⁺) or the protonated ascorbate radical (AH⁻). It should be apparent that similar arguments will apply to any enzyme for which ascorbic acid is an effective electron donor.

The term "concerted proton/electron transfer" is meant to emphasize the distinct roles of H⁺ and e⁻ and not to imply a particular mechanism. In terms of mechanism, one could imagine that, when ascorbate passes an electron to the hemeprotein, the proton is transferred to a protonatable group on the cytochrome. One could also imagine that H⁺ is transferred not to the protein but to water. The cytochrome may bind ascorbate in such a way (e.g., ionic bonding between ascorbate and a cationic group on the cytochrome) that the pKₐ of bound ascorbate is considerably lower than pKₐ of free ascorbate. This would facilitate the dissociation of the second proton from ascorbate and thereby enhance electron transfer.

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