The Energy Landscape for Ubihydroquinone Oxidation at the Qo Site of the bc1 Complex in Rhodobacter sphaeroides*

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Activation energies for partial reactions involved in oxidation of quinol by the bc1 complex were independent of pH in the range 5.5–8.9. Formation of enzyme-substrate complex required two substrates, ubihydroquinone binding from the lipid phase and the extrinsic domain of the iron-sulfur protein. The activation energy for ubihydroquinone oxidation was independent of the concentration of either substrate, showing that the activated step was in a reaction after formation of the enzyme-substrate complex. At all pH values, the partial reaction with the limiting rate and the highest activation energy was oxidation of bound ubihydroquinone. The pH dependence of the rate of ubihydroquinone oxidation reflected the pK on the oxidized iron-sulfur protein and requirement for the deprotonated form in formation of the enzyme-substrate complex. We discuss different mechanisms to explain the properties of the bifurcated reaction, and we preclude models in which the high activation barrier is in the sec- ond electron transfer or is caused by deprotonation of QH2. Separation to products after the first electron transfer and movement of semiquinone formed in the Qo site would allow rapid electron transfer to heme b1. This would also insulate the semiquinone from oxidation by the iron-sulfur protein, explaining the efficiency of bifurcation.

The ubihydroquinone:cytochrome c oxidoreductase (EC 1.10.2.2) (bc1 complex)† family of proteins is the central component of all major energy-conserving electron transfer chains (1–5). Crystallographic structures for mitochondrial complexes from three vertebrate species in six different crystals forms, with and without inhibitors, have recently been solved (6–8). Although the mitochondrial structures have 10 or 11 subunits, the mechanism is determined by a catalytic core consisting of three subunits, cytochrome (cyt) b, cyt c1, and the Rieske iron-sulfur protein (ISP), which are highly conserved between mitochondria and the α-proteobacteria from which they evolved and are the only subunits in some species (9, 10). These enzymes operate through a modified Q-cycle (4, 11, 12), involving catalytic sites for oxidation of ubihydroquinone (quinol or QH2), reduction of ubiquinone (quinone or Q), and reduction of cyt c (in mitochondria) or cyt c2 (in photosynthetic bacteria). The oxidation of quinol occurs at the Qo site through the bifurcated reaction (13), so called because it involves two 1-electron transfers to separate acceptor chains, one to the high potential chain (the Fe2S2 cluster of the ISP, cyt c1, cyt c, or cyt c2), and the second to the low potential chain (heme b1, heme b1T, and the quinone or semiquinone acceptor at the Qo site).

The reaction at the Qo site is remarkable because of the high efficiency of the bifurcation, which ensures that the second electron is delivered to the low potential chain despite the strong thermodynamic gradient favoring delivery to the high potential chain. The reaction can be initiated either by addition of quinol to the oxidized complex or by delivery of an oxidizing equivalent to the complex in the presence of quinol. In the latter case, the equivalent is brought to heme c1 through cyt c (or cyt c2) and is carried from there to the Qo site by a constrained movement of the extrinsic domain (head) of the ISP (5, 7). In addition to the three catalytic sites for binding of the external substrates (QH2, Q, and cyt c, or cyt c2), two additional catalytic interfaces for the reactions of the ISP must be included in the overall mechanism. For all mechanisms, the movement of the ISP during the catalytic cycle between reaction sites on cyt c1 and cyt b requires that the extrinsic domain of the ISP should be considered as a second substrate in formation of the enzyme-substrate complex at the Qo site, as shown in Reaction 1.

Much speculation has centered on the mechanism determining the efficiency of bifurcation and on the role of the ISP movement (14–16). The structures show that the Qo site has a volume with two domains, one proximal and the other distal with respect to heme b1 (5–8). In co-crystals, UHDBT or stigmatellin, both of which change the apparent midpoint potential and EPR spectrum of the Fe3S2 cluster (17, 18), bind in the distal domain, forming an H bond to the ISP docked at the interface on cyt b. Occupants in this domain are somewhat distant (≈11 Å between nearest conjugated atoms of stigmatellin and heme b1) from the acceptor for the second electron, the lower potential heme of cyt b (heme b1). The proximal domain is occupied by the pharmacophores of myxothiazol or MOA-stilbene in co-crystals with these inhibitors (6–8, 15, 16, 19), and these occupants are closer to heme b1 (≈6.3 Å nearest atom distances). The volumes occupied by both classes of in-
hibitor overlap in the region constrained by a narrow exit channel, occupied by the hydrophobic tails, to the lipid phase (19). A wealth of biochemical evidence suggests that the inhibitors act by displacing substrate from the Q$_o$ site (18–21).

As we have discussed elsewhere (16, 19), evidence from the structures, mutational studies, competition between inhibitors, and previous kinetic studies support mechanisms involving a single quinone/quinol species in the catalytic mechanism at the Q$_o$ site. Although double occupancy models (14, 20, 21) cannot be excluded, we believe that there are serious difficulties in explaining the data using such schemes, and we will concentrate our discussion on single-occupancy models (5, 7, 8, 15, 16, 19, 22–24). These fall into two main groups, depending on which of the two electron transfer steps precedes dissociation to products.

Crofts and Wang (22) showed that the high activation barrier and rate-limiting step were after formation of the enzyme-substrate complex. Because no semiquinone could be detected at the Q$_o$ site in Rhodobacter sphaeroides under conditions in which its formation would be strongly favored, they suggested that the mechanism likely involved a high positive $\Delta G^\circ$ for formation of the intermediate. Junemann et al. (24) have performed similar experiments with mitochondrial preparations and reached a similar conclusion. We have more recently suggested that dissociation to products (semiquinone and ISP$_{red}$) occurs at this stage (5, 7, 16, 19).

Alternatively, Link (23) has suggested that the first electron transfer occurs spontaneously within the reaction complex between quinol and the ISP$_{ox}$ to give a semiquinone-ISP$_{red}$ complex as a relatively stable intermediate state. The properties of this intermediate would suggest that the activated step must be elsewhere in the reaction mechanism. Brandt and colleagues (14, 25–27) suggested, from a pH dependence of the activation energy in the alkaline range, that deprotonation of QH$_2$ explained the activation barrier. Iwata et al. (8) have extended these ideas, using earlier suggestions from Brandt and von Jagow (13, 28), to accommodate three different configurations of the ISP in a three-state catalytic switch mechanism. In this model, the activation barrier is in the binding of quinol through prior deprotonation to the anion, and in the second electron transfer, and dissociation to products (quinone and ISP$_{red}$) occurs later. Kim et al. (15) have suggested an alternative catalytic switch model, in which the two electron transfer reactions occur “simultaneously.”

In this paper, we report on the temperature and pH dependence of the partial reactions leading to quinol oxidation in the bc$_1$ complex of the cyclic photosynthetic chain of Rh. sphaeroides. In this experimental system, the reactions of the complex can be initiated by flash illumination, since the photochemical reaction generates both substrates through reactions of secondary donor (cyt c$_2$) and acceptor (Q) molecules, and the kinetics of a single turnover can be followed on a rapid time scale. By judicious use of redox poising, specific inhibitors, and choice of appropriate wavelength, the quinol oxidation reaction can be dissected so as to provide separate measurement of the kinetic parameters defining the partial reactions. We have measured the activation barriers for each of these as a function of pH, and we estimated binding constants for each of the substrates. We show that the process with a high activation energy is after binding of substrates and most likely in the first electron transfer reaction. We discuss constraints on mechanism from the structure and from electron transfer theory, and we suggest that the restricted movement of substrates (the extrinsic domain of the ISP and the semiquinone intermediate) allows electron transfer over distances that would otherwise be forbidden by the energy-gap law of Marcus (reviewed in Ref. 29).

**EXPERIMENTAL PROCEDURES**

Measurements of kinetics of partial reactions and their temperature dependence were as described previously (22), with the following modifications. The kinetic spectrophotometer used was of conventional single-beam design, as described (30). The cuvette was stirred from the bottom using a stirring bar, and an electromagnetic driver was constructed in-house. Stirring was turned off during measurement of a kinetic trace. The sample was kept in the dark between measurements, and the measuring beam was opened ~100 ms before the flash excitation. Base-line subtraction was through an automated circuit. Control of shutter opening, stirring, flash excitation, base-line correction, and wavelength change were through a computer interface and software designed in-house. A conventional mercury bulb thermometer inserted through a rubber bung closing the top of the cuvette measured the temperature of the reaction mixture, and the reaction mixture was maintained anaerobic by a flow of argon. Redox potential was controlled by addition of small aliquots of ferricyanide or dithionite, and equilibration with the measuring redox electrodes was facilitated through added mediators as described previously (12).

Unless otherwise indicated, experiments were carried out under conditions of ambient redox potential such that the quinone pool was poised 30% reduced, and the high potential chain was completely reduced, before flash illumination. The poise was adjusted by changing the redox potential, taking account of the variation of $E_m$ with pH and with temperature, as described by Crofts and Wang (22).

Residues are numbered as in the chicken mitochondrial complex (Ref. 7 and Protein Data Bank code 1bcv) or as in Rh. sphaeroides (31) if in italic. General characterization of some mutant strains of Rh. sphaeroides has been discussed previously (E295G, Y297F, W296L, W296F in Ref. 32, and H111N, H111D, H212D in Ref. 33), and molecular engineering methods used were as described (31). Molecular engineering, thermodynamic, and kinetic properties of Rh. sphaeroides strains with mutations at Tyr-165 in the ISP (Y156H, Y156F, Y156L, Y156W) are discussed in detail. The strain 199T in which an extra threonine was inserted in helix D of cyt b leading to a modified $E_m$ for heme $b_1$ was described (35).

Data were plotted using the program Origin (version 5.0, Microcal Software, Inc., Northampton, MA), and curve fitting was done using the built-in routines. The dependence of rate on parameters of the Marcus relationship was explored using a Visual Basic computer program that plotted $\log_{10}$ (rate constant) as a function of $\Delta G$, $\lambda$, and distance using the equation of Moser et al. (36). Parameters contributing to the two separate electron transfer reactions could be varied interactively to determine the rates expected under different conditions and models (see legend to Fig. 11).

**RESULTS**

Kinetics of Quinol Oxidation Measured through Cytochrome $b_H$ Reduction—Under conditions where the quinone pool is partially reduced, the kinetics of reduction of cyt $b_H$ in the presence of antimycin are determined by the limiting reaction at the Q$_o$ site (4, 12, 22). The kinetics of cyt $b_H$ reduction as a function of temperature, shown at three different pH values in Fig. 1, indicate clearly that the rate varied strongly with both temperature and pH. In Fig. 2, Arrhenius plots of data similar

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2 M. Guergova-Kuras, R. Kuras, N. Ugulava, I. Hadad, and A.R. Crofts, submitted for publication.

3 The program for PC-compatible computers is available from ARCG on request. Marcus.exe is a stand-alone program; other files available are the Visual Basic source code and an explanatory text.
Fig. 1. Quinol oxidation assayed through reduction of cyt $b_{\text{L}}$. Kinetics of cyt $b_{\text{L}}$ reduction in the presence of antimycin, measured through the difference kinetics at 561–569 nm, at three pH values. Temperatures were (from bottom trace up in each set) 10, 15, 20, 25, 30, 35, and 40 °C. Chromatophores from $Rb.$ sphaeroides strain Ga (at 0.5 μm reaction center) were suspended in 100 mM KCl, 50 mM buffer (MES (pH 5.5–6.5); MOPS (pH 7.0); HEPES (pH 7.5); HEPPS (pH 8.0)) containing 10 μM antimycin, with the following reduct mediators: 1 μM each of 2-OH-1,4-NQ and pyrocyanin; 2 μM each of DAD, PES and PMS; 10 μM each of p-BQ, 1,2-NQ, 1,4-NQ, and DQ. Also present were 2 μM each of valinomycin and nigericin and 1 mM ferric sodium EDTA. Traces shown were the average of 20 separate traces, with 60 s between measurements. The instrument response time was <10 μs. The ambient redox potential in the cuvette was adjusted to maintain values.

Fig. 2. Arrhenius plots of cyt $b_{\text{L}}$ reduction at different pH values. Data from experiments similar to those in Fig. 1. Representative slopes for the best linear fit to the data at pH 5.5 and 7 are shown. Note that the plots are nonlinear at all pH values, indicating that more than one process contributes to the activation barrier, but they show a similar shape. The activation barrier was higher at low temperature than at high temperature. The linear best fits were similar throughout the pH range explored. The slopes drawn in the figure are linear fits to the data, assuming a single barrier, and show values for the activation energy of $\sim 45 \text{kJ mol}^{-1}$ (using $E_{\text{act}} = -k \text{slope} \times R$). This mean slope was similar over the entire pH range explored. The slopes at low temperature were fit by values $\sim 60–65 \text{kJ mol}^{-1}$, those at the high end of the range by $\sim 30 \text{kJ mol}^{-1}$, similar to the values previously reported (22).

Fig. 3. Kinetics of quinol oxidation measured through cyt $b_{\text{H}}$ reduction. A, kinetics of reduction of heme $b_{\text{H}}$ were measured by subtracting contributions from heme $b_{\text{L}}$ and the reaction center from traces measured at 566 nm, as suggested previously (12). Reaction conditions were as for Fig. 1, and pH was 8.5. B, Arrhenius plot of the data in A. Values for rate constants (ms$^{-1}$) were taken from the time constant of the reduction after the lag, measured using a single exponential component, assuming pseudo-first-order kinetics.
Quinol Oxidation by the bc₁ Complex

**Kinetics of Quinol Oxidation Measured through the Electrogenic Processes of the Complex**—A, kinetic traces at pH 8.0, at different temperatures. The electrochromic carotenoid band shift was measured from the absorbance change at 503 nm. Concentrations of mediators, and buffers used, were the same as for Fig. 1, except that antimycin, valinomycin, and nigericin were omitted. The redox poise of mediators, and buffers used, were the same as for Fig. 1, except that antimycin was omitted, and myxothiazol added, where indicated, at 10 μM.

Because the rate-determining step is the reaction at the Qo site, the initial rate reflects the rate of quinol oxidation. Fig. 4 shows the kinetics of cyt c (slow phase, cyt c₁; fast phase, cyt c₂) oxidation following flash excitation in the absence (a) and presence (b) of myxothiazol. Reaction conditions as in Fig. 1, except that antimycin was omitted, and myxothiazol added, where indicated, at 10 μM.

**Activation Barriers in the High Potential Chain**—In order to determine the location of the activation barrier, and any effect of pH, we have also measured the rates of reactions in the high potential chain as a function of temperature and pH. Fig. 5 shows the kinetics of cyt c₁ plus cyt c₂ as a function of temperature, measured at three values of pH, in the presence or absence of myxothiazol. The $E_h$ for cyt c₁ oxidation could be separated from those of cyt c₂ because of the separation of their rate constants (44). The instrument used did not have the kinetic resolution to allow us to determine the rate of cyt c₂ oxidation accurately but was fast enough to allow a clear separation from the slower rise kinetics of cyt c₁ oxidation. As observed previously, the oxidation of cyt c₁ showed a weaker temperature dependence than the reduction of cyt b₅₅₃, although the activation energy was higher than previously reported (22). Note the relatively weak dependence on pH of the rapid rise kinetics for cyt c oxidation in the presence of myxothiazol (Fig. 5b) and the strong dependence on
pH and temperature of the re-reduction phase in the uninhibited system (Fig. 5a). The latter is linked to oxidation of quinol (12), and the apparent pH dependence of the rate seen for the data in the absence of inhibitor (Fig. 6) reflects a convolution of the reduction kinetics (see "Discussion").

Transient Kinetic Features Associated with the Role of the ISP in Electron Transfer—We have noted elsewhere that information about the reactions of the ISP can be obtained by analysis of transient effects in the kinetics of both high potential chain and cyt b reduction (43). Because the extrinsic domain of the ISP must move between catalytic interfaces on cyt c1 and cyt b to ferry oxidizing equivalents to the Q_o site, any contribution of these reactions to the rate limitation would be readily apparent.

We have previously observed a transient overshoot on the kinetics of cyt c1 oxidation, which we attributed to a lag in reduction by the ISP (22, 43, 44). The overshoot was seen in the difference kinetics between the myxothiazol-inhibited system and the uninhibited complex (Fig. 7, A and B). We interpreted the overshoot as showing that, in the myxothiazol-inhibited system, the ISP was more rapidly available as a reductant than in the uninhibited system. This pattern of behavior was consistent with the crystallographic data showing the ISP close to cyt c1 in myxothiazol and MOA-stilbene containing structures (16, 19, 43). A similar position is suggested by the recent observation of loss of iron density from the cyt b docking interface on binding myxothiazol or MOA-stilbene (15). Both groups show a preferential occupancy of the cyt c1 docking domain in the presence of these inhibitors. The slowed reduction of cyt c1 observed when the pool was initially oxidized and ISP was reduced was consistent with much evidence from EPR spectroscopy of formation of a complex, indicated by the g_x = 1.800 line, between quinone and the reduced ISP (19–21, 45), presumably at the cyt b docking interface.

FIG. 6. Activation barriers in the high potential chain. Arrhenius plots of data from experiments similar to those in Fig. 5 to show the activation barrier for the oxidation of cyt c. Rate constants were calculated by fitting the oxidation phase of the kinetic curves using two first-order functions. The first component, accounting for about half the trace, was in the range <10 μs, representing the time constant of the apparatus, and was assumed to be due to cyt c2 oxidation (44). The second component was in the range 40–500 μs (depending on conditions), and was attributed to cyt c1 oxidation. The time constant of this component was used to derive the rate constants (ms⁻¹) shown. At higher temperatures and pH values, the re-reduction phase truncated the oxidation phase, leading to an artifically high estimate for rate constant. No attempt was made to deconvolute this component. Symbols indicate values from experiments under the following conditions: □, no inhibitor, pH 5.5; ▲, no inhibitor, pH 6.5; ●, no inhibitor, pH 7.0; ×, no inhibitor, pH 8.0; ○, with myxothiazol, pH 5.5; ▼, with myxothiazol, pH 6.5; +, with myxothiazol, pH 7.0; and Δ, with myxothiazol, pH 8.0. Best linear fits are shown for representative data.

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FIG. 7. Temperature dependence of transient kinetics associated with reactions of the ISP. Difference kinetics for traces with and without myxothiazol at pH 5.5 (A) or at pH 7.0 (B), taken from experiments similar to those of Fig. 5. C, Arrhenius plots for the lag phase in the cyt bH reduction. The lag before cyt bH reduction was measured from experiments similar to those of Fig. 1. The lag in milliseconds from the time of flash activation was determined by extrapolating the best straight line fit to the initial linear portion of the reductive phase to the base-line level before the flash. The value plotted is ln(1/1lag) (lag in ms), to give units comparable to those for ln(k). Representative slopes from best linear fits to the data at pH 5.5 and 7 are shown.
Although the transient kinetics of the overshoot phenomenon cannot be unambiguously deconvoluted without reference to a specific model (43), the transient was marginally smaller at lower temperature, indicating a weaker temperature dependence than for cyt c1 oxidation (Fig. 7, A and B). The transient was not obviously dependent on pH. We have not attempted to make an Arrhenius analysis of these data.

In the reduction of cyt b$_{552}$, a substantial lag before the onset of a maximal rate was previously reported (12, 46). The lag when the pool was initially oxidized (~1 ms) was attributed to the release and diffusion of the QH$_2$ from the reaction center. With the pool reduced, the lag was much shorter (~150 ms) and was attributed to the time needed to deliver an oxidizing equivalent from the reaction center through the high potential chain. This latter process involves the reactions of the ISP, including an expected movement from the cyt c$_1$ to cyt b interfaces. A major fraction of the lag was attributable to the ~150-ms rise time for cyt c$_1$ oxidation. In addition, because the redox potential of ISP over most of the pH range ($E_m$ ~300 mV at pH ≤7.0) is higher than that of cyt c$_1$ ($E_m$ ~270 mV), the redox poise of the former would be expected to lag the latter even if the kinetics were much more rapid than the delivery of oxidizing equivalents. The effects of temperature and pH on the lag can be seen in the traces of Fig. 1. It is apparent from the Arrhenius plots, shown on Fig. 7c, that the lag was much less affected by temperature than the rate of cyt b$_{552}$ reduction but that lowering the pH induced a substantial increase in the lag.

**Activation Barriers for the Partial Reactions as a Function of pH**—In Fig. 8A, Arrhenius plots for the electron transfer reactions at pH 7.0 are shown, with slopes labeled with the activation energies calculated. In Fig. 8B, the activation energies derived from Arrhenius plots similar to those in Figs. 2–7 for all the partial reactions are plotted as a function of pH. For none of the partial reactions measured did the activation energy show any substantial dependence on pH in the range tested (compare the linear fits to the data with the slope of ~5.7 kJ mol$^{-1}$ assuming the dissociation of one proton, shown by the dotted line). At all pH values, the reaction showing the highest activation barrier was quinol oxidation, as measured by cyt b$_{552}$ or cyt b$_{559}$ reduction, or through the kinetics of the electrogenic processes. The activation barrier was independent of the degree of reduction of the quinone pool and was the same when measured through reduction of either heme b$_{552}$ or b$_{559}$. The process showing the lowest activation barrier was the lag before reduction of heme b$_{553}$. Oxidation of cyt c$_1$ showed an intermediate barrier height, which was independent of inhibition by myxothiazol, showing that the contribution of movement of the ISP to electron donation did not modify the barrier.

**Dependence of the Rate of Quinol Oxidation on $E_m$ of ISP and on pH**—In Fig. 9A, traces at fixed temperature from experiments similar to those of Fig. 1 are plotted to show the relative rates of quinol oxidation as a function of pH. The rates from these curves are plotted as a function of pH (Fig. 9B) or of the fraction of the deprotonated form of ISP$_{ox}$ (Fig. 9C), calculated from the $pK_a$ value of 7.6 (47). As previously observed the rate of quinol oxidation increases to reach a plateau at pH ~7.5 (27, 48).

The effect of the $E_m$ of the Fe$_5$S$_4$ center on the rate of quinol oxidation at pH 7.0 was plotted using a logarithmic scale in Fig. 10. Values are taken from our own recent work on mutant strains in which the $E_m$ value of the center was changed in Rh. sphaeroides (Y156H, Y156L, Y156F, Y156W), and the more extensive studies from Denke et al. (49) of mutant strains in yeast. For each set, the data were normalized to the rate observed in the wild-type strain. The data fall close to a common curve, with a slope of ~0.0091 mV$^{-1}$. However, it should be noted that this value includes possible inhibitory contributions not attributable to the effect of change in $E_m$ and is biased by the lowest point, represented by a double mutation; the slope in the range of $\Delta E$ closer to the wild-type value is ~0.0051 mV$^{-1}$.

### DISCUSSION

In order to account for the high efficiency of the bifurcated reaction, it is necessary to explain how the second electron transfer is directed almost exclusively to the low potential chain. We have recently discussed a modified version of the mechanism proposed by Crofts and Wang (22), in which the first electron transfer to the ISP$_{ox}$ occurs from an enzyme-substrate complex with QH$_2$ formed after docking of the ISP$_{ox}$ on cyt b (7, 19). Our present results confirm that this is the step with high activation energy. We have suggested that the semiquinone intermediate, formed at low concentration, might move from the site of its production in the distal domain to the...
proximal domain before the second electron transfer (5, 7, 16, 19, 48). This would bring it closer to heme $b_L$ to facilitate rapid electron transfer and also remove it from the ISP docking site, so as to hinder the second electron transfer to the high potential chain. We suggested that changes in conformation of the protein around the Qo site on occupation of the proximal domain, seen in the configuration of myxothiazol (or MOA-stilbene)-containing structures, might help to insulate the pocket so as to limit reaction with external acceptors such as the ISPox and O$_2$ (19). The mechanism proposed by Iwata et al. (8), which derives from earlier models suggested by Link (23) and Brandt and colleagues (13, 27, 28), has three main postulates as follows: (i) deprotonation of QH$_2$ provides an activation barrier that precedes electron transfer; (ii) a relatively stable complex containing semiquinone and ISPr-$\Delta$ is formed as a result of the first electron transfer; (iii) the second electron transfer occurs from this complex and may also be rate-limiting. In the mechanism favored by Kim et al. (15), movement of the semiquinone was explicitly excluded, since the two electron transfers were suggested to occur simultaneously. This was based on the suggestion of Jünemann et al. (24) that movement of the semiquinone was not compatible with their failure to detect the intermediate. Both these latter models require that electron transfer to heme $b_L$ would occur from the distal domain, since they specifically require electron transfer from the enzyme-substrate complex, or an intermediate state, involving ligation with the ISP. Both groups have suggested that the bifurcation is directed by gating mechanisms (the “catalytic switch”) involving conformational changes triggered by redox events. In the Kim et al. (15) model, these are outside the quinol oxidation reactions, in the $b$-heme chain.

As discussed below, the kinetic evidence, interpreted in the light of the structural information now available, constrains proximal domain before the second electron transfer (5, 7, 16, 19, 48). This would bring it closer to heme $b_L$ to facilitate rapid electron transfer and also remove it from the ISP docking site, so as to hinder the second electron transfer to the high potential chain. We suggested that changes in conformation of the protein around the Qo site on occupation of the proximal domain, seen in the configuration of myxothiazol (or MOA-stilbene)-containing structures, might help to insulate the pocket so as to limit reaction with external acceptors such as the ISPox and O$_2$ (19). The mechanism proposed by Iwata et al. (8), which derives from earlier models suggested by Link (23) and Brandt and colleagues (13, 27, 28), has three main postulates as follows: (i) deprotonation of QH$_2$ provides an activation barrier that precedes electron transfer; (ii) a relatively stable complex containing semiquinone and ISPr-$\Delta$ is formed as a result of the first electron transfer; (iii) the second electron transfer occurs from this complex and may also be rate-limiting. In the mechanism favored by Kim et al. (15), movement of the semiquinone was explicitly excluded, since the two electron transfers were suggested to occur simultaneously. This was based on the suggestion of Jünemann et al. (24) that movement of the semiquinone was not compatible with their failure to detect the intermediate. Both these latter models require that electron transfer to heme $b_L$ would occur from the distal domain, since they specifically require electron transfer from the enzyme-substrate complex, or an intermediate state, involving ligation with the ISP. Both groups have suggested that the bifurcation is directed by gating mechanisms (the “catalytic switch”) involving conformational changes triggered by redox events. In the Kim et al. (15) model, these are outside the quinol oxidation reactions, in the $b$-heme chain.

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Quinol Oxidation by the bc$_1$ Complex

The mechanistic possibilities and excludes both the latter models as currently formulated. We will discuss the mechanism with reference to Fig. 11, which shows a schematic representation of possible features of the energy landscape of reactions at the Q$_o$ site. In this scheme, the equilibria numbered 1–6 are from previous work (22) or from this paper, and the ranges indicated by the arrows about the species 7 and 8 represent possible intermediate states discussed below.

**The Activation Barrier Does Not Depend on Deprotonation of QH$_2$**—In contrast to the Crofts and Wang (22) mechanism, Brandt and colleagues (14, 25–27) have suggested that deprotonation of QH$_2$ could explain a pH dependence of the activation barrier they measured in steady-state assays, and this aspect of the mechanism was incorporated into the Iwata et al. (8) three-state model. Brandt and Okun (27) concluded that the dissociating species must have a pH well above the range of assay and identified this with the pK for the dissociation of free QH$_2$ to QH$^-$, which has a value $>$11.3 in aprotic solvents (50). Since this value is appropriate for the unbound species, their hypothesis would imply that, in contrast to most enzymatic reactions, formation of the reaction complex did not lower the activation barrier. Our present results are clearly in contrast to the results of Brandt and Okun (27). However, it is difficult to compare the two sets of data, since Brandt and Okun (27) obtained their results using a steady-state assay of the mitochondrial complex. Since turnover involves the concerted participation of five catalytic interfaces, the steady-state assays could not allow any discrimination between the different partial reactions. Furthermore, the redox midpoint potentials of the quinone/quinol couple as substrate, and several of the prosthetic groups, show pH dependencies over the range tested, so their relative activities and the equilibrium constants for the electron transfer reactions would likely have varied with the initial conditions. In our own work, the separate partial reactions were measured, and care was taken to poised the reactants at the same initial concentration before flash activation. The results are therefore unambiguously associated with the separate partial reactions, and the rates measured could be expected to reflect accurately the rate constants for those processes.

**The Activation Barrier Is After Formation of the Enzyme-Substrate Complex**—In this work we confirm our previous observation that the activation barrier is independent of the reduction of ISP$_{ox}$ and cyt $b_L$ by QH$_2$ (12); 7 and 8, possible intermediate states with ranges shown by the arrows. B and C, theoretical curves. The curves were generated by plotting the equation of Moser et al. (36), using the values shown below (slope, $E_{1/2}$, $E_{inact}$ are in V). Superimposed on the curves are vertical bars showing the energy levels for the first electron transfer (state 3 $\rightarrow$ 7 or 8), the second electron transfer (state 7 $\rightarrow$ 8 $\rightarrow$ 5), and the overall reaction from the ES state (3 $\rightarrow$ 5). The arrows point to the intercept between these energy levels and the Marcus curve for the electron transfer reaction, and the values for rate are calculated from $k$ & $\lambda$ from Table I. The occupancy as detailed in Table I, taken from the structures with stigmatellin (11 Å nearest atom distance to heme $b_L$) or myxothiazol (6.3 Å nearest atom distance to heme $b_L$). Slopes below are values for $d(\log k)/d(-\Delta G)$ (with $\Delta G$ in electrical units, and the conventional sign to give negative values for spontaneous processes) at the intercepts of $\Delta G$ values for each reaction with the Marcus curve. B, a Link (23) model is assumed (the intermediate state labeled 7 in A, corresponding to association to products after the second electron transfer), with the following parameters: $\Delta G_{overall}$ = 0.04, $\Delta G_1$ = 0, $\Delta G_2$ = 0.04, $\lambda_1$ = 2.6, $\lambda_2$ = 0.95, $E_{1/2}$ = 0.65, $E_{inact}$ = 0.26, $R_1$ = 6 Å, $R_2$ = 6 Å, $v_1$ = 2.19 × 10$^3$ s$^{-1}$, $v_2$ = 2.39 × 10$^3$ s$^{-1}$, slope at $\Delta G_1$ = 0.0062/mV, slope at $\Delta G_2$ = 0.0065/mV. C, a modified Crofts-Wang model is assumed (the intermediate state labeled 6 in A, corresponding to dissociation to products after the first electron transfer), with the following parameters: $\Delta G_{overall}$ = 0.04, $\Delta G_1$ = 0.275, $\Delta G_2$ = −0.253, $\lambda_1$ = 2.01, $\lambda_2$ = 1.2, $E_{1/2}$ = 0.65, $E_{inact}$ = 0.26, $R_1$ = 6 Å, $R_2$ = 6.3 Å, $v_1$ = 2.23 × 10$^3$ s$^{-1}$, $v_2$ = 1.34 × 10$^3$ s$^{-1}$, slope at $\Delta G_1$ = 0.0076/mV, slope at $\Delta G_2$ = 0.0055/mV. See text for discussion.
degree of reduction of the quinone pool (22). Our present results extend the range of validity for this conclusion for an initial poise of the pool from fully oxidized to 90% reduced. We have argued elsewhere that the pH dependence of the rate of electron transfer in the acidic range shows that the formation of the enzyme-substrate complex depends on deprotonation of the ISP_{ox} (47, 48). If this is so, then the lack of dependence of activation energy on pH when the quinol concentration was kept constant also shows that the barrier is not affected by the concentration of the second substrate, deprotonated form of the oxidized ISP. Both these results are fully consistent with our previous conclusion that the activation energy is after formation of the enzyme-substrate complex (22).

**Binding Constants for Substrates**—The rate of quinol oxidation increases with degree of reduction of the pool (increasing [QH_{2}]) to reach saturation with the pool still substantially oxidized (12, 22, 42, 46, 51). Crofts and Wang (22) could fit their kinetic data at different redox potentials with a model in which QH_{2} was bound preferentially over Q, with a ~50-fold tighter binding constant. In contrast, redox titrations of the amplitude of the g_{s} = 1.800 band in the EPR spectrum of the ISP, associated with formation of a complex between quinone and ISP_{red} titrated near the mid-point of the quinone pool (20, 21), implying similar binding constants for Q and QH_{2}. This apparent paradox is readily resolved by noting that the complexes are different. For oxidation of quinol, the enzyme-substrate complex is formed between quinol and ISP_{ox}, and the relative affinities would be expected to differ from those assayed through the g_{s} = 1.800 complex, where the apparent E_{m} reflects the differential binding between quinone or quinol and ISP_{red}. Recent refinement of structures of the chicken mitochondrial complex in the presence of stigmatellin4 have shown that Glu-272 of the conserved –PEWY– loop is a second ligand to quinol but not to ISP_{ox}. The rate of quinol oxidation remained the same, and similar to the apparent pH dependence was exaggerated. When similar experiments were performed with the quinone pool oxidized before the flash, no re-reduction occurred over the 1-ms range, and this artifact of convolution was avoided. Then, the pH dependence of oxidation was similar to that in the presence of myxothiazol (not shown, but see Fig. 8A for pH 7.0 values). In all cases, the activation barrier for cyt c_{1} oxidation remained the same, and similar to the apparent activation energy for quinol oxidation in the higher temperature range. This suggests that the mean linear slope (~45 kJ.mol^{-1}) may underestimate the true activation barrier, which likely is that seen at lower temperature, and this should be taken into account in the quantitative arguments below. The dashed arrow in Fig. 11 extends to the upper limit of the likely range (~65 kJ.mol^{-1}).

In the treatment below we ignore the separate contributions of enthalpy and entropy to the free energy of activation. The entropic contributions of binding of substrates and unbinding on dissociation to products are unlikely to contribute to the activated step so that, for the reaction from the enzyme-substrate complex state, it is likely that E_{act} ~ ΔG^{‡} (29). However, assignment of the dissociation to products to a particular partial reaction will determine the entropic contribution to the energy levels on the product side of the scheme in Fig. 11. We recognize that this lack of partitioning is a simplification, but we do not believe that a more rigorous treatment is justified by the results or would modify the conclusions below.

**Constraints from Theory**—Marcus theory has been tested in the context of electron transfer reactions in photochemical redox reaction centers and similar “solid-state” systems, and the expected constraints on electron transfer due to distance and the interplay between ΔG^{‡}, ΔG^{2}, and reorganization energy, λ, have been convincingly demonstrated (36, 54). The results have been encapsulated in the empirical formula suggested by Moser et al. (36) (Dutton’s ruler) and the Marcus energy-gap law (29, 55) given in the legend to Table I. Application of Marcus theory to the reaction at the Q_{o} site is complicated by...
### Table I

| $K^b$ | $\Delta K^b$ | $E_b^H/QH_2$ | $E_b^Q/QH_1$ | $\Delta G/RT^b$ | occupancy$^{a,%}$ | $R^b$ | $V^b$ | $s^{-1}$ | Rate$^k$ |
|------|-------------|--------------|--------------|----------------|------------------|------|------|--------|--------|
|      |             |              |              |                |                  |      |      |        |        |
| A. Mechanisms with electron transfer to heme $b_1$ after dissociation of the reaction complex |
| $2 \times 10^{-4}$ | $-218$ | 528 | $-268$ | $-178$ | 0.01 | 11 | 0.5 | $5.7 \times 10^7$ | $5.7 \times 10^5$ |
| $2 \times 10^{-4}$ | $-218$ | 528 | $-268$ | $-178$ | 0.01 | 6.3 | 0.5 | $3.8 \times 10^{10}$ | $3.8 \times 10^9$ |
| $2 \times 10^{-4}$ | $-218$ | 528 | $-268$ | $-178$ | 0.01 | 11 | 1.0 | $2.0 \times 10^6$ | $2.0 \times 10^5$ |
| $2 \times 10^{-4}$ | $-218$ | 528 | $-268$ | $-178$ | 0.01 | 6.3 | 1.0 | $1.3 \times 10^9$ | $1.3 \times 10^8$ |
| $2 \times 10^{-4}$ | $-218$ | 528 | $-268$ | $-178$ | 0.01 | 11 | 2.0 | $1.8 \times 10^6$ | $1.8 \times 10^5$ |
| $2 \times 10^{-4}$ | $-218$ | 528 | $-268$ | $-178$ | 0.01 | 6.3 | 2.0 | $1.2 \times 10^6$ | $1.2 \times 10^5$ |
| $2.3 \times 10^{-5}$ | $-274$ | 584 | $-324$ | $-234$ | 0.005 | 11 | 0.5 | $9.1 \times 10^5$ | $9.1 \times 10^4$ |
| $2.3 \times 10^{-5}$ | $-274$ | 584 | $-324$ | $-234$ | 0.005 | 6.3 | 0.5 | $6.0 \times 10^9$ | $6.0 \times 10^8$ |
| $2.3 \times 10^{-5}$ | $-274$ | 584 | $-324$ | $-234$ | 0.005 | 11 | 1.0 | $3.8 \times 10^6$ | $3.8 \times 10^5$ |
| $2.3 \times 10^{-5}$ | $-274$ | 584 | $-324$ | $-234$ | 0.005 | 6.3 | 1.0 | $2.5 \times 10^5$ | $2.5 \times 10^4$ |
| $2.3 \times 10^{-5}$ | $-274$ | 584 | $-324$ | $-234$ | 0.005 | 11 | 2.0 | $2.5 \times 10^{15}$ | $2.5 \times 10^{14}$ |

B. Mechanisms with electron transfer to heme $b_1$ from the reaction complex |

| $1.0$ | $0$ | $310$ | $-50$ | $40$ | $0.5$ | $11$ | $0.5$ | $3.9 \times 10^5$ | $1.95 \times 10^6$ |
| $1.0$ | $0$ | $310$ | $-50$ | $40$ | $0.5$ | $11$ | $1.0$ | $1.1 \times 10^5$ | $5.5 \times 10^4$ |
| $0.01$ | $-118$ | 428 | $-168$ | $-78$ | 0.01 | $11$ | $0.5$ | $2 \times 10^7$ | $2 \times 10^6$ |
| $0.01$ | $-118$ | 428 | $-168$ | $-78$ | 0.01 | $11$ | $1.0$ | $5.8 \times 10^9$ | $5.8 \times 10^8$ |

C. Mechanisms with electron transfer to heme $b_1$ from the transition-state complex or from semiquinone at a concentration determined by the transition state |

$2 \times 10^{-8}$ | $-466$ | 776 | $-516$ | $-426$ | $2 \times 10^{-8}$ | $11$ | $0.5$ | $2.3 \times 10^8$ | $4.6$

### Footnotes
1. For reaction either (i) $E_{b_1}/QH_2$ $\rightarrow E_{b_1}/QH_1$ $\rightarrow$ HIS$^{+}$ or (ii) $E_{b_1}/QH_2$ $\rightarrow$ HIS$^{+}$ $\rightarrow$ $E_{b_1}/QH_1$ + HIS. Since neither semiquinone nor reduced ISP were detectable, their concentration is put at the detection limit. For [QH], this is taken as <1%. We assume that the enzyme-substrate complex is fully populated under oxidant-induced reduction conditions. If an intermediate complex is formed (i) and has a concentration given by the limit of detection then $K$ (Equation 2) is 0.01.

$$K = \frac{[E_{b_1}/QH^{\text{HIS}^{+}}]}{[E_{b_1}/QH_{2}^{\text{ISP}^{+}}]}$$

If, under normal forward values, semiquinone can be formed at concentrations higher than those detected, but is masked under conditions of oxidant-induced reduction (24), then higher values for $K$ could be expected. An example assuming $K = 1$ is included. If dissociation to products occurs (ii), then (assuming uncertainties in detection of 1 and 2% for QH and ISP, then $K$ (Equation 3) is $2 \times 10^{-4}$.

$$K = \frac{[E_{b_1}/QH^{\text{HIS}^{+}}]}{[E_{b_1}/QH_{2}^{\text{ISP}^{+}}]}$$

If the semiquinone product of Reaction (i) or (ii) determines the activation barrier, the value for $K$ comes from $\Delta G = -RT \ln K$, to give $K \approx 2 \times 10^{-8}$ for an activation energy of 45 kJ mol$^{-1}$. See ‘Discussion’ for explanation of the value for $K$ of $2.3 \times 10^{-5}$.

2. From $\Delta E = (RT/s) \ln K$, where $\Delta E$ is $E_m$ (ISP) $- E_m$ (QH/QH) (bound).

3. $E_m$ values for the bound semiquinone couple, using $K$, $E_m$ for ISP of 310 mV; $E_m$ for QH2 (bound) of 130 mV (shifted from $E_m$ for QH2 pool of 90 mV because of preferential binding of QH$_2$).

4. $E_m$ for cyt bd$_2$ of ~90 mV, and $E_m$ (QH/QH') calculated from the values above.

5. Occupancy of semiquinone: if the occupancy were determined by Reaction (ii) in Footnote 1, the maximal occupancy would be given by the detection limit, since $[E_{bd_1}/QH]$ and [HIS$^{+}$] are separate terms and interaction of spins would be unlikely. For $K$ of $2.3 \times 10^{-5}$, occupancy was set at $10%$.

6. In models with electron transfer from a reaction complex at the distal domain, $E_{bd_1}/QH_{2}^{\text{HIS}^{+}}$ is a single species, $K = [E_{bd_1}/QH_{2}^{\text{HIS}^{+}}]/[E_{bd_1}/QH_{2}^{\text{ISP}^{+}}]$, and occupancy is given by $K$, and is $K$ if occupancy of the enzyme-substrate complex is close to 1. If the intermediate complex is assumed to quench the EPR signals of the contributing species, $K$ might well be higher than indicated by the detection limit.

7. With $K$ derived from the activation energy, $E_{bd_1}/QH_{2}^{\text{HIS}^{+}}$ is a single species, and the occupancy is given by $K = [E_{bd_1}/QH_{2}^{\text{HIS}^{+}}]/[E_{bd_1}/QH_{2}^{\text{ISP}^{+}}]$.

8. For nearest atom (edge-to-edge) distances to heme $b_1$, $R = 11$ Å for stigmatellin (distal domain); $R = 6.3$ Å for myxothiazol (proximal domain).

9. All complexes with ISP must form at the distal domain.

10. The relation between $\Delta G$, $\Delta G^*$, and $\Lambda$ is given by the energy-gap law of Marcus theory (29, 55) ($\Delta G = (\Lambda + \Delta G^*)^2/\lambda$), see Devault (29) for a comprehensive review. Typical values for $\Lambda$ in the literature for solid-state systems fall in range 0.5–1.4 eV. However, for activation energy of 45–60 kJ mol$^{-1}$ of the quinol oxidation reaction, a value of $\sim 2.5$ eV is appropriate, since $\Delta G^*$ for the reaction ($\sim 0$ kJ mol$^{-1}$) is $<\Lambda$.

11. Rate for transfer of the second electron, expressed as mol$^{-1}$ (mol $b_1$)$^{-1}$.s$^{-1}$, using $K \times$ occupancy. The values in bold are compatible with the measured rate. Those in italic are too slow, and those in normal text are marginal.

A Visual Basic program (Marcus) for exploration of these parameters is available (see footnote 3).
the fact that the two electron transfers occur between two different pairs of reaction partners, but the two reactions are intimately coupled through the common semiquinone intermediate. A value for $\Delta G^0\text{sl}$ for the overall reaction can be readily determined from the $E_{\text{m}}$ values of the reactants (4, 12) and corrected for binding of substrates to give a value with respect to the enzyme-substrate complex (Fig. 11). The partitioning of the overall free-energy change between the two partial reactions then has to take account of the possibilities provided by the different models proposed. Although specific aspects of the mechanism proposed by Iwata et al. (8) can be excluded (see above), with variation of particulars the Iwata et al. (8), Crofts and Wang (22), and Kim et al. (15) models represent examples from a continuum of possibilities in which the intermediate semiquinone is more or less stable. Because the 2-electron transfer reaction involves a semiquinone intermediate, there is an interesting trade-off between $\Delta G^0\text{sl}$ values for the two 1-electron transfers and semiquinone concentration. Both these terms are important in determining the rates of reaction. In addition, the two different domains of occupancy of inhibitors found in the structures (6–8) open the possibility of different distances over which electron transfer could occur if the intermediate semiquinone moved (16, 19). Finally, the energy-gap law provides a relation between $\Delta G^0\text{sl}$, $\Delta G^0$, and $\lambda$, that, together with distance, constrains values for the rate constants. As a consequence, the rates of the two reactions will depend on the contribution of semiquinone stability to both occupancy and the $\Delta G^0$ values for the electron transfers, on distance and on $\lambda$, as determined by the activation energy and $\Delta G^0\text{sl}$. Sets of representative values for different classes of mechanism are shown in Table I. In the discussion below, subscripts 1 and 2 to $\Delta G^0\text{sl}$, $\lambda$, etc. indicate values for the first and second electron transfer steps, respectively. We recognize that a more rigorous treatment of tunneling pathways might be appropriate in the future, but, as Williams has pointed out (56), imprecision in experimental data, together with poorly defined pathways, preclude a more detailed discussion; the simplifications used here will serve for discussion.

**Mechanisms in Which the Activation Barrier Is in the Second Electron Transfer Are Precluded by Distance and Activation Energy**—For both the Iwata et al. (8) model and that favored by Kim et al. (15), the second electron transfer to heme $b_1$ has to occur from semiquinone in a complex with ISPred. Since the reaction complex suggested involves a tight liganding to the ISP, it must be formed at the distal end of the Q$_b$ pocket. In the mechanism suggested by Link (23), oxidation of quinol proceeded through an intermediate complex in which the first electron transfer produced a relatively stable semiquinone-ISPred state. This feature was adopted by Iwata et al. (8) in their 3-state mechanism. Although Iwata et al. (8) followed Brandt (25–27) in placing the activation barrier in the deprotonation of QH$_2$ on binding, our results above make this seem unlikely. We can consider a similar mechanism, involving a stable intermediate semiquinone, in which the activation barrier is elsewhere. The most obvious step would be in the second electron transfer reaction (see Ref. 8). In the Kim et al. (15) mechanism, since the two electrons are transferred simultaneously, the activation barrier is implicitly in the electron transfer to heme $b_1$, and we must assume that the semiquinone is at or close to the transition state (22). From the structures we can estimate a maximal rate constant for the electron transfer from semiquinone to heme $b_1$, using the crystallographic distances and Dutton's ruler (36). For $R \sim 11$ Å, the distance between stigmatellin and the heme edge, appropriate for reaction from the distal domain, $k_{\text{max}} = 2.51 \times 10^8$ s$^{-1}$ when $-\Delta G = \lambda$. This might seem to indicate that electron transfer would always be fast enough to meet the expectations from experiment ($k \sim 1.7 \times 10^9$ s$^{-1}$, see Ref. 22). However, more detailed consideration shows that this is not the case for two reasons. (i) The structures show that the intervening protein is mainly from helix C, perpendicular to the path, and with poor contacts to the head group of the distal domain occupant. A possible path via the –PEWY– loop through a poor contact between heme $b_1$ and Tyr-274 is unlikely to be of importance, because a mutation to this residue in *Rb. sphaeroides* (Y297F) showed minimal effects on electron transfer rate (32). From the lack of a suitable path, the $k_{\text{max}}$ value above is likely too high a value. (ii) The actual rate constant will depend on the matching between $\Delta G$ and $\lambda$, which are related to the activation barrier through the Marcus energy-gap law (see legend to Table I) Since $\Delta G^\text{overall}$ is close to 0 ($K_m \sim 0.3$ for reduction of heme $b_1$ and the ISP ox by bound QH$_2$, see Ref. 22) and in the Iwata et al. (8) and Link models, $\Delta G_1$ for the first electron is also close to 0, $\Delta G_2$ would also be close to 0, and so $\ll \lambda$. An approximate value is therefore given by $\lambda \sim \Delta G^2$ or a reorganization energy $-2$ V for 45–50 kJ·mol$^{-1}$ (from the slope of the linear fit). With these values ($r = 11$ Å, $\Delta G^0 = 0$, $\lambda = 2$ V), we obtain a rate constant of $-158$ s$^{-1}$ for electron transfer through 11 Å, an order of magnitude lower than the measured rate. Because of the low value for rate constant calculated using $\lambda$ of 2 V or higher, no obvious combination of reaction parameters would provide a rate for reduction of heme $b_1$ as fast as that measured, if the reaction involved the activation barrier and the distance was 11 Å (Table I, part B).

**Kinetic Constraints from Effects of Mutation**—Additional constraints can be provided by consideration of data from mutant strains with modified $E_m$ values for ISP or cyt $b_1$. In principle, the change of rate on change of $E_m$ could arise from a number of effects. Most obviously, lowering the redox midpoint potential of the ISP ($E_m^\text{ISP}$) will decrease and raising the $E_m^\text{ISP}$ would increase the driving force ($-\Delta G^0$) for the overall reaction, and change the rate of the controlling reaction through the Marcus effect (29, 55). However, since the overall $\Delta G^0$ is partitioned between the two partial processes, more detailed theoretical considerations become model-dependent. In this partitioning, the profile of the Marcus curve, and hence rate constant, will depend on values for $\lambda$, $\Delta G^0\text{sl}$, and $\Delta G^0$ for the individual reactions, and $\Delta G^0\text{sl}$ for the partial processes depends on the semiquinone stability, which also determines occupancy, and hence rate. In principle, if the activation barrier for one of the steps was much higher than the other, then that step would show the largest control on rate when $\Delta G^0\text{sl}$ is varied. The interplay between $\Delta E_m$ and activation energy can be discussed in the context of two approaches as follows: (i) classical Eyring, Randall-Wilkins extension of the Arrhenius equation (discussed in Ref. 29); and (ii) substitution of the Marcus relationship into the Arrhenius equation. In the latter case, differentiation gives the control coefficient of DeVault (29) or the slopes at the intercepts of the curves obtained by plotting the Moser et al. (36) equation, in which quantum mechanical contributions are included in the numerical constants (see Fig. 11).

From the detection limit of semiquinone, Crofts and Wang (22) concluded that the value for the equilibrium constant of the first electron transfer was very low, and they suggested as the most parsimonious hypothesis that the activation barrier might be determined by the large positive $\Delta G^0\text{sl}$ for formation of semiquinone, i.e. that the semiquinone was the activated state. The Kim et al. (15) mechanism seems to imply a similar situation. Mutations that lower $E_m^\text{ISP}$ (Fig. 10) provide a test of this hypothesis. The slowing of rate on lowering $E_m^\text{ISP}$ seen in a wide range of ISP mutants (49, 57, 58) is as expected if this was the limiting step. If the transition state was determined by
semitriquinone occupancy, and if \( E_m \) for the semitiquinone couples was unaffected by mutation, a decrease in \( E_{m(ISP)} \) would increase \( E_{act} \) proportionately, to give a decrease in rate constant approaching that expected from the Arrhenius relationship. The decrease in rate with change in \( E_m \) of ISP (a slope of between 0.0051 and 0.0091 mV\(^{-1}\) in Fig. 10) was rather less than that expected from a simple Arrhenius treatment (a slope of 0.017 mV\(^{-1}\) at 25 °C is expected from \( \log(k/\bar{k}) \propto -(E_{act} - E_{act})/59 \)). From this, it is unlikely that the activation barrier is determined solely by the probability of formation of semitiquinone. By using the Marcus relationship, the slopes at the intercept \( \Delta G_i \) in Fig. 11 show values –0.006 when \( \Delta G_i = 0 \) (Fig. 11B) and –0.007 when \( \Delta G_i \) is more positive (Fig. 11C), both compatible with the data. The steeper slope of Fig. 10 would require a positive \( \Delta G_i > 0.55 \) V, at the limit of the realistic range but still less than the probable activation barrier. The large effect observed strongly suggests that the first electron transfer is the limiting process and is quite compatible with the experimental finding of an undetectable level of semitiquinone.

In the original Link (23) model, changing the \( E_{m(ISP)} \) would be expected to change the stability of the semitiquinone-ISP\(_{red} \) complex and \( \Delta G \) for formation of this state, but since this step was not rate-determining and did not contain the activation barrier, the strong effect observed would not be expected. A modified Link mechanism in which the activation barrier is in the first electron transfer step, but with \( \Delta G_i \) close to 0, is considered below and would overcome this objection to the model.

If the activation barrier were in the second electron transfer, this step would be rate-determining at saturating substrate. The \( E_m \) value of ISP would not contribute directly to the driving force \( \Delta G_i \), but a change in \( E_m \) for heme \( b_L \) would be expected to change the rate constant through a Marcus effect and hence the overall rate. The complexes from bacteria (–90 mV), chloroplasts (–150 mV), and mitochondria (–40 mV) show wide variation in \( E_m \) of heme \( b_L \) (as indicated by the values in parentheses) but operate at similar maximal rates and with similar \( E_m \) values for the other substrates. We have previously characterized mutant bacterial strains with \( E_m \) for heme \( b_L \) shifted to higher values, which showed similar or slower rates (W296L and W296F in Ref. 32; H111N, H111D, and H121D in Ref. 33; and 199T in Ref. 35). Since the shift of \( E_{m(bL)} \) to higher values would give a more negative \( \Delta G \) for the second electron transfer, the rate would be expected to increase. Both sets of data suggest that the value for \( E_{m(bL)} \) is not critical. This point is underscored by the fact that the activation energy for quinol oxidation was the same whether measured through reduction of heme \( b_H \) or heme \( b_L \) (see Ref. 22 and see Figs. 1–3 and 8B). The operational \( E_m \) of heme \( b_L \) would likely be lower when heme \( b_H \) was pre-reduced, because of coulombic interactions over the 21-Å Fe-Fe distance between the hemes (cf., Ref. 33). The lack of any major effect on the rate or the activation energy could therefore be taken as indicating that a change in \( E_{m(bL)} \) does not affect the barrier determining the overall rate constant. Because the rate and activation energy were not strongly dependent on \( \Delta G_2 \), it seems very unlikely that this is the rate-determining process.

Clearly it would be interesting to explore the effects of change in \( \Delta G \) on rates for the partial reactions more fully, since more precise values would provide additional constraints through comparison with the theoretical curves. It is worth noting that an increase in \( \Delta G_i \) (for the first step) increases the slope of the Marcus curve at the intercept with \( \Delta G_i \), whereas the need to maximize the rate constant to achieve compatible rates for the second step pushes the intercept with \( \Delta G_i \) toward the limiting value at which \( d\log k/d(\Delta G_i) \) approaches 0 (as the driving forces approaches \( \Delta G_i \) (22). Interestingly, as this limit is approached, the lack of effect of \( \Delta G_i \) on rate would be explained without the need to have an intrinsic rate faster than the limiting rate.

The first electron transfer must occur from a configuration in which quinol and ISP\(_{red} \) are at a similar distance to that indicated by the stigmatellin structure, with ∼7 Å from the quinol to the Fe₂S₂ cluster (7, 53). Electron transfer would be through the bridging His-161, and the intrinsic rate constant would be expected to be high and would provide minimal constraint on the rapidity of the reaction. However, this raises the question of why the reaction is so slow. For mechanisms in which the barrier is in the first step, the measured activation energy and the distance shown by the stigmatellin structure determine the choice of parameters. The distance chosen depends on the role of the bridging histidine. By using the Moser et al. (36) equation, an activation energy of 0.65 V, a distance of 2.9 Å for the H bond between stigmatellin and His-161, and \( \beta \) calculated using the activation barrier and any reasonable value for \( \Delta G \), the rate constant would be too large to give a rate as slow as that observed. Distances more than ∼6 Å would be compatible with the measured rate for a range of plausible values for \( \Delta G \), suggesting that the bridging histidine is not tightly coupled electronically to the cluster. For activation energies <0.6 V (∼58 kJmol⁻¹), a rate constant determined using values for \( \beta \) calculated with any plausible \( \Delta G \) for the first electron transfer, and reasonable stigmatellin-iron nearest atom distance (oxygen to iron distances, 7.1 for –C=O; 6.65 for –O-Me), would be too high for the rate observed. This suggests that the true activation energy is at the high end of the range measured (as observed at lower temperature) and that His-161 and the bonds to iron and to quinol provide a path with a \( \beta \) coefficient measured over the value of 1.4 incorporated into Dutton’s ruler (36).

Although the first reaction would be rapid, if the concentration of semitiquinone product was as low as indicated by experiment (22, 24), the rate constant for the second electron transfer could be limiting, especially if the distance involved were that from the distal domain. This is because, in addition to the constraint provided by \( \beta \), the rate of the second electron transfer reaction would be determined by the product of the rate constant and the concentration of the semitiquinone substrate. As noted above, estimations of these values are not independent.

It is useful to distinguish between mechanisms in which dissociation of the ISP\(_{red} \) occurs after the first electron transfer and those in which it occurs after the second electron transfer.

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5 B. Barquera, R. Gennis, and A. R. Crofts, unpublished observations.
The latter class of mechanism (8, 15, 23) would open the possibility of occupancy by semiquinone at concentrations higher than the detection limit of the EPR experiments, since semiquinone might be undetected because of interaction between neighboring spins (24). A number of scenarios could then provide realistic rates (Table I, part B). If the second electron transfer were after the activation barrier, the value for $\lambda$ would certainly be lower than for the first. However, with the semiquinone bound in the distal domain, electron transfer would have to occur over the 11-Å distance, and the interplay between the Marcus parameters and occupancy would limit the options. With occupancy close to 1, and if the intrinsic rate for the second electron transfer had to be ~10-fold more rapid than the measured rate (see discussion of mutant strains with changed $E_m$ in the above), $\lambda$ would have to be <1.2; occupancy of the semiquinone at 0.01 would require a $\lambda$ <0.8 V. Although the positive $\Delta G_1$ associated with low occupancy would make $\Delta G_2$ for the second electron transfer more negative, operation in this region would imply a much lower activation energy over a longer distance than that for the first electron and operation in the region where $-\Delta G_2$ approaches $\Delta G_1$.

Mechanisms in the former class (16, 19), with the dissociation to products after the first step, open up the possibility of movement of the semiquinone product and thereby provide a wider range of rapid pathways for the second electron transfer (Table I, part A). However, after separation of products, the possibility that semiquinone went undetected because of interaction between spins (24) is lost, so occupancy would be equal to or less than the detection limit. If the semiquinone moved to the proximal domain (R = 6.3 Å, from myxothiazol or MOA-stilbene structures), the maximal rate constant would be about 3 orders of magnitude higher than from the distal domain, and occupancy and reorganization energy would be less critical. It has been suggested that the failure to detect semiquinone at the Q$_b$ site under conditions of “oxidant-induced reduction” excludes this type of mechanism (15, 24). However, this is clearly not the case. Indeed, if $\lambda$ was much greater than 1 V (if occupancy was ~1) or 0.5 V (if occupancy was less than the assumed detection limit), the measured rate would require movement to the proximal domain. Even if the concentration of semiquinone was limited to that calculated from the mean activation energy (~45 kJ-mol$^{-1}$), a rate compatible with the measured rate could be achieved at a reasonable value of $\lambda$ (~3 $\times$ 10$^{-5}$ s$^{-1}$ at $\lambda$ ~0.5) (Table I, part C), but this would require operation at the limiting rate constant ($-\Delta G \sim \lambda$). If activation energy were much higher, the calculated occupancy would be too low to achieve the measured rate for the second step.

Fig. 11, B and C, shows theoretical curves that fit the kinetic data. They illustrate models in which the first electron transfer has $\Delta G_1$ ~0, and dissociation to products is after the second electron transfer (23) (in Fig. 11B), or in which $\Delta G_2$ is positive, and dissociation to products occurs after the first electron transfer (22) (in Fig. 11C). Intermediate scenarios provide plausible parameters, but as the value for $\Delta G_2$ is made more positive, $\lambda_2$ has to be made smaller to accommodate the 11-Å constraint.

Double Occupancy— Several groups (14, 20, 61) have considered the mechanism of the Q$_b$ site in the context of the structure and a double occupancy by two quinone species. We have discussed some of the difficulties presented by double occupancy elsewhere (16, 19, 32). However, we should note here that we cannot exclude the possibility of double occupancy and that such mechanisms provide a ready solution to the problem of electron transfer over the distance between the distal domain occupant and heme $b_1$, through a bridging function of a second quinone in the proximal domain. Clearly, double occupancy would be a more attractive alternative if single occupancy models failed under the constraints discussed above. Since this is not the case, further discussion must await compelling evidence for double occupancy and a more detailed quantitative model.

Conclusions—Our results suggest that the choice of mechanistic models should be constrained to those with the following properties. 1) The activation barrier is after formation of the enzyme substrate complex. 2) The activation barrier is not determined by deprotonation of any group with a $pK_a$ in the physiological range or higher. 3) The activation barrier is in the first electron transfer step. 4) The intermediate semiquinone is not the activated state. 5) From the results reported here, two scenarios for the role of intermediate states seem plausible. (a) For a modified Link (23) mechanism, the kinetics are compatible with a mechanism in which dissociation to products occurs after the second electron transfer. However, since the second electron transfer has to occur over the 11-Å distance from semiquinone bound in the distal domain of the Q$_b$ site to heme $b_1$, realistic mechanisms require a high occupancy of the intermediate state. Since no semiquinone is detectable, a plausible case for a mechanism with $\Delta G_1$ ~0 requires demonstration that the ISP$_{red}$ and semiquinone are formed, but undetected by EPR, under conditions of oxidant-induced reduction. (b) For a modified Crofts-Wang (16, 19) mechanism, this model is in line with the experimental evidence showing that semiquinone and ISP$_{red}$ are undetected under conditions of oxidant-induced reduction (22, 24). However, because of the low concentration of intermediate semiquinone, movement of the semiquinone to the proximal domain from the site of its formation in the distal domain may be necessary to allow rapid reduction of heme $b_1$ in the second electron transfer reaction. Such a movement has not been demonstrated but is compatible with evidence from the structures and the consequences of mutation (5, 16, 19).

As we have noted elsewhere (16, 19), movement of the semiquinone intermediate to the proximal domain would be advantageous for reasons other than the kinetic arguments above. The efficiency of bifurcation between the high and low potential chains is a prerequisite for energy conservation through the Q-cycle. The efficiency depends on the discrimination between heme $b_1$ and ISP$_{red}$ as potential acceptors for the second electron. The thermodynamic gradient clearly favors transfer to the high potential chain, so the discrimination must be mechanistic. In contrast to Link (23), who suggested that the $E_m$ of ISP$_{red}$ was raised into an unreactive range by interaction with semiquinone, Brandt and von Jagow (28) argued for a catalytic switch in the Q$_b$ site, which controlled the physical interaction between reaction partners, whereas Jünemann et al. (24) argued for a purely kinetic model, based on the low occupancy. In the model we favor, both these latter elements are incorporated. In addition to providing a favorable kinetic pathway for reduction of heme $b_1$, movement of the semiquinone to the proximal domain would remove it from the point of access for reaction with the ISP$_{red}$. As we have discussed elsewhere, the structures with inhibitors in place show substantial differences in conformation that might be considered as models for the configurations adopted when quinone species occupy the different domains of the Q$_b$ site. In particular, in the myxothiazol and MOA-stilbene structures, conformational changes around the channel through which ISP$_{red}$ interacts with stigmatellin close the aperture, thus insulating the occupant (19).

Insulation of semiquinone from interaction with exogenous acceptors might be important in another context. Production of superoxide anion by reduction of oxygen at the Q$_b$ site of the mitochondrial bc$_1$ complex is a major source of the oxidative damage that is now thought to be largely responsible for cellu-
lar aging (62). In a wider context, the appearance of O$_2$ in the biosphere some 1.5–2 billion years ago would have challenged existing organisms, and it seems highly likely that refinement of the bc$_1$ complex mechanism to minimize O$_2$ production was a key to survival for species that adapted to this new condition. From the redox chemistry of the O$_2$/O$_2$ couple (34), a strong reductant is needed, and the semiquinone at the Q$_o$ site is an obvious candidate. Clearly, the low occupancy of the semiquinone would minimize the rate of this reaction. In addition, movement of the semiquinone, and insolation by the conformational changes discussed above, would reduce the probability of collision with O$_2$ compared with exposure at the vacant ISP docking interface. These secondary arguments provide a rationale for the evolution of the bifurcated volume of the Q$_o$ site and explain the effects of mutations that suggest that occupation of the proximal domain is important in function (16, 19).

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