# Evidence for an Essential Histidine Residue in the Ascorbate-Binding Site of Cytochrome $b_{561}$

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ABSTRACT: Cytochrome $b_{561}$ mediates equilibration of the ascorbate/semidehydroascorbate redox couple across the membranes of secretory vesicles. The cytochrome is reduced by ascorbic acid and oxidized by semidehydroascorbate on either side of the membrane. Treatment with diethyl pyrocarbonate (DEPC) inhibits reduction of the cytochrome by ascorbate, but this activity can be restored by subsequent treatment with hydroxylamine, suggesting the involvement of an essential histidine residue. Moreover, DEPC inactivates cytochrome $b_{561}$ more rapidly at alkaline pH, consistent with modification of a histidine residue. DEPC does not affect the absorption spectrum of cytochrome $b_{561}$ nor does it change the midpoint reduction potential, confirming that histidine modification does not affect the heme. Ascorbate protects the cytochrome from inactivation by DEPC, indicating that the essential histidine is in the ascorbate-binding site. Further evidence for this is that DEPC treatment inhibits oxidation of the cytochrome by semidehydroascorbate but not by ferricyanide. This supports a reaction mechanism in which ascorbate loses a hydrogen atom by donating a proton to histidine and transferring an electron to the heme.

Cytochrome $b_{561}$, a membrane protein found in secretory and synaptic vesicles, regenerates ascorbic acid needed by intravesicular enzymes such as dopamine $\beta$-monooxygenase and peptidylglycine α-amidating monooxygenase. The cytochrome reacts with ascorbic acid and its oxidized product, semidehydroascorbate (SDA),[1] on both sides of the vesicle membrane, so it maintains redox equilibrium between cytoplasmic and intravesicular pools of ascorbate and SDA (*1*).

Ascorbic acid is an unusual reducing agent in that it normally functions as a donor of single reducing equivalents and is oxidized to its radial anion semidehydroascorbate (*2*). Because the reaction between cytochrome $b_{561}$ and ascorbic acid involves only a single reducing equivalent and is not coupled to other reactions, the cytochrome presents a simple model for studying the enzymatic oxidation of organic substrates. At physiological pH, the oxidation of ascorbate to semidehydroascorbate yields the equivalent of a hydrogen atom. The cytochrome's heme takes an electron, leaving a proton to be released. We have proposed that the cytochrome binds ascorbic acid in such a way that it facilitates the removal of this proton (*1*, *3*).

Among the amino acids that might function in proton transfer, a likely candidate is histidine with a p$K_a$ of 6.5. Diethyl pyrocarbonate, which ethoxyformylates the imidazole side chain of histidine residues, has been widely used to probe for essential histidine residues in enzymes (*4*, *5*). In this study, we show that diethyl pyrocarbonate inhibits the reaction between ascorbic acid and cytochrome $b_{561}$ and explore the mechanism of this inhibition.

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[1] Abbreviations: DEPC, diethyl pyrocarbonate; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDA, semidehydroascorbate.

## EXPERIMENTAL PROCEDURES

*Preparation of Chromaffin Vesicle Ghosts.* Chromaffin vesicles were isolated from bovine adrenal medulla as described by Kelley and Njus (*6*). After resuspension in 0.3 M sucrose and 10 mM Hepes, pH 7.0, the vesicles were layered over 1.6 M sucrose and 10 mM Hepes, pH 7.0, and purified by centrifugation at 90000*g* for 36 min at 4 °C (*7*). The pellet was resuspended in buffer A (0.1 M potassium phosphate, 0.1 M KCl, pH 7.0), and one-eighth volume of 30% glycerol and 70% buffer A (v/v) was added to lyse the vesicles (*8*). After 20 min at 4 °C, the membrane suspension was frozen and stored at −80 °C until needed. Protein concentrations were determined using the biuret assay.

*DEPC Inactivation of Cytochrome $b_{561}$.* Chromaffin vesicle ghosts were thawed, centrifuged at 27000*g*, and resuspended in buffer B (0.133 M potassium phosphate, 0.2 M KCl, pH 7.0). The ghosts were divided in two, and one portion was treated with DEPC (14 mM final concentration) at room temperature for 60 min. To terminate the DEPC reaction, methylamine hydrochloride (0.1 M final concentration) was added to both portions. The DEPC-treated and untreated samples were then centrifuged at 48200*g* for 20 min, and each pellet was resuspended in 2 mL of buffer B. The samples were dialyzed against the same medium for four changes at 4 °C. Following dialysis, each sample was divided into two equal portions. To one DEPC-treated sample and one untreated sample was added hydroxylamine to a final concentration of 0.5 M (adding concentrated KOH to keep the pH at 7.0). All samples were again dialyzed for four changes against buffer B at 4 °C. Then each was diluted to 8 mL with 0.2 M KCl, 10 mM methylamine hydrochloride, 250 $\mu$M KCN, and 10 mM Hepes, pH 7.0.

Cytochrome reduction was monitored at 561 nm relative to an isosbestic point at 569 nm using an SLM-Aminco DW-

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2000 spectrophotometer operated in the dual wavelength mode. The ghost sample was mixed at room temperature with an equal volume of 10 mM ascorbate in buffer B in an Aminco-Morrow stopped-flow apparatus.

*Redox Titration.* Chromaffin vesicle ghosts were thawed, centrifuged, and resuspended in buffer A. Following dialysis against four changes of the same medium, the ghosts were divided into two equal portions and diluted to 15 mL each with buffer A. DEPC (14 mM final concentration) was added to one sample, and both were incubated at room temperature for 30 min. Then histidine (20 mM final concentration) was added to each sample to terminate the DEPC reaction. Finally, all samples were dialyzed for 2−3 h against buffer A to remove ethoxyformylated and free histidine.

Samples were placed in the spectrophotometer in a custom cuvette (1 cm path, 15 mL volume), redox mediators (2.7 $\mu$M $FeCl_2$, 0.27 $\mu$M EDTA, and 1.3 $\mu$M duroquinone) were added, and the sample was kept under $N_2$ to exclude oxygen. Each cytochrome sample was fully reduced by adding sodium dithionite to a final concentration of ~700 $\mu$M. The reduced cytochrome $b_{561}$ was then titrated by addition of 5−50 $\mu$L aliquots of 100 mM $K_3Fe(CN)_6$. The sample was allowed to equilibrate after each addition. Then the absorption spectrum was recorded from 505 to 590 nm in the SLM-Aminco DW 2000 spectrophotometer, and the reduction potential was measured using a platinum electrode and a calomel reference electrode.

*Oxidation by Semidehydroascorbate.* Chromaffin vesicles were lysed in 0.1 M ascorbate and 0.1 M potassium phosphate, pH 6.5 (*6*). These were dialyzed against four changes of the same medium and then centrifuged onto a layer of 15% Ficoll (*8*) to remove external ascorbate. Ghosts were taken up in 10 mL of 0.1 M KCl, 0.1 M potassium phosphate, and 388 $\mu$M KCN, pH 7.0. The sample was divided, and DEPC (14 mM final concentration) was added to one half. After 30 min, histidine (20 mM final concentration) was added to both samples to terminate the DEPC reaction. Cytochrome reduction in each sample was assayed by dual wavelength spectrophotometry (561−569 nm). Oxidation was initiated by adding 10 units of ascorbate oxidase (to generate semidehydroascorbate from residual external ascorbate) and then 400 $\mu$M $K_3Fe(CN)_6$ (final concentration).

*Kinetics of the DEPC Reaction.* Chromaffin vesicle ghosts were thawed, centrifuged, and resuspended in 0.1 M KCl and 5 mM potassium phosphate, pH 7.0. Following dialysis against two changes of the resuspension medium, the ghosts were titrated to pH 7.2 or 6.2. DEPC (6.4 mM final concentration) was added to 0.5 mL aliquots. Histidine (20 mM final concentration) was added to terminate the reaction at specified times (0−6 min). Samples were then mixed with 0.5 mL of assay medium (0.2 M KCl, 10 mM methylamine hydrochloride, 200 $\mu$M EDTA, 50 mM potassium phosphate, pH 7.0), and each sample was assayed by dual-wavelength spectrophotometry (561−569 nm). After a baseline absorbance was recorded (0% reduction), ascorbate (5 mM final concentration) was added and reduction was followed for 2 min. Finally, sodium dithionite was added to determine 100% reduction. The data points between ascorbate and dithionite additions were fit by the least-squares method to the function $A = A_1 - A_2 \exp(-k_{sr}t)$ to determine the rate constant for the slow phase of reduction by ascorbate.

*pH Dependence of Inactivation.* Chromaffin vesicle ghosts were thawed, centrifuged, and resuspended in 0.1 M KCl and 5 mM potassium phosphate, pH 7.0. Following dialysis against two changes of the resuspension medium, the sample was divided into seven equal volumes. Each portion was titrated to a specific pH (between 6.2 and 8.0) and then split in two. DEPC (5.8 mM final concentration) was added to one half; the other remained untreated. After incubation for 9 min at room temperature, 0.5 mL of each sample received 50 $\mu$L of 200 mM histidine to terminate ethoxyformylation. To return the pH to 7.0, 0.5 mL of assay medium (0.2 M KCl, 10 mM methylamine hydrochloride, 200 $\mu$M EDTA, 50 mM potassium phosphate, pH 7.0) was added. Each sample was then assayed by dual-wavelength spectrophotometry (561−569 nm). After a baseline absorbance was recorded (0% reduction), ascorbate (5 mM final concentration) was added and reduction was followed for 2 min. Finally, sodium dithionite was added to determine 100% reduction. The rate constant ($k_i$) for inactivation by DEPC was calculated as $k_i = [\ln(A_0/A_i)]/t$, where $A_i$ is the absorbance change following ascorbate addition in the DEPC-treated sample, $A_0$ is the corresponding absorbance change in the untreated sample, and $t$ is the time of incubation with DEPC.

*Protection by Ascorbate.* Chromaffin vesicle ghosts were thawed and centrifuged and then resuspended in and dialyzed against four changes of buffer A. Following dialysis, the sample was split into four equal parts and treated as follows: (A) no additions, (B) 25 mM ascorbate, (C) 14 mM DEPC, and (D) 25 mM ascorbate and 14 mM DEPC. After incubation at room temperature for 30 min, each sample received 100 $\mu$L/mL 200 mM histidine to inactivate nonreacted DEPC. The samples were dialyzed first against buffer A to remove ascorbate, then against 5 mM $K_3Fe(CN)_6$ in buffer A for 1 h to reoxidize the cytochrome, and finally against three changes of buffer A to remove $K_3Fe(CN)_6$. Cytochrome reduction was assayed by dual-wavelength spectrophotometry (561−569 nm). After a baseline absorbance was recorded, 2.5 mM ascorbate and 200 $\mu$M sodium dithionite were added at the times indicated.

## RESULTS

When chromaffin vesicle membranes are mixed with ascorbic acid, cytochrome $b_{561}$ is quickly reduced (Figure 1A). Treatment of the membranes with DEPC completely inhibits subsequent reduction by ascorbate (Figure 1B). The ethoxyformyl adduct formed when DEPC modifies a histidine residue is removed by treatment with hydroxylamine. Hydroxylamine treatment does, in fact, reverse the effect of DEPC on the reducibility of cytochrome $b_{561}$ (Figure 1C), indicating that the site of DEPC action is a histidine residue. This reversal may not be complete, however, raising the possibility that DEPC also modifies other sites (*11*).

DEPC could be acting on cytochrome $b_{561}$ either by affecting a heme directly or by interfering with the action of ascorbic acid at its binding site. To test for an effect of cytochrome $b_{561}$ on heme, we examined effects of DEPC both on the absorption spectra and on the midpoint reduction potential. DEPC treatment has no effect on the absorption spectrum of cytochrome $b_{561}$ in either the oxidized or the reduced form (data not shown). Moreover, DEPC has no significant effect on the midpoint reduction potential (Figure

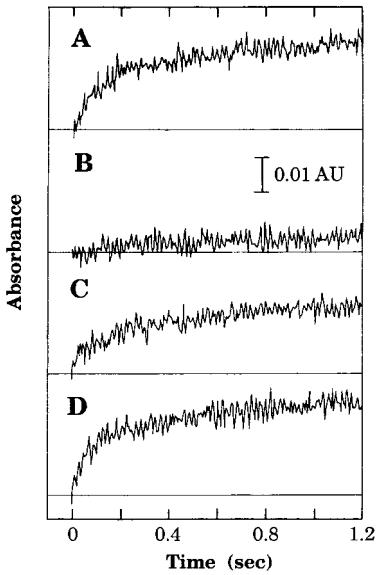


FIGURE 1: Inactivation of cytochrome $b_{561}$ by DEPC and reversal by hydroxylamine. Ascorbic acid (5 mM final concentration) and cytochrome $b_{561}$ were mixed by stopped flow at $t = 0$ and reduction followed by monitoring absorbance. (A) Nontreated cytochrome $b_{561}$. (B) Cytochrome $b_{561}$ treated with 14 mM DEPC. (C) Cytochrome $b_{561}$ treated with 14 mM DEPC followed by 0.5 M hydroxylamine. (D) Cytochrome $b_{561}$ treated with 0.5 M hydroxylamine but not DEPC.

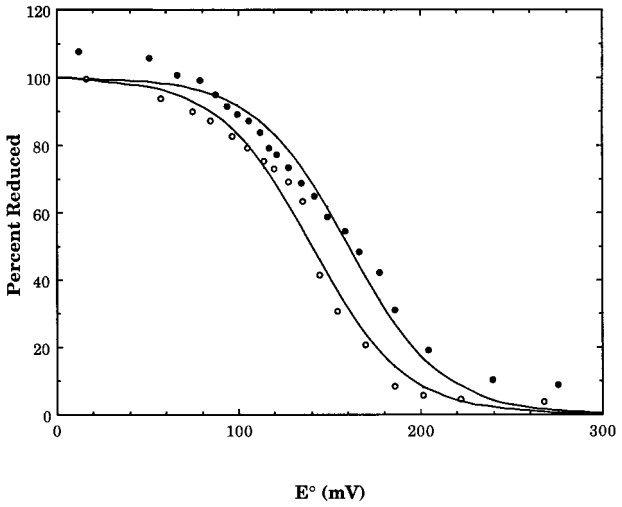


FIGURE 2: DEPC treatment does not lower the reduction potential of cytochrome $b_{561}$. The redox state of of untreated (○) and DEPC-treated (●) cytochrome $b_{561}$ was followed spectrophotometrically as the reduction potential was titrated by adding ferricyanide to dithionite-reduced cytochrome. Absorbance measurements are normalized to 100% at fully reduced and 0 at fully oxidized. The lines are least-squares best fits to the Nernst equation.

2). In three separate titrations, the DEPC-treated cytochrome had a midpoint reduction potential 18 ± 34 mV more positive than the native cytochrome, which has a potential of +140 mV (*9*, *10*). Thus, DEPC treatment does not significantly change the reduction potential, and the small observed positive shift would make DEPC-treated cytochrome $b_{561}$ slightly more rapidly reducible than native, not less.

If DEPC inhibits reduction of the cytochrome by ascorbate but does not change the midpoint potential, then DEPC should also inhibit the reverse reaction, oxidation of the cytochrome by external semidehydroascorbate. This may be tested using chromaffin vesicle ghosts loaded with ascorbate.

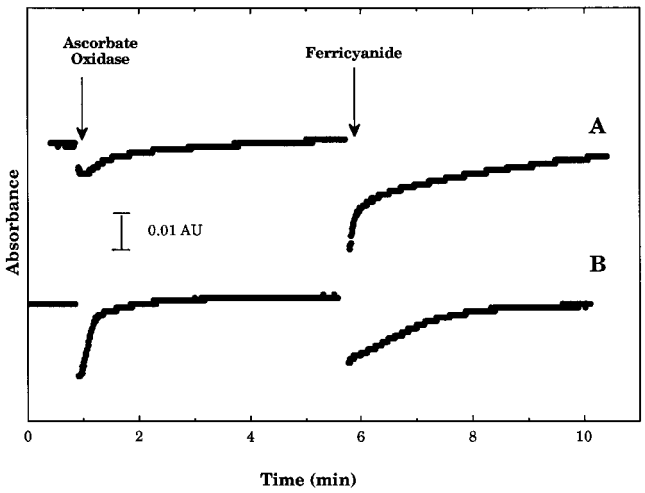


FIGURE 3: Effect of DEPC treatment on oxidation by semidehydroascorbate. Ascorbate-loaded chromaffin vesicle ghosts were either treated with DEPC (A) or untreated (B). The redox state of cytochrome $b_{561}$ in each ghost suspension was followed spectrophotometrically. Oxidation was initiated by addition of ascorbate oxidase (to generate semidehydroascorbate) and ferricyanide at the times indicated.

In these ghosts, cytochrome $b_{561}$ is normally in the reduced state (*6*). When ascorbate oxidase is added to the ghost suspension, the enzyme generates semidehydroascorbate externally, causing a transient oxidation of the cytochrome (Figure 3). When either external ascorbate or oxygen is depleted, semidehydroascorbate generation ceases, and the cytochrome is reduced again by internal ascorbate. In this assay, prior treatment with DEPC inhibits the extent of oxidation caused by semidehydroascorbate (Figure 3). By contrast, subsequent oxidation by external ferricyanide is not inhibited, indicating that DEPC interferes specifically with the reaction between cytochrome $b_{561}$ and external ascorbate/semidehydroascorbate.

To characterize further the effect of DEPC on cytochrome $b_{561}$, it is necessary to quantitate the inhibition. As the cytochrome is treated with DEPC for longer times, its reducibility by ascorbic acid diminishes (Figure 4). Taking the initial absorbance as 0% reduction and the absorbance following the addition of dithionite as 100% reduction, reducibility may be quantified as the percentage of the absorbance change caused by ascorbic acid. A complicating factor is that DEPC appears to change the kinetics of reduction by ascorbate as well as the extent of reduction; a slow reduction phase appears following DEPC treatment. Thus, it is not readily apparent whether reducibility by ascorbate should be measured immediately after ascorbate addition or later after reduction has reached a constant level.

Cytochrome reduction measured immediately after ascorbate addition (rapid reduction) disappears as an exponential function of the time of DEPC treatment (Figure 5A). By contrast, the extent of reduction measured at the plateau (total reduction) is inhibited only after a lag period. This suggests that a single DEPC-sensitive site is responsible for inhibition of the rapid reduction, while multiple sites must be modified to totally inhibit reduction by ascorbate.

The slow component of cytochrome $b_{561}$ reduction changes not only in magnitude but also in rate. To quantify this, absorbance traces (as in Figure 4) were fit to the function $A = A_1 - A_2 \exp(-k_{sr}t)$ to determine the rate constant $k_{sr}$ for

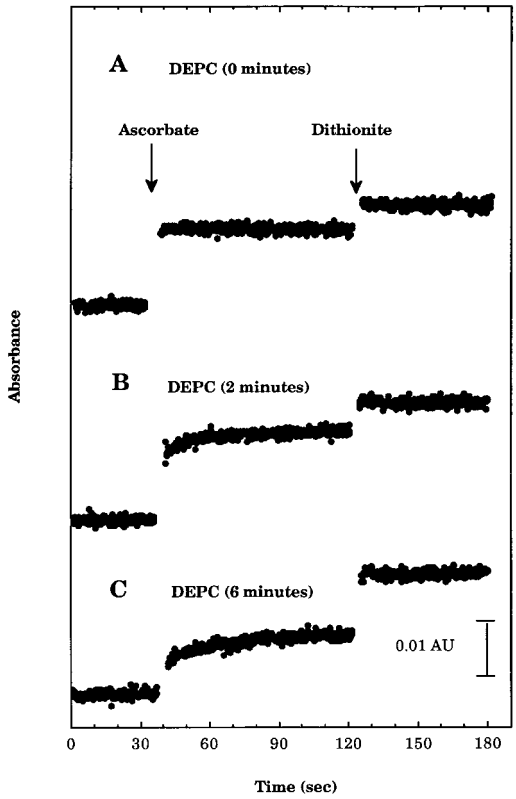


FIGURE 4: Effect of DEPC treatment on kinetics of reduction by ascorbate. Chromaffin vesicle ghosts were treated with 14 mM DEPC at pH 7.2. Histidine was added to terminate the reaction after 2 min (panel B), after 6 min (panel C), or before DEPC addition (panel A). The redox state of the cytochrome in each sample was then monitored spectrophotometrically at pH 7.0. Ascorbate and sodium dithionite were added at the times indicated.

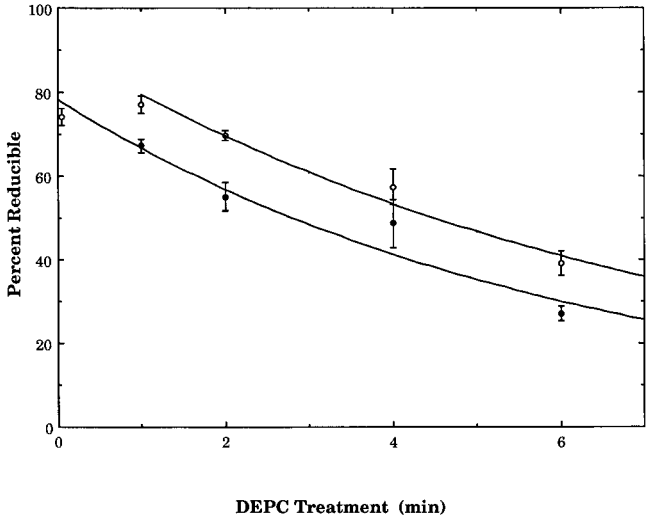


FIGURE 5: Time course of DEPC inhibition. Chromaffin vesicle ghosts were treated with DEPC for specified periods of time, and the samples were assayed as shown in Figure 4. Percent reducible is measured as the change in absorbance following ascorbate addition relative to the total change observed following dithionite addition. Rapid reduction was measured 5 s after ascorbate addition (●), and maximum reduction was measured after 80 s (○). Each point is the average (±SD) of three replicate samples. Lines are exponential curves best fitting the data points.

the slow phase of reduction by ascorbate. Interestingly, this rate constant decreases with the length of DEPC treatment (Figure 6A); the slow rate of reduction gets slower as the

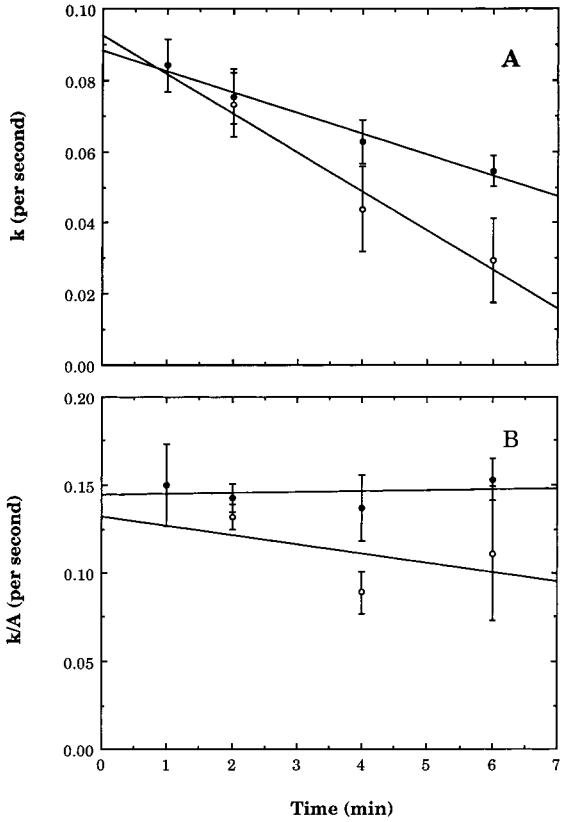


FIGURE 6: Kinetics of the slow phase of reduction. Chromaffin vesicle ghosts were treated with DEPC for specified periods of time at pH 6.2 (●) or pH 7.2 (○), and the samples were assayed at pH 7.0 as shown in Figure 4. (A) The slow increase in absorbance following ascorbate addition was fit to an exponential function by a least-squares method to obtain the rate constant *k*. (B) Each value of *k* was normalized by dividing by the percent of cytochrome $b_{561}$ reducible in 5 s (rapid reduction) as shown in Figure 5. Each point is the average (±SD) of three replicate samples. Lines are least-squares best fits to the data points.

length of DEPC treatment increases. Since the fraction of rapidly reducible cytochrome also decreases with length of DEPC treatment, $k_{sr}$ is roughly proportional to the rapidly reducible fraction and their ratio is approximately constant (Figure 6B). The rate constant for slow reduction seems to correlate with cytochrome reducibility rather than with time of treatment. If the length of DEPC treatment is kept constant, but the pH is varied, the slow phase (assayed at pH 7.0) has a slower rate constant in pH 7 treated ghosts than in pH 6 treated ghosts (data not shown). As discussed below, DEPC inactivates cytochrome $b_{561}$ more effectively at pH 7 than at pH 6.

DEPC reacts with imidazole in the unprotonated but not the protonated form, so the reaction of DEPC with histidine residues is pH dependent (*4*). Consistent with this, the rate of inactivation of cytochrome $b_{561}$ by DEPC is faster at higher pH and slower at lower pH (Figure 7). This result was obtained whether reducibility was measured immediately after ascorbate addition or after the plateau was reached.

Because DEPC does not affect the heme(s), it is likely that the modified site is involved in the reaction with ascorbic acid. Reduction of cytochrome $b_{561}$ by ascorbate exhibits Michaelis−Menten kinetics with a $K_m$ of 0.34 mM at pH 7.0 (*9*). Thus, the cytochrome has a specific ascorbate-binding site on its extravesicular face. If the amino acid

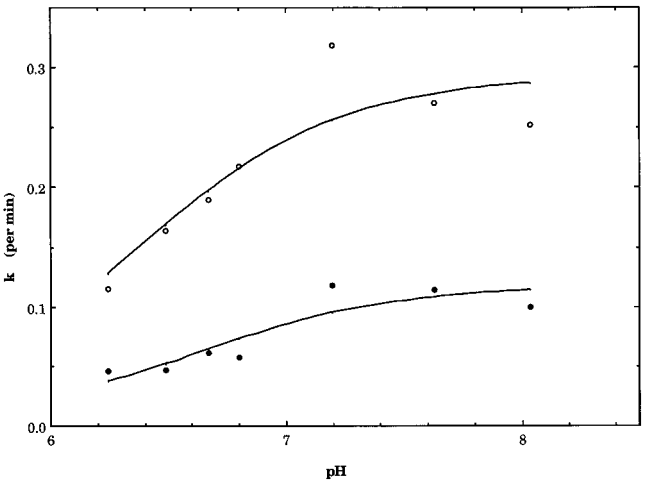


FIGURE 7: pH dependence of inactivation. Chromaffin vesicle ghost suspensions were titrated to different pH values and then treated with 5.8 mM DEPC. After 9 min, histidine was added to stop the reaction. Reduction by ascorbate was monitored spectrophotometrically as in Figure 4. Rapid reduction was measured 5 s after ascorbate addition (○), and maximum reduction was measured after 80 s (●). The rate constant for inactivation by DEPC ($k_i$) was calculated as described in Experimental Procedures. Each point is the average (±SD) of three replicate samples.

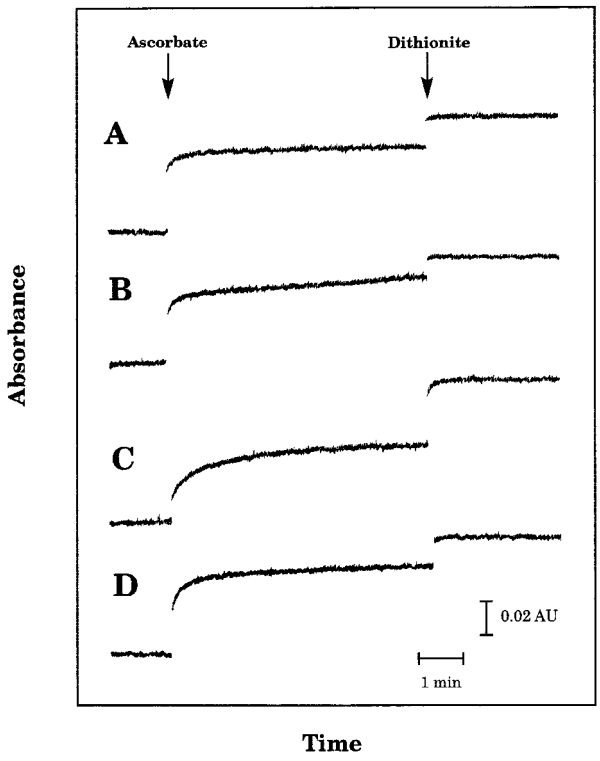


FIGURE 8: Ascorbate protects cytochrome $b_{561}$ from inactivation by DEPC. Cytochrome $b_{561}$ was reduced by ascorbate and dithionite at the times indicated by arrows. (A) Untreated cytochrome $b_{561}$. (B) Cytochrome $b_{561}$ pretreated with ascorbate alone. (C) Cytochrome $b_{561}$ pretreated with DEPC. (D) Cytochrome $b_{561}$ pretreated with DEPC in the presence of ascorbate.

residue modified by DEPC lies in the ascorbate-binding site, then ascorbic acid might protect the cytochrome from inactivation by DEPC. To test this hypothesis, chromaffin vesicles were pretreated with 100 mM ascorbate before addition of DEPC. Since the ascorbate would be occupying the binding site, DEPC would not be able to modify the histidine. The addition of ascorbate did preserve the activity of cytochrome $b_{561}$ (Figure 8). This was true whether measured immediately after ascorbate addition or after reaching a plateau (Table 1).

To eliminate the possibility that ascorbate interferes nonspecifically with the reaction between ascorbate and imidazole, we compared the ethoxyformylation of histidine in the presence and absence of ascorbate. Ascorbate has no effect on the reaction between DEPC and histidine as monitored by observing the absorbance of the product at 240 nm (data not shown).

Table 1: Protection of Cytochrome $b_{561}$ by Ascorbate[a]

| pretreatment | rapid reduction (%) | maximum reduction (%) |
|---|---|---|
| none | 55.5 ± 7.4 | 71.3 ± 8.2 |
| DEPC | 19.3 ± 3.5 | 50.1 ± 9.9 |
| ascorbate | 60.8 ± 8.4 | 80.5 ± 8.7 |
| ascorbate + DEPC | 37.0 ± 8.5 | 72.9 ± 7.5 |

[a] From traces obtained as shown in Figure 8, the extent of reduction observed immediately after ascorbate addition (rapid reduction) and that observed just prior to dithionite addition (maximum reduction) were compared as a percentage of reduction observed following dithionite addition. Each value is the average (±SD) of traces obtained in three separate experiments.

## DISCUSSION

Diethyl pyrocarbonate treatment inhibits the extent of cytochrome $b_{561}$ reduction by external ascorbate. It also introduces a slow reduction phase giving rise to biphasic kinetics. DEPC could create the slow phase by (1) modifying the rate of the normal reduction reaction or (2) unmasking another reduction pathway with slower kinetics.

The first alternative is unlikely. If modification by DEPC slows the rate of reduction by ascorbate, then it must also slow the rate of oxidation by semidehydroascorbate since the midpoint reduction potential of the cytochrome does not change. Tsubaki et al. (*11*) have reported that DEPC treatment does not affect the kinetics of cytochrome $b_{561}$ oxidation by semidehydroascorbate, although the extent of oxidation is inhibited. In addition, to totally inhibit reduction of cytochrome $b_{561}$ and cause slower reduction with a range of rate constants, DEPC would need to modify a number of sites. Bovine cytochrome $b_{561}$ has only seven histidine residues; only three (possibly four) are on the external surface and one or more of these are liganded to the heme. This makes it unlikely that DEPC could modify enough histidine residues to inactivate the cytochrome and cause the complicated reduction kinetics observed. DEPC could, however, slow reduction by modifying another amino acid (*11*).

Another possibility is that there is an alternative pathway for cytochrome $b_{561}$ reduction, which becomes observable when the fast pathway is inhibited. The slow reduction pathway could be intramolecular, involving ascorbate reaction at a second site on cytochrome $b_{561}$. Alternatively, it could be intermolecular, involving reduction of an inhibited cytochrome by an unmodified cytochrome. Intermolecular electron transfer is supported by several observations. First, the slow phase and the fast phase seem to share common characteristics in terms of inhibition by DEPC. In particular, both pathways are protected by ascorbate and have the same pH dependence for inactivation, being more sensitive to

DEPC above pH 7. This indicates that the fast and slow pathways must involve either very similar sites on a single cytochrome or the same site on different cytochrome molecules. Second, DEPC inhibits the slow phase only after a delay of about 1 min, suggesting that multiple sites are involved. This would be unlikely on a single cytochrome but natural for electron transfer among multiple cytochromes. Finally, the apparent rate constant for the slow phase decreases in proportion to the amount of active cytochrome. This is consistent with an intermolecular but not an intramolecular mechanism.

Intermolecular electron transfer is possible because of the high density of cytochrome $b_{561}$ in the chromaffin vesicle membrane. From a cytochrome content of 2.3 nmol/mg of protein (*12*) and a surface area of 3800 $cm^2$/mg of membrane protein (*13*), a mean cytochrome-to-cytochrome distance of 20 nm may be calculated. The rate constant for the slow phase (0.09 $s^{-1}$) corresponds to a half-time of about 10 s. For the cytochrome to diffuse 20 nm in 10 s, its two-dimensional diffusion constant would have to be approximately $10^{-13}$ $cm^2/s$. This is within the range commonly observed for membrane proteins and well below the value of $4 \times 10^{-9}$ $cm^2/s$ measured for rhodopsin (*14*, *15*), a membrane protein comparable to cytochrome $b_{561}$ in size and number of transmembrane domains.

The possibility of intermolecular electron transfer suggests that cytochrome $b_{561}$ will tend to distribute reducing equivalents evenly around the chromaffin vesicle membrane. Functionally, this means that reducing equivalents will be available to reduce internal semidehydroascorbate even if intravesicular monooxygenases and extravesicular ascorbate regeneration are localized near different parts of the vesicle membrane.

A number of investigators have reported phenomena suggesting the presence of more than one heme in cytochrome $b_{561}$ (*10*, *16*−*18*). These observations include multiple EPR signals and a redox titration Nernst plot having a sigmoid shape. In light of the possibility of electron transfer between cytochrome $b_{561}$ molecules, these data need to be reevaluated to see whether they can be explained by heme/heme interactions occurring intermolecularly rather than intramolecularly.

Cytochrome $b_{561}$ has an essential histidine residue. That DEPC is affecting a histidine residue is indicated by several factors. First, the inhibition is reversed by hydroxylamine. Second, inhibition by DEPC occurs most rapidly above pH 7.0, and the rate decreases below pH 7.0, consistent with reaction with a residue having a p$K_a$ near neutrality.

The essential histidine residue does not affect the heme, as indicated by a lack of effect on either absorption spectra or midpoint reduction potential. The essential histidine residue does seem to affect the ascorbate binding site. First, ascorbate protects the cytochrome from inactivation by DEPC. Second, although reaction of the cytochrome with ascorbate and semidehydroascorbate is blocked by DEPC, reaction with ferricyanide is not.

Tsubaki et al. (*11*) have argued that DEPC inhibits cytochrome $b_{561}$ reduction by ascorbate but not oxidation by semidehydroascorbate. Their data show, however, that while the rate of oxidation by SDA is not affected by DEPC, the extent of oxidation is greatly reduced. We suggest that DEPC treatment totally inhibits oxidation by SDA so the observable effect is on extent of oxidation rather than on rate. The fact that DEPC does not alter the reduction potential of the cytochrome also argues that reduction by ascorbate and oxidation by SDA must be inhibited in parallel.

Using mass spectrometry, Tsubaki et al. (*11*) have identified residues modified by DEPC as Lys85, His161, and either His88 or His92. These studies were done with purified cytochrome $b_{561}$, and our experiments used cytochrome $b_{561}$ in the native membrane. Nevertheless, since these modified residues are all on the external side of the vesicle membrane (*19*−*21*), it is likely that they are modified in our studies as well. This would suggest that either His88, His92, or His161 is located in the external ascorbate binding site, and its modification by DEPC blocks reduction by external ascorbate.

A number of possible functions of the essential histidine residue might be imagined. It could function simply in binding ascorbate to the active site. Ascorbate must lose a proton as well as an electron to oxidize to semidehydroascorbate, however, so it is possible that the histidine residue also functions as a proton acceptor.

In solution, ascorbic acid is a natural donor of single hydrogen atoms (*2*), because its reduced and radical forms are both monoanions at physiological pH. The ascorbate monoanion is a poor electron donor ($E^\circ = +0.766$ V), because it must pass through an unstable free radical intermediate to oxidize to the radical anion. The ascorbate dianion ($E^\circ = +0.076$ V) is a much more powerful electron donor than the monoanion because the dianion oxidizes directly to the semidehydroascorbate anion. The p$K_a$ for loss of the second proton is 11, however, so the fraction in the dianionic form is exceedingly small. We have argued before that cytochrome $b_{561}$ oxidizes the ascorbate monoanion by a concerted proton/electron transfer mechanism (*1*, *3*); the cytochrome has a mechanism for accepting both an electron and a proton from the ascorbate monoanion. Histidine, with a p$K_a$ of 6.5, is a likely mediator of proton transfer. This hypothesis is supported by the existence of an essential histidine residue in the ascorbate binding site of cytochrome $b_{561}$ and by the fact that blocking this histidine interferes with the reaction of the cytochrome with hydrogen atom carriers such as ascorbate/semidehydroascorbate but not with electron carriers such as ferricyanide.

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