Physicochemical Aspects of the Movement of the Rieske Iron Sulfur Protein during Quinol Oxidation by the bc1 Complex from Mitochondria and Photosynthetic Bacteria

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ABSTRACT: Crystallographic structures for the mitochondrial ubihydroquinone:cytochrome c oxidoreductase (bc1 complex) from different sources, and with different inhibitors in cocrystals, have revealed that the extrinsic domain of the iron sulfur subunit is not fixed [Zhang, Z., Huang, L., Shulmeister, V. M., Chi, Y.-I., Kim, K. K., Hung, L.-W., Crofts, A. R., Berry, E. A., and Kim, S.-H. (1998) Nature (London), 392, 677–684], but moves between reaction domains on cytochrome c1 and cytochrome b subunits. We have suggested that the movement is necessary for quinol oxidation at the Qo site of the complex. In this paper, we show that the electron-transfer reactions of the high-potential chain of the complex, including oxidation of the iron sulfur protein by cytochrome c1 and the reactions by which oxidizing equivalents become available at the Qo site, are rapid compared to the rate-determining step. Activation energies of partial reactions that contribute to movement of the iron sulfur protein have been measured and shown to be lower than the high activation barrier associated with quinol oxidation. We conclude that the movement is not the source of the activation barrier. We estimate the occupancies of different positions for the iron sulfur protein from the crystallographic electron densities and discuss the parameters determining the binding of the iron sulfur protein in different configurations. The low activation barrier is consistent with a movement between these locations through a constrained diffusion. Apart from ligation in enzyme–substrate or inhibitor complexes, the binding forces in the native structure are likely to be ≤RT, suggesting that the mobile head can explore the reaction interfaces through stochastic processes within the time scale indicated by kinetic measurements.

Structures for the ubiquinol:cytochrome c oxidoreductase (bc1 complex) from several mitochondrial sources have recently been solved crystallographically (1–5). Xia et al. (1) identified the location of the [2Fe–2S] center in their crystals of the beef heart mitochondrial complex, but the protein was disordered, and they were unable to assign structure. The center was further from cyt c1 than expected from the rapid electron transfer reactions observed experimentally (6–10). They discussed several possible mechanisms for redox exchange over this distance, including electron transfer through the protein, a substantial conformational change between oxidized and reduced forms of the ISP, and the possibility that the disorder might arise from a mobility of the subunit that could be functionally important. In our native structures from chicken, rabbit, and two different crystal forms of the beef complex, the ISP was relatively well ordered, and the extrinsic domain (the mobile head) was close to cyt c1. However, the distances from the [2Fe–2S] center to the heme Fe varied by as much as 4 Å between different structures (2), being closest in hexagonal P6322 crystals of the beef mitochondrial complex, where the His-161 was within H-bonding distance of the heme c1 propionate. We suggested that this was the likely configuration for electron transfer, and Iwata et al. (3) have reached the same conclusion from similar P6322 crystals. After

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5 Abbreviations: bc1 complex, ubihydroquinone:cytochrome c (or c2) oxidoreductase; bh, higher potential cytochrome b heme; bl, lower potential cytochrome b heme; p-BQ, p-benzoquinone; cyt, cytochrome; DAD, 2,3,5,6-tetramethyl-p-phenylenediamine; DQ, duroquinone; EDTA, ethylenediaminetetraacetic acid; g<sub>e</sub> = 1.80 signal, electron paramagnetic resonance spectral band at this g value associated with the presence of quinone and reduced iron sulfur protein together in the bc1 complex; ISP, iron sulfur protein; ISP<sub>ox</sub> and ISP<sub>red</sub>, oxidized and reduced forms of the iron sulfur protein; ISP<sub>b</sub> or ISP<sub>c</sub>, the iron sulfur protein docked at cytochrome b or cytochrome c1 interfaces, respectively; 2-OH-1,4-NQ, 2-hydroxy-1,4-naphthoquinone; MOA, -methoxyacrylate, or similar group, the pharmacophore of a class of inhibitors acting at the Q<sub>i</sub> site; NQ, naphthoquinone; P<sup>e</sup> and P, oxidized and reduced forms of the primary donor of the photochemical reaction center; PES, N-ethylphenazonium ethosulfate; PMS, N-methylphenazonium methosulfate; QH<sub>2</sub>, ubihydroquinone or quinol; Q, ubiquinone or quinone; Q<sub>i</sub> site, quinone reducing site; Q<sub>c</sub> site, quinol oxidizing site; RC, photosynthetic reaction center; SQ, semiquinone; UHDBT, 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazol; UHNNQ, 3-n-undecyl-2-hydroxy-1,4-naphthoquinone.
The complex is shown in six different states. The state vacant-ISPox+c1− is the complex with a vacant Qo site and ISPox oxidized; upon binding quinol the reaction complex, QH2-ISPox+c1−, is formed, with quinol in the distal domain of the Qo site, and oxidized ISP docked on cyt b; SQP-HISPox+c1− is the intermediate formed after the transition state, in which H-transfer has occurred, the semiquinone has flipped to the proximal domain, and the reduced ISP has moved to cyt c1; QoISPox+c1 is the product-bound complex; vacant-ISPox+c1− is the complex after dissociation of quinone and oxidation by cyt c1+. The state Qo-ISPox+c1 represents the complex between quinone and reduced ISPox that forms the g, = 1.80 signal, which is not in the reaction cycle.

cocrystallization with stigmatellin, a Qo site inhibitor known to interact strongly with the reduced form of the ISP (ISPred) (11), the ISP was found in a different position, close to the Qo site (2). The conserved liganding domain (head) had moved by a rotational displacement from a site in contact with cyt c1 in the native structure to a concave interface on cyt b close to the binding pocket containing the inhibitor. This position brought the [2Fe-2S] center close to that found in the native structures from the work of Xia et al. (1). Connections between the ISP and the inhibitor in our electron density maps have been modeled as H-bonds between Ne of His-161 and carbonyl and methoxy O-atoms of the stigmatellin ring (2). From the distance between reaction partners, and the constraints on rates of reaction, none of the structures so far available would be competent in all partial reactions necessary for quinol oxidation. We have therefore suggested that the movement of the ISP mobile domain between interfaces on cyt c1 and cyt b is a necessary part of the catalytic mechanism, as summarized in Figure 1 (2, 5, 12, 13). An additional position for the ISP mobile domain has been demonstrated by Iwata et al. (3) in a new crystal form of the bovine complex, P65. One monomer had the ISP in a new position, intermediate between those found in previous structures (1, 2), and the other had a disordered ISP. Kim et al. (4) have recently located the ISP structure in the crystals of Xia et al. (1) and also noted displacements of the [2Fe-2S] cluster induced by inhibitor binding.

We have discussed in companion papers (13, 25) aspects of the structure and the movement of the ISP head that relate to mechanism. Several groups have discussed mechanisms in which a “catalytic switch” controls delivery of reducing equivalents to the ISPred and suggested that the movement of the ISP is controlled by protein binding changes linked to redox events outside the immediate reactions of quinol oxidation (3, 4). In this paper we report measurements of the kinetics of electron transfer through the bc1 complex in chromatophores from the photosynthetic bacterium Rhodobacter sphaeroides. The mitochondrial complexes have evolved from those in ancestors from the α-proteobacteria (14), and the catalytic subunits have been well conserved across the bacterial–mitochondrial divide. The bacterial complexes have a simpler subunit complement (15, 16), but the complex performs the same function as in the mitochondrial chain, oxidizing ubiquinol through a modified Q-cycle mechanism (6, 17–21). However, the kinetics can be more readily measured in situ on a rapid time scale because the electron-transfer chain can be populated with stoichiometric amounts of substrates (QH2 as reductant and ferricyt c2 as oxidant) by flash illumination to activate the photochemical reaction center. The partial reactions contributing to reactions at each of the catalytic sites of the bc1 complex can be separately measured through use of redox poising and specific inhibition of one or the other site. We show here that the reactions that involve movement of the ISP are rapid compared to the rate-limiting step. We measure the activation energies of these processes and conclude that they do not determine the overall activation barrier measured for quinol oxidation. The occupancies of the ISP in different configurations of the complex have been estimated from the crystallographic electron densities of the extrinsic domain in native crystals and in cocrystals with stigmatellin, UHDBT, myxothiazole, or MOA-type inhibitors. We discuss values for the binding constants determining occupancy, calculated from the electron densities in different states. We conclude that, except for the ligation involved in formation of enzyme–substrate complexes or with the Qo site occupant, the forces constraining the ISP to the positions observed in native crystals are relatively weak. We discuss the mobility of the complex in the light of these binding forces and a stochastic model in which the mobile head of the ISP explores the reaction domain through a simple constrained diffusion.

**MATERIALS AND METHODS**

**Estimation of Occupancies at Different ISP Positions.** The CCP4 program ICOEFL was used to determine what linear combination of the sets of calculated structure factors best fits the observed amplitudes for the native and inhibitor containing crystals (22–25). For any defined set of electron densities, the program calculates a scale factor and a B-factor, where the scale factor is proportional to occupancy and the B-factor is derived from the temperature factor, which is a measure of the disorder at each atom. Negative B-factors indicate that the density is better ordered in the partial structure than in the overall model. For calculation of occupancy, the data from residues 71–196 (the mobile head) and the [2Fe-2S] cluster were used. The ISP membrane anchor was included in the “non-ISP” portion.
and the structural parameters were assigned to five different groups of atoms: (1) protein other than ISP but including the anchoring span of ISP (residues 1–70) (non-ISP) (2) ISP<sub>h</sub>, the ISP mobile head (residues 71–196) in the proximal (to heme <i>b</i>) position in monomer 1; (3) ISP<sub>c</sub>, the ISP mobile head in the position close to cyt <i>c</i>1 in monomer 1; (4) ISP<sub>th</sub>, the ISP mobile head in the proximal position in monomer 2; and (5) ISP<sub>c</sub>, the ISP mobile head in the position close to cyt <i>c</i>1 in monomer 2. Non-ISP was fixed at unit occupancy.

**Measurement of Cytochrome Kinetics.** Kinetic traces were obtained essentially as previously described (6), with specific conditions as detailed in the figure captions. All experiments were performed in an anaerobic cuvette, with the redox potential monitored, and changed by addition of small aliquots of dithionite or ferricyanide to reduce or oxidize the suspension. Redox mediators, inhibitors, etc., when present are described in the figure legends. For experiments determining the activation energy, traces were recorded at different temperatures, as monitored by a thermometer in the cuvette. Rate constants were estimated from the kinetic traces by fitting the curves using exponential functions (one component for cyt <i>b</i> reduction, two components for cyt <i>c</i> oxidation), assuming pseudo-first-order kinetics, using the graphics program Origin 5.0 (Microcal Software, Inc., Northampton, MA). For cyt <i>b</i> reduction, relative rate constants were also measured by recording the maximal rate after the lag phase. The relative rate constant for quinol oxidation was determined from the rate of cyt <i>b</i> reduction (6, 18, 19). The redox potential was adjusted so that the initial concentration of quinol in the membrane was the same before each experiment, and flash excitation generated oxidant in the same relative stoichiometry. The initial concentration of reactants was therefore the same, and the rate therefore directly reflected the rate constant. For cyt <i>b</i> kinetics, only the portion of the curve after the lag phase was used.

The activation energies found in this work differ somewhat from those previously reported. We attribute the difference primarily to a better measurement of the temperature of the reaction medium in the present experiments. In the earlier work, a thermistor was placed outside the cuvette and between it and the water jacket. Although the thermostated cuvette housing was enclosed by insulation, the stirring was from above, leaving an uninsulated surface. In the present experiments, the stirring was from below, and the temperature was monitored directly by a thermometer in the medium.

**RESULTS AND DISCUSSION**

**Modified Q Cycle, and Role of the High-Potential Chain in Oxidation of Quinol.** The reactions involved in oxidation of quinol have been discussed at length in companion papers (13, 25). The reactions are catalyzed by three subunits: cyt <i>b</i>, containing two <i>b</i>-type hemes, <i>b</i>1 (lower potential heme) and <i>b</i>2 (higher potential heme); cyt <i>c</i>1, containing heme <i>c</i>1; and the iron sulfur protein, containing a [2Fe-2S] center. Quinol is oxidized at the <i>Q</i>o site of the complex. Quinol oxidation occurs in a bifurcated reaction, in which one electron is transferred to a high-potential chain and the other to a low-potential chain. The high-potential chain, consisting of the ISP, cyt <i>c</i>1, and cyt <i>c</i> (or <i>c</i>2), transfers the first electron...
from quinol to an acceptor (cytochrome oxidase in mitochondria, the oxidized photochemical reaction center in photosynthetic systems), leaving a semiquinone at the $Q_o$ site. The semiquinone is undetectable during normal turnover, so the reaction at the $Q_o$ site appears to be a concerted electron transfer to the high- and low-potential chains. The low-potential chain consists of the two cyt $b$ hemes, which serve as a pathway through which electrons are transferred across the coupling membrane from the $Q_o$ site to the $Q_c$ site, at which quinone is reduced to quinol. To provide the two electrons at the $Q_c$ site required for reduction of quinone, the $Q_c$ site oxidizes 2 equiv of quinol in successive turnovers. The first electron at the $Q_c$ site generates a relatively stable semiquinone, which is reduced to quinol by the second electron.

**Kinetics in the High-Potential Chain in the Presence of Myxothiazole or UHDBT.** In *Rb. sphaeroides*, oxidizing equivalents from the primary donor ($P^+/P$) of photochemical reaction center (RC) are delivered to cyt $c_1$ by cyt $c_2$, with a $t_{1/2}$ of about 100–200 $\mu$s, depending on temperature and cyt $c_2$ content of the chromatophores (3, 13, 20). The electron-transfer from the Rieske center to cyt $c_1$ cannot be resolved, because it is not rate-determining. A minimal value for the rate constant has been determined from the kinetics of cyt $c$ turnover, taking advantage of the fact that inhibitors of class I (stigmatellin, UHDBT, or UHNQ), prevent the [2Fe-2S] center from reducing cyt $c_1$ (by binding preferentially to the reduced form and raising the $E_{m,3}$ value; 11, 26–28), but inhibitors of class II (myxothiazole and MOA-type inhibitors) do not (27–29). Both classes of inhibitor prevent quinol oxidation by binding at the site normally occupied by the quinol ($1\sim4, 11, 26\sim33$). In the presence of either class, since quinol is prevented from binding and delivering electrons to the reactants, the electron transfer chain is simplified so that only the high-potential chain is available for photooxidation. Because excitation with a sufficiently short and intense flash activates all reaction centers, but only once, electrons and holes are introduced into the chain in stoichiometric amounts. In chromatophores from wild-type cells under normal growth conditions, the ratio of RC:cyt $c_2$:bc1 complex is close to 2:1:1 (18, 19), so that a flash provides enough holes to oxidize ~2 one-electron carriers in each chain:

$$2P^+ + 2 \text{ferrocyan} c_2 = 2P + 2 \text{ferrocyan} c_2^+ (~1 \mu s) \quad (1a)$$

$$[2\text{Fe-2S}]\text{ferrocyan} c_1 + \text{ferrocyan} c_2^+ = [2\text{Fe-2S}]\text{ferrocyan} c_1^+ + \text{ferrocyan} c_2 (~150 \mu s) \quad (1b)$$

$$[2\text{Fe-2S}]\text{ferrocyan} c_1^+ = [2\text{Fe-2S}]^+\text{ferrocyan} c_1 (~150 \mu s) \quad (1c)$$

$$[2\text{Fe-2S}]^+\text{ferrocyan} c_1 + \text{ferrocyan} c_2^+ = [2\text{Fe-2S}]^+\text{ferrocyan} c_1^+ + \text{ferrocyan} c_2 \quad (1d)$$

Overall, in the presence of myxothiazole:

$$2P^+ + [2\text{Fe-2S}]\text{ferrocyan} c_1 = 2P + [2\text{Fe-2S}]^+\text{ferrocyan} c_1^+ \quad (1)$$

With UHDBT or stigmatellin present, and the properties of the [2Fe-2S] center modified (shown by [2Fe-2S]*):

$$2P^+ + \text{ferrocyan} c_2 + [2\text{Fe-2S}]^*\text{ferrocyan} c_1 = 2P + \text{ferrocyan} c_2^+ + [2\text{Fe-2S}]^*\text{ferrocyan} c_1^+ \quad (2)$$

The electrons will come from the components with lowest $E_{m}$ value. In the presence of myxothiazole, the electrons come from cyt $c_1$ and the [2Fe-2S] center, which then remain oxidized on the 100 ms time scale. The cyt $c_2$, although it is initially rapidly oxidized by $P^+$, becomes reoxidized as the lower $E_{m}$ components are oxidized (reaction 1 above) (6, 7). In the presence of UHDBT (or stigmatellin or UHNQ), no electrons are available from the [2Fe-2S] center, so the flash-induced change is an oxidation of cyt $c_1$ and cyt $c_2$ only (reaction 2) to give an absorbance change of larger amplitude when measured at 551–542 nm, at which both cyt cytochromes absorb with a similar extinction coefficient (6, 7, 11, 33; and see Figure 2). The extra amplitude of the flash-induced cyt $c$ change after addition of UHDBT is due to the inhibition of electron transfer from the [2Fe-2S] center (11, 33). This contribution can be assayed from the difference between the kinetics with myxothiazole present and those with UHDBT present (Figure 2). In the traces shown, the chromatophore preparation had less cyt $c_2$ than in “standard”
preparations (RC2;3:bc1 of 2:0.8:1, instead of 2:1:1), so the 
$t_{1/2}$ for oxidation of cyt c1 was a little greater (170 μs at 30
°C), some cyt c2 remained oxidized in the presence of
myxothiazole, and the increased amplitude induced by
UHDBT was less than we have previously reported (6, 7,
33, 29).

If the electron donation from [2Fe-2S] to cyt c1 were slow
compared to the oxidation of cyt c1, one would expect that
the c-type cytochrome oxidation kinetics would first rise to
the level seen with UHDBT present and then decay to the
myxothiazole level, with a rate reflecting the transfer of
electrons from the reduced [2Fe-2S] center. No trace of any
such a transient is observed in the changes measured in the
presence of myxothiazole alone (6, 29). We have interpreted
this as indicating that the rate of electron transfer from the
[2Fe-2S] center to cyt c1 is rapid compared to the rate of
oxidation. Because the $E_m$ value for the ISP is higher than
that for cyt c1 (7, 11), the rate of reduction by ISPred must
match the rate of oxidation by cyt c2 when the reactants for
the former process are far from favorable, requiring that the
rate constant is greater by a factor of at least 10. In a kinetic
model of these reactions, Crofts and Wang found that a rate
constant for oxidation of the [2Fe-2S] center in the range
$>10^7$ s$^{-1}$ provided a good fit (6). Tsi et al. (8) concluded
that the rate was also rapid in mitochondrial complexes.
The activation energy for these reactions in *Rb. sphaeroides*,
assayed from the kinetics in the presence of myxothiazole,
and the difference kinetics with UHDBT, was much lower
than for quinol oxidation (5).

**Contribution of the ISP Movement to the Kinetics of the
High-Potential Chain.** In the work of Crofts and Wang (6)
and in the earlier work of Meinhardt and Crofts (29), it was
observed that the kinetics of cyt c oxidation ± myxothiazole
in the 100 μs range differed, so that the rise kinetics appeared
to be slower in the presence of the inhibitor. This effect was
then unexplained, but the new structural results have
prompted us to reexamine this phenomenon. The contribution
of the ISP to the kinetics of the high-potential chain could
be observed from the differences in kinetics in the presence
and absence of myxothiazole or UHDBT (Figure 2). At $E_b$
$\sim 200$ mV in the absence of inhibitor, since the quinone
pool is oxidized before the flash, the Qo site cannot turn over
until a quinol arrives from the reaction center, with a delay of
$\sim 1$ ms (18). The kinetics of the cyt c changes in the first
millisecond therefore reflect only the activity of the high-
potential chain. The changes (cyt c$_{total}$) were measured at
551–542 nm, where both cyt c1 and cyt c2 contribute equally.
The fast phase ($t_{1/2} < 10$ μs) was due to cyt c2 oxidation,
and the slower oxidation phase reflects cyt c1 oxidation.
However, the amplitude at $\sim 3$ ms reflects the oxidation of
only $\sim 1$ equiv of cyt c, since the oxidation of cyt c1 occurs
at the expense of cyt c2, and their extinction coefficients are
nearly the same at this wavelength pair. In these experiments,
the difference kinetics ± myxothiazole in the $\sim 200$ μs range
showed a small overshoot (see Figure 2). The crystal-
lographic evidence suggests that, in the presence of myx-
thiazole, most of the ISP is in the ISPc configuration (see
below for data and discussion of the association constant),
from which reaction occurs in the $\sim 10$ μs time scale. In the
absence of myxothiazole, some fraction of the ISP will likely
be in the ISPb position, since the ISP is reduced and the
quinone pool is oxidized, and these are the conditions in
which the ISP interacts with quinone to give a changed EPR
spectrum, with a band at $g_\perp = 1.800$ (30–32). In terms of
the states shown in Figure 1, the most straightforward
interpretation of the overshoot is therefore that, in a fraction
of complexes, the arrival of the electron on cyt c1 from the
ISP is delayed because of the association of the ISP in
formation of this complex. Since the complex requires
ligation of quinone in the Qo site with ISPred in the ISPb
position, the delayed electron transfer would reflect the
dissociation of this complex and movement from ISPb to
ISPc positions. The amplitude of the overshoot was about
20% of the change attributable to cyt c1. However, this
amplitude could be explained either by a model in which the
overshoot represents a small fraction of centers in this
configuration, with a relatively slow reaction time, or a model
in which a large fraction is initially in ISPb, and has a
relatively rapid reaction time. If all complexes had the ISP
denoted in the ISPb configuration, then the reaction time
indicated by the small overshoot would suggest a process
with a half-time in the range $\sim 30$ μs. Alternatively, the
kinetics may indicate that $\sim 20\%$ of complexes are in
the ISPb configuration and that the complex between Q and
ISPred is relatively long-lived ($t_{1/2} \sim 3$ ms). The rise kinetics
of the overshoot obviously cannot be faster than the rate of
oxidation of cyt c1 and do not measure directly the kinetics
of oxidation of the ISP.

When similar experiments were performed at $E_b$ $\sim 100$
mV (quinone pool $\sim 30\%$ reduced, Qo site $>80\%$ occupied
by QH2), the kinetics showed a rereduction of cyt c as
electrons were delivered to the high-potential chain from the
Qo site. Under these conditions, the overshoot was still
observed but was more transient and reversed in $\sim 200$ μs
(Figure 2). The fraction of centers in which quinone would
be complexed with ISPb$r$ at $E_b$ $100$ mV compared to $E_b$
200 mV could be estimated from the apparent $E_m$ of the $g_\perp$
= 1.800 signal ($\sim 90$ mV, similar to that of the pool). From
this value, 0.7% of the centers with the $g_\perp$ = 1.800 signal at
$E_b$ 200 mV would have the signal at 100 mV. Centers with
the $g_\perp$ = 1.800 signal would have ISP initially in the ISPb
position, and the mobile head would have to have moved to
the ISPc position in order to transfer an electron to cyt c1
during the 200 μs transient.

It should be noted that the complex between quinol and
ISPb$r$ is not an intermediate of the normal catalytic cycle,
and the relatively long decay time of the overshoot seen when
the pool is initially oxidized does not have a significance in
normal turnover.

**Kinetics of Quinol Oxidation.** In addition to the measure-
ments of cyt c kinetics above, the kinetics of turnover of the
Qo site also provide constraints on the reactions and
displacements of the ISP. The oxidation of quinol can be
followed in chromatophores from *Rb. sphaeroides* by
observing the rate of reduction of cyt bH following flash
illumination (6, 7, 34; and see Figure 3). Since the reactions
in the b-heme chain are much more rapid than the delivery
development to the chain from the Qo site (6, 34), the electron
transfer from the Qo site can be assayed when electrons are
prevented from passing from cyt bH to the Qo site by antmycin.
The kinetics show a second-order dependence on quinol concentration, and the rate saturates as the pool
becomes reduced. Maximal rates ($t_{1/2} \sim 1$ ms at 20 °C, pH
7) are seen with the pool partially oxidized, suggesting a
tighter binding of QH$_2$ than Q. Under these experimental conditions ($E_0 \sim 100$ mV), the reactions at the Q$_b$ site are delayed by a short lag ($\sim$150 $\mu$s). This lag reflects the time taken for an oxidizing equivalent produced in the photochemical reactions to reach the site through turnover of the high-potential chain, including the movement from the ISP$_b$ to the ISP$_c$ position (see below for further discussion). For the reverse displacement, the transient overshoot in cyt c oxidation discussed above limits the time to <200 $\mu$s when the pool is partly reduced (see above). These values are maximal times for the entire electron-transfer process, including binding and unbinding, diffusion, and electron transfer. Times for these processes can also be estimated from a consideration of the turnover of the complex in the absence of inhibitor. Under uninhibited conditions, 2 equiv of QH$_2$ are oxidized at the site following a single-turnover flash (18), and the overall process can be followed through the electrogenic reactions associated with electron transfer between Q$_b$ and Q$_c$ sites through the low-potential chain (18, 35). These can readily be measured through the slow phase of the electrochromic carotenoid absorbance change ($t_{1/2} \sim 2$ ms). The electrogenic reactions follow a continuous monotonic kinetics, without any indication of a kinetic break, suggesting that all processes contributing to the second turnover, including the displacements of the ISP in the high-potential chain to regenerate an oxidant at the Q$_b$ site, and replacement of Q by QH$_2$, are much faster than the rate-determining step.

**Activation Barriers in Quinol Oxidation.** Crofts and Wang (6) explored the temperature dependence of the reactions leading to quinol oxidation in chromatophores from *Rb. sphaeroides*. They showed that the oxidation of quinol had the same value for activation energy for different states of the quinone pool. With the pool 30% reduced, the kinetics were assayed at pH 7 through reduction of cyt $b_{1H}$, while at pH 8.9, the kinetics were measured by reduction of cyt $b_{1C}$. Both reactions showed a similar maximal rate and activation energy. Under conditions in which the pool was initially oxidized, turnover of the Q$_b$ site (assayed through cyt $b_{1H}$ reduction) was slower and occurred with a $\sim$1 ms lag, attributed to delivery of QH$_2$ from the reaction center, as discussed above. However, the activation barrier was the same as when the pool was partly reduced. Because the local concentration of quinol did not affect the activation energy, Crofts and Wang (6) concluded that the reactions associated with binding of quinol could be excluded as the source of the activation barrier. Crofts and Wang (6) also measured the reactions of the high-potential chain in the presence of myxothiazole or UHDBT and showed that the oxidation of cyt $c_{total}$ in the presence of either inhibitor and the difference kinetics with UHDBT and myxothiazole (arising from the availability of an electron on the ISP in the presence of myxothiazole) all had relatively small activation barriers. They therefore excluded these processes as the location of the larger activation barrier seen in the cyt $b_{1H}$ reduction kinetics.

In light of the new structural information, we have re-investigated the activation barriers of the partial reactions of quinol oxidation in order to see if the movement of the ISP from cyt $c_1$ to cyt $b$ contributes to the activation barrier, as has been recently suggested (36). Kinetic traces showing the temperature dependence of oxidation of cyt $c_{total}$ in the presence or absence of myxothiazole, and the “overshoot” revealed in the difference kinetics, are shown in Figure 4. The oxidation rates, with or without inhibitor, increased over the range from 10 to 40 $^\circ$C, with an activation energy of $\sim 25$ kJ mol$^{-1}$, but were substantially faster over this entire range than cyt $b_{1H}$ reduction.

The overshoot was little affected by temperature change. If the movement of the ISP had a higher activation barrier than the reactions leading to oxidation of cyt $c_1$, the overshoot would have increased in amplitude on lowering the temperature, rather than the small loss of amplitude observed at lower temperatures. The results show that the contributing processes had either lower activation barriers or kinetics much faster than the rate-determining step (the $\sim 150$ $\mu$s oxidation of cyt $c_1$).

Although no direct information about the activation energy of these more rapid processes can be obtained from these traces, it is clear that oxidation of the ISP and its movement are more rapid than the rate-limiting step at all temperatures and will not contribute to the activation barrier for quinol oxidation.

In addition to the direct measurements from cyt $c$ kinetics, the kinetic lag before onset of reduction of reduction of cyt $b_{1H}$ (to monitor quinol oxidation; 6, 18) provides hitherto unnoted information about the reactions delivering oxidizing equivalents to the Q$_b$ site. These include the movement from
The strong temperature dependence over the range 10–40 °C was 47 kJ mol\(^{-1}\), activation energy derived by fitting the best straight line to values in similar experiments varied between 40 and 56 kJ mol\(^{-1}\). Some of the points were 47 kJ mol\(^{-1}\), activation energy derived by fitting the best straight line to values in similar experiments varied between 40 and 56 kJ mol\(^{-1}\).

The temperature dependence of cyt \(b\) reduction, measured with the quinone pool ~30% reduced, is shown in Figure 3. The strong temperature dependence over the range 10–40 °C is obvious from inspection. In the experiment shown, the activation energy derived by fitting the best straight line to the points was 47 kJ mol\(^{-1}\), but this was biased by the stronger temperature dependence at lower temperatures, and values in similar experiments varied between 40 and 56 kJ mol\(^{-1}\). The value from the slope between 15 and 40 °C for cyt \(b\) reduction was ~37 kJ mol\(^{-1}\), closer to that previously reported (6). The nonlinearity of the Arrhenius plot is discussed further below. In contrast to the temperature dependence of the rate of cyt \(b\) reduction, the traces show that the lag before onset of reduction was not strongly temperature-dependent. The lag was 180 ± 50 µs at 15 °C and 130 ± 50 µs at 40 °C, and the activation energy was ~14 kJ mol\(^{-1}\). Much of the lag can be attributed to the time taken for oxidizing equivalents to arrive at [2Fe-2S] (1/12 ~150 µs for oxidation of cyt \(c_1\)). Since the \(E_m\) value for cyt \(c_1\) (~270 mV) is a little lower than that for the [2Fe-2S] center (~290–300 mV at pH 7), oxidation of the latter lags a little behind that of the former. This constrains estimation of the contribution of movement to the lag and suggests that diffusion from the ISP\(c\) to the ISP\(b\) position must occur in ~100 µs. Since neither the lag nor the overshoot in cyt \(c_1\) oxidation shows an activation energy comparable to that for cyt \(b\) reduction, it seems unlikely that the movement of the ISP contributes to the activation barrier for quinol oxidation. This result is therefore in contradiction to the observation of Tian et al. (36), who noted an inhibited rate and increased activation energy for strains with mutations in the linker region of the ISP, when electron transfer was assayed by a steady-state approach. The authors did not indicate how they identified the contribution to the partial reactions involving the ISP, or if they had eliminated other possible contributions of the mutation to the properties of the ISP. Mutations that lower the \(E_m\) value of the cluster also show an inhibited rate (37–39) and an increased activation energy (38), but this is attributable to the direct contribution to the \(\Delta G^\circ\)** for formation of the semiquinone (6, 38).

The Arrhenius plot for cyt \(b\) reduction was not linear and showed a much steeper slope at lower temperatures, suggesting that the reaction is not simple. At 10 °C, the kinetics were slower than expected from the slope obtained at higher temperatures. The lower slope to the Arrhenius plot at higher temperatures may in part be due to the fact that the reactions of the high-potential chain, because they have a lower
activation energy, contributed increasingly to limitation of the kinetics as the temperature was raised (6).

In summary, the kinetic evidence suggests that none of the reactions involved in delivery of oxidizing equivalents to the Qo site is rate-limiting in quinol oxidation and none has a high enough activation energy to account for the value seen in cyt b reduction.

Role of Distance in Determining the Rate of Electron Transfer from the \([2Fe-2S]\) Center to cyt c1. The positions of the ISP in different structures, and the distance between the \([2Fe-2S]\) center and reaction partners, have been discussed at length elsewhere (2, 13, 25; PDB files 1bcc, 2bcc, and 3bcc). Three positions of the \([2Fe-2S]\) center are of importance in the present discussion: the stigmatellin-induced position in the chicken complex (which is close to that in the beef complex of Xia et al. (1), as calculated from the data they provided], the position in the native chicken complex, and the position in the native beef hexagonal complex, which shows the closest approach to cyt c1.

The structural context of electron transfer theory has been much studied recently, and a consensus has emerged on the parameters of importance in determining rate constants. Three factors are of critical importance: distance, the standard free-energy change for the reaction, and the coupling, or reorganization energy, \(\lambda\), from Marcus theory (see ref 40 for review). An important point on the energy-v. reaction coordinate surface is the condition under which the products parabola intersects the reactant parabola at its minimum (when \(E_{\text{act}}\) is zero). Under these conditions \(\lambda = -\Delta G^0\) and the reaction proceeds at its maximal rate, with rate constant \(k^e_{\text{ET}}\) normalized to this condition. Values for \(k^e_{\text{ET}}\) can be found experimentally by measuring the rate constant for a reaction under conditions giving different values for \(\Delta G^0\). Moser et al. (9) have measured reactions in photochemical reaction centers and adjusted values so as to span the range about the \(\Delta G^0\) giving this maximal value for \(k^e_{\text{ET}}\). From these results, and an extensive literature on other reactions, they have produced an empirical equation (Dutton’s ruler) relating rate to distance:

\[
\log k^e_{\text{ET}} = 15 - 0.6R - 3.1(\Delta G^0 + \lambda)^2/\lambda
\]

Here \(R\) is the edge-to-edge distance in angstrom units, and \(\Delta G^0\) and \(\lambda\) are expressed in electron volts. Similar work from Gray and Winkler (10) has led to some refinement of this picture in terms of secondary structure but with similar values for critical terms defining the ruler.

Applying this formula to the distance between centers in the structure of Xia et al. (1) under the condition when \(\lambda = -\Delta G^0\), we obtain a maximal rate constant of \(2.5 \times 10^{-4} \text{ s}^{-1}\) for the 31 Å center-to-center distance and \(2.5 \times 10^{-3} \text{ s}^{-1}\) when a 4 Å adjustment is made for the edge-to-edge distance. The corresponding distances in the native structure of Zhang et al. (2), from the chicken heart complex give rate constants of 166 \(\text{s}^{-1}\) and 1.66 \(\times 10^5 \text{ s}^{-1}\), respectively, and the other complexes solved by Zhang et al. (2), which have shorter distances, have correspondingly more rapid rate constants. Clearly, the only distances that would permit electron transfer on the time scale measured are the values from the Zhang/Berry structures, including the 16.3 Å edge-to-edge value for the chicken heart complex and the closer distances in the beef and rabbit structures. The closest possible distance from the structure of Xia et al. (1) gives a maximal rate 5 orders of magnitude too slow. These values represent the fastest possible reaction times; more realistic estimates of the rate constant need to take account of probable reorganization energies, \(\lambda\) (7, 27, 33). Taking the distance from the beef hexagonal crystals between Ne2 of H161 of the Rieske protein, which provides one of the ligands to the \([2Fe-2S]\) cluster, and an oxygen atom of heme propionate D (\(\sim 3.4\) Å), or the edge of the heme \(\pi\)-bonded system at the Ne2 atom (8.1 Å), we can calculate approximate rates of electron transfer from the \([2Fe-2S]\) center to the heme of cyt c1 of \(\sim 3 \times 10^9 \text{ s}^{-1}\) and \(\sim 10 \times 10^6 \text{ s}^{-1}\), respectively, assuming nonadiabatic electron tunneling with a reorganization energy of 1.0 electron volts and \(\Delta G\) of zero. These rates are significantly faster than measured rates for this reaction (6).

In the native chicken crystals the distance is 14.4 Å, which would give a rate of \(\sim 1.8 \times 10^7 \text{ s}^{-1}\) with the same assumptions, considerably slower than the rates observed in the presence of myxothiazole. If these values are realistic, they suggest that the variation in distances between crystal structures must reflect a dynamic situation, in which the ISP can reach the more intimate docking with the cyt c1 with high probability during the \(<10 \mu\text{s}\) reaction time observed. In the crystals with stigmatellin (2), or in the tetragonal beef crystals (1) the shortest distance from the cluster or its ligands to the heme tetrapyrrole ring is 27 Å, giving a rate of \(\sim 10^{-4} \text{ s}^{-1}\) (with the same assumptions), making it very unlikely that the enzyme could function if this conformation represented a static structure.

The treatment above is appropriate for a solid-state system in which the redox centers are fixed and the reorganization energy pertains to reactant and product sharing a common protein environment. It may therefore be appropriate for reaction from a complex of lifetime long compared to the reaction rate. It seems very likely that such a long-lived reaction complex is formed between QH2 and ISP\(^{\text{ox}}\) at the interface on cyt b (4, 35, 36) (see Figure 1) and that reaction to form the semiquinone proceeds from such a complex. However, the movement of the mobile head introduces a more dynamic element into the overall reaction mechanism, with properties of both first- and second-order processes. Because the movement is locally constrained, the concentration terms will be unitary, and the reaction rate will not vary with concentration of the bc1 complex and will appear to be first-order. However, the local mechanism will include a collisional component, and the overall rate at either reaction interface will be determined by the probability of forming a reaction complex and will therefore be determined by reaction parameters, including local concentration of substrates and binding coefficients, and by diffusion. The extent to which the rates of partial reactions will be determined by these stochastic processes will depend on the stability of the reaction complexes.

Diffusional Constraints. The displacement of the ISP from the position in native crystals to the position in stigmatellin-containing crystals does not involve any significant displacement of the membrane anchor but rather an extension of the connecting loop and a rotation of 57–63°. To a first approximation, this can be modeled as a constrained diffusion, with root-mean-square displacement given by \(\langle \Delta x^2 \rangle = 2Dt\) for the one-dimensional case. With a diffusion coefficient of \(\sim 10^{-6} \text{ cm}^2\text{s}^{-1}\) appropriate for a small protein in an
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aqueous environment, a displacement of this order (∼16–22 Å) would require ∼13–25 ns. This gives a minimal time for the reaction. A more complex displacement than the simple rotation about a virtual axis that seems to account for the movement, or some element of higher dimensionality in the diffusional space, would increase this time. The time for reaction will include this minimal time but be greater by a factor representing stochastic exploration of the binding domains to find configurations needed for efficient electron transfer (the on rate constants), the probabilities for dissociation from bound states (the off rate constants), and changes in on and off rate constants as the nature of the reaction complex changes on electron transfer. Some appreciation of the relative values for some of these parameters can be obtained by a consideration of the binding constants involved.

Estimates for Equilibrium Constants for Binding of the Iron Sulfur Protein at Different Sites. The order of magnitude of some of the forces involved in constraining the ISP can be derived from data in the literature. These include the binding energies associated with the change in $E_{m,7}$ of the [2Fe-2S] center induced by inhibitors, and estimates of the occupancy of the state giving rise to the $g_x$ = 1.800 EPR signal of the ISP. Some of the different states of binding for the ISP in the $bc_1$ complex are represented in the scheme below. We assume that the lower potential redox centers of the complex ($b_H$, $b_L$) are in the oxidized state, and the Q$_x$ site is initially vacant, conditions appropriate for both sets of crystallographic data.

\[
\begin{align*}
\text{ISP}^{\text{ox}}_{\text{C}*} & \Leftrightarrow \text{ISP}^{\text{ox}}_{\text{C}} \Leftrightarrow \text{ISP}^{\text{ox}}_{\text{free}} \Leftrightarrow \text{ISP}^{\text{ox}}_{\text{B}} \\
\text{ISP}^{\text{red}}_{\text{C}*} & \Leftrightarrow \text{ISP}^{\text{red}}_{\text{C}} \Leftrightarrow \text{ISP}^{\text{red}}_{\text{free}} \Leftrightarrow \text{ISP}^{\text{red}}_{\text{B}} \\
\end{align*}
\]

For binding of quinone species to the reduced ISP in the ISP$_B$ position:

\[
\begin{align*}
\text{ISP}^{\text{red}}_{\text{B}} & \Leftrightarrow \text{ISP}^{\text{red}}_{\text{B}} \Leftrightarrow \text{ISP}^{\text{red}}_{\text{Q}} \\
\text{ISP}^{\text{red}}_{\text{B}} & \Leftrightarrow \text{ISP}^{\text{red}}_{\text{B}} \Leftrightarrow \text{ISP}^{\text{red}}_{\text{QH}_2} \\
\end{align*}
\]

In the scheme, we indicate that the mobile head of the ISP can be free or specifically associated with reaction interfaces on cyt $b$ or on cyt $c_1$. The superscripts $o$ and $r$ refer to the redox state of the [2Fe-2S] center of the protein (oxidized or reduced, respectively); the redox states can be interrelated through reaction with an arbitrary redox couple:

\[
D + \text{ISP}^{\text{ox}}_{\text{free}} \Leftrightarrow \text{ISP}^{\text{red}}_{\text{free}} + D^+ \quad K_{ovr}
\]

Some uncertainty in the attribution of binding constants comes from uncertainty as to the redox status of the complexes during crystallographic analysis. The uncertainty is compounded by the possibility that reductant may be generated in the crystals by X-ray irradiation. In the discussion below, it should be recognized that any relation between equilibrium constants for reactions with partners in mixed redox states includes implicitly a $K_{ovr}$ term of unknown value.

Apart from $K_{ovr}$, all $K$ terms denote association constants. The subscripts $C*$, $C$, free, $B$, and $B$-nnn (where nnn is stg, UHDBT, Q, or QH$_2$) designate the binding states for the ISP head as follows: $C*$, bound at the cyt $c_1$ interface, with an additional force from differential effects of crystal contacts between $C$ and $B$ interfaces; $C$, bound at the cyt $c_1$ interface; free, not specifically bound; $B$, bound at the cyt $b$ interface; $B$-nnn, liganded by the occupant of the Q$_x$ binding pocket (stigmatellin, UHDBT, Q, or QH$_2$).

The forces constraining the ISP head to a particular state are represented by equilibrium (association) constants, so that the transition from the free state to any particular bound state can be represented by the product of equilibrium constants for partial reactions, as shown in the scheme above and discussed below. The terms $K_{cyt}$ are included to cover differences between data from different labs attributable to differences in crystals used. These could include different crystal contacts (likely the dominant term), different redox states, and different amounts of quinone in native crystals. The scheme could be expanded to include terms for the complex in different redox states of the cyt $b$ and cyt $c_1$ hemes, but few data are available to justify the additional complexity.

Calculation of Occupancy from the Crystallographic Data. We have used ICOEFL from the CCP4 suite of programs (22–24) to estimate the occupancy of the ISP in different positions in different crystals, as summarized in Tables 1–3. This set of data makes it possible to estimate some of the binding constants from the scheme above. We recognize that the treatment used here is somewhat simplistic, but we note that the calculation of occupancy for the stigmatellin-induced position gave a value close to 1, as expected from the strong ligand between the ISP and the inhibitor. With this as a mark of confidence, we might expect that occupancies for other states would sum to 1. For the weaker binding cases, it would not be surprising to find a fraction of the ISP head in the free configuration (and therefore disordered), and we suggest that this is the simplest explanation for the missing occupancy in the native, myxothiazole, MOA-inhibitor, and UHDBT crystals. With this assumption, we can calculate association constants directly but recognize that these values should be used with appropriate caveats. One source of error might be an overestimation of occupancy from inclusion of the anchored tail in the calculation. We have eliminated this by using data from the mobile head alone (Tables 1–3). Another source of error would arise from the averaging of occupancies for the two ISP positions within a dimer, since the crystal packing is not the same for both monomers. We have looked for evidence for this by using two different methods of calculating occupancies for the two monomers independently (Tables 2 and 3), and we conclude that there is not a large difference between the monomers in our structures (but see ref 3). We have made similar calculations after separate refinement of five contributions to the structure, with the ISP in the four separate potential locations in the two monomers treated separately. Errors in values derived from the crystallographically estimated association constants could...
arise from additional liganding terms. Although both groups prepared their crystals under aerobic conditions and assumed that the redox centers were oxidized, the possibility exists of reduction by electrons arising from X-ray irradiation. The uncertain redox status of the complex in the crystals and the variability of quinone content might also contribute to the variability of the data and the differences between our structures (2) and that of Xia et al. (1). Despite these considerations, the results show an encouraging internal consistency, which suggests that the values are reasonable.

**Association Constants for the Oxidized ISP.** The distribution of the oxidized ISP is determined by its association constants for occupancy of interfaces on cyt b and cyt c₁ and by the changes in these values due to crystal contacts or other liganding interactions. The ISP subunit was disordered in the crystals of Xia et al. (1), which did not have crystal contacts in this region. This lack of contacts likely contributed to the disorder but perhaps also allowed the ISP to bind to the cyt b interface. The ISP in each of the structures of Zhang et al. (2) showed crystal contacts, but these contacts were slightly different in different structures and between the ISP subunits of different monomers. In the P₆₃ crystals of Iwata et al. (3), the monomer with the intermediate structure showed crystal contacts. The data of Tables 2 and 3 suggest that differences between monomers in our structures were not large and that relative association constants are $K_{c}^{b} < 1$, $K_{c}^{cyt} > 1$, $K_{c}^{cyt} > K_{c}^{cyt}$, and $K_{c}^{c} < K_{c}^{cyt}$.

In the crystals of Xia et al. (1), the density of the [2Fe-2S] center measured at the Fe edge was 11.1, compared with 20.7 for heme b₄ (the iron with the highest density). Since the [2Fe-2S] center has two iron, the density was 0.28 times that expected, assuming an occupancy of 1 for heme b₄. Kim et al. (4) report a value of 0.18 from a different data set. These values give occupancies for the ISP₆ configuration, from which a range of values for the association constant (0.22–0.39) can be calculated, assuming that all the unaccounted ISP was free. For the crystals of Zhang et al. (2), the data of Table 1 show occupancies of 0.46–0.7 and 0.003–0.118 for the ISP₆ and ISP₈ configurations, respectively. Assuming that the unaccounted density reflects unbound ISP heads (in the ISP₈ state), we obtain the following (means from seven values for native structures treated by the different methods): $K_{c}^{b}K_{c}^{c} = 1.47 ± 0.55$ and $K_{c}^{cyt} = 0.15 ± 0.09$, which can be compared to the value, $K_{c}^{cyt} = 0.22–0.39$, calculated from the iron densities of Xia et al. (1).

We have considered above that the difference between the occupancies in the structures of Xia et al. (1) and our own might be explained by crystal contacts that introduce differential stability to the protein on the cyt b or cyt c side of the complex. Since the occupancy of the ISP₆ position was undetected in the structure of Xia et al. (1) (but see ref 4), we cannot make any estimate of the relative affinities of the two positions. Nevertheless, the occupancy of the ISP₈ position in our crystals was substantially less than in the crystals of Xia et al. (1), so it seems likely as argued above that a major contribution to the term $K_{c}^{b}K_{c}^{c}$ must come from $K_{c}^{cyt}$. From this and from the relative occupancies in the crystals of Xia et al., we can assume that $K_{c}^{c} < 1$. The values of association constants for the oxidized ISP for both of its docking interfaces are therefore in the range of unity, given binding forces $< RT$. It therefore seems probable that the barriers to exchange between states are small and that thermal energy will be sufficient to allow the ISP to explore all states with some probability of occupancy in a time scale of the measured reaction time.

**Relative Affinities of the ISP for the Interface on Cyt b in the Presence of Inhibitors.** The change in $E_{m}$ of the [2Fe-2S] center induced by stigmatellin or UHDBT reflects the differential binding to the reduced and oxidized forms of the ISP (21, 23, 24). Since neither binding constant is known independently, we do not know the absolute value of either. The changes in redox midpoint potential reported are $ΔE_{m,2} \approx 220$ mV induced by stigmatellin and $ΔE_{m,2} \approx 80$ mV induced by UHDBT. From these values, $K_{c}^{b}/K_{c}^{b} \approx 5000$, and $K_{c}^{UHDBT}/K_{c}^{UHDBT} \approx 20$. In the presence of stigmatellin in the chicken complex, all the ISP (within error) was found at the cyt b interface, as would be expected from the high affinity for the reduced ISP shown above. Crystals of the chicken complex containing UHDBT showed a weaker occupancy of the ISP position than those with stigmatellin (see Table 1), from which $K_{c}^{b} < K_{c}^{cyt}$, $K_{c}^{c} < K_{c}^{cyt}$, and $K_{c}^{UHDBT} \approx K_{c}^{cyt}K_{c}^{c} / K_{c}^{cyt}$. From the values in Table 1 and using the same assumptions as for the native crystals, we can calculate association constants for ISP₆ for UHDBT crystals for comparison with those in the native crystals, $K_{c}^{UHDBT}K_{c}^{c} = 1.46 ± 0.76$. This is in good agreement with the value from the native complex, suggesting that UHDBT does not affect the affinity for the cyt c₁ interface. The affinity for the cyt b interface was markedly increased, to give $K_{c}^{UHDBT}K_{c}^{cyt} = 0.99 ± 0.38$. By use of the average value for $K_{c}^{cyt}$ given above, $K_{c}^{UHDBT} \approx 6.53$, which can be compared with the relative affinities of the inhibitor for reduced and oxidized forms of the ISP given above (a value of $\sim 20$).

The values for binding constants in the presence of stigmatellin and UHDBT are appropriate only if the binding species was the reduced form. Addition of stigmatellin or UHDBT at ambient potential to the bc₁ complex in situ or in detergent suspension induces a reduction of the ISP, presumably from endogenous reductant, in response to the removal of the reduced form from the redox equilibrium of the free form. We might suppose that this reduction by adventitious donor also occurred in the cocrystals with stigmatellin or UHDBT.

Values for association constants similar to those for the native complex were obtained from the data in the presence of myxothiazole or the MOA-type inhibitors (Tables 1 and 3). A trend in the occupancy values for the ISP₈ position toward a loss of occupancy from the cyt b interface was apparent, suggesting that either occupancy of the proximal domain or displacement of quinone by these inhibitors might weaken the affinity of the cyt b interface for ISP₈. Kim et al. (4) observed a more dramatic loss of the anomalous Fe density from the cyt b docking configuration in crystals with MOA-stilbene. The structural difference associated with myxothiazole binding, showing a closing of the access between the Qₐ pocket and the aqueous phase at the ISP-binding interface (35), might contribute to this differential binding of the ISP₈. Other data from the recent analysis by Kim et al. (4) of occupancy of the [2Fe-2S] cluster in structures with and without inhibitors, using the anomalous difference data, show a similar pattern of effects.
of inhibitors to that reported here.

Relative Binding Constants for Oxidized and Reduced ISP at the Cyt b Interface. The change in EPR spectrum of the ISP to give a band at $g_s = 1.80$ is ascribed to the ligation of the reduced ISP by quinone bound in the distal domain of the Qo site (25). De Vries et al. (41) analyzed the spectrum of the ISP under conditions in which the $g_s = 1.80$ signal was formed and could fit the spectrum with two components. Component 1 included a sharp peak at $g_s = 1.8046$, which broadened on reduction, to give a shape more similar to that of component 2. De Vries et al. (41) interpreted the two spectral components seen when the pool was oxidized as two separate species. If we interpret the two components as two forms of the same species, corresponding to ISPred liganded with Q (component 1) and unbound ISPred (component 2), the results suggest that less than half the ISPred was in the ISPb configuration, to give $K_A \approx 0.8 \pm 1$. The relatively small amplitude of the overshoot in the experiments of Figures 2 and 4 could indicate a value as low as 0.2.

Preliminary results from fluorescence quenching experiments using labeling of specifically engineered cyt562 residues on ISP and cyt b with complementary fluorophores (42) suggest a value for $K_A \approx 2.3$, giving $K_{QOc} \approx 0.2 \pm 2.3$.

Since the $E_m$ value at which the $g_s = 1.80$ signal appears on redox titration is close to that of the quinone pool, the binding constants of the site under these conditions (ISPred) for Q and QH$_2$ must be similar, suggesting that $K_{Q} \approx K_{QH2}$. However, it is unlikely that the ISPred binds with the same affinity when QH$_2$ rather than Q occupies the pocket. Fluorescence quenching data from FRET experiments (42) show that the ISPred moves away from cyt b on reduction of the quinone pool, suggesting that ISPred does not form a ligand with QH$_2$ at the Qo site. A more complete description requires partitioning of the binding equilibria into partial contributions and introduction of additional terms:

$$Q + \text{vacant} \cdot b + \text{ISP}^{\text{red}} \overset{K_{Q}^{\text{assn}}}{\longleftrightarrow} Q \cdot b + \text{ISP}^{\text{red}} \overset{K_{Q}^{\text{lig}}}{\longleftrightarrow} Q \cdot b \cdot \text{ISP}^{\text{pred}} \quad (3)$$

If the ligand between QH$_2$ and ISPred is absent or much weaker than that with Q, then the apparent equal affinity of the site for Q and QH$_2$ would indicate that the protein provides an additional binding free energy for the QH$_2$ to compensate for the differential ligation by ISPred:

$$QH_2 + \text{vacant} \cdot b + \text{ISP}^{\text{red}} \overset{K_{QH2}^{\text{assn}}}{\longleftrightarrow} QH_2 \cdot b + \text{ISP}^{\text{red}} \overset{K_{QH2}^{\text{lig}}}{\longleftrightarrow} QH_2 \cdot b \cdot \text{ISP}^{\text{pred}} \quad (4)$$

The apparent $E_m$ value observed in titrations of the occupancy of the Qo site when the ISP is reduced will include the right-hand terms in eqs 3 and 4. Since $K_{QH2}^{\text{overall}} \approx K_{QH2}^{\text{assn}}$:

$$E_m(QH_2, \text{pool}) \approx E_m(Q \cdot b \cdot \text{ISP}^{\text{red}} b / QH_2 \cdot b \cdot \text{ISP}^{\text{pred}} b)$$

If $K_{Q}^{\text{overall}}$ includes a liganding force, $K_{Q}^{\text{lig}}$, then $K_{QH2}^{\text{assn}}$ must be greater than $K_{Q}^{\text{assn}}$ by a value equal to $K_{Q}^{\text{lig}} / K_{ISP}^{\text{c}}$. It seems possible that this might account for some of the higher apparent association constant for QH$_2$ seen in kinetic experiments. As noted in the companion paper (25), from recently refined structures of the complex with stigmatellin (2bcc and revised 3bcc), Glu-272 provides a ligand to the QH group of the inhibitor, with the latter acting as H-bond donor. We have suggested that this side chain might also provide a ligand to quinol, with the OH of quinol as H-bond donor. In the native crystals, the side chain is rotated out of the site. We suggest that this is the configuration when quinone occupies the site, since the carbonyl group will not provide a H-bond donor. The differential binding ($K_{QH2}^{\text{assn}} / K_{Q}^{\text{assn}}$) likely reflects this H-bond. Mutant strains with Glu-272 changed to D or G (E295D or -G in Rb. sphaeroides numbering) showed apparent $K_m$ values for quinol substrate greater by 1.54- and 2.4-fold respectively, suggesting that this was a relatively weak contribution.

As suggested by Crofts and Wang (6) and discussed in a companion paper (25), the activated step in electron transfer, and the rate-limiting step at saturating substrate concentration, is the oxidation of bound quinol. We have suggested that the reaction proceeds from an ES complex formed between ISP, docked in the ISPb position, and quinol in the distal domain of the Qo site (12, 25). To account for the maximal rates of quinol oxidation observed when the pool is substantially oxidized, Crofts and Wang (6) modeled the reaction with a differential binding of QH$_2$ and Q of 30-50-fold. It seems likely that the structure with stigmatellin demonstrates the location of all complexes formed between the Qo site occupant and the ISP, including the reaction complex (12, 21, 25, 43). The bond between the carbonyl group of the inhibitor and His-161 of the ISP might mimic that between quinone and ISPred. However, the ES complex would involve a bond between the OH of the quinol and His-161 of the ISP* (25, 42, 44). Since the H-bond provided by Glu-272 likely provides only a factor of ~2.5 of the 30-50-fold differential binding of quinol (see above), we suggest that the major part, a factor of 10-20, is contributed by the liganding of quinol by the ISP* through His-161.

CONCLUSIONS

We have demonstrated that all the reactions of the high-potential chain through which oxidizing equivalents are delivered to the quinol oxidizing site are rapid compared to the rate-limiting step of quinol oxidation. For those reactions accessible to direct measurement, the activation energies were much lower than that of the overall process. For those reactions for whose rate constant we can only estimate a lower limit, it is clear that there is no contribution to the rate-limiting step and neither therefore to the activation barrier. The movement of the ISP between catalytic interfaces must therefore occur much more rapidly than the rate-limiting steps for the partial reactions measured. From the lack of transient overshoot in the high-potential chain inhibited by myxothiazole, the electron donation from the [2Fe-2S] in the ISPc position is < 10 μs. From the 150 μs lag before cyt b reduction and the rise times of the constituent processes, the transfer of an oxidizing equivalent from ferricyt c$_1$ to the Q$_b$ site occurs in < 50 μs. We have shown that diffusion
of the mobile head of the ISP is expected to be rapid, with a time for root-mean-square displacement over the ~16–22 Å diffusion distance in the range ~25 ns. We have estimated the binding constants for the interactions of the ISP with its two interfaces and shown that with the exception of specific ligands between occupant and the ISP, the association constants are ~2. Absolute values for on and off rate constants are not available, but we anticipate that the on rate constants will be determined by the collisional frequencies and an additional time for stochastic exploration of the docking interfaces. Since these will reflect the rapid diffusion constrained to a local domain by the tethering of the head, we can expect an efficient exploration of the docking domains to find a suitable reaction configuration. Since the apparent association constants calculated from the crystallographic occupancies are all within the range <2, the off rate constants must have values similar to the on rate constants. The range of calculated values for the processes associated with the movement of the ISP are therefore in accord with the limits on rate determined from kinetic measurements. Both on and off rate constants are certainly greater than the values >2 × 10^4 s^-1 indicated by our measurements of the overall processes.

The kinetic picture that emerges from these considerations is of a rapid process, in which electrons are transferred efficiently over a distance, without the entropic loss of diffusion in the volume of the aqueous phase. By tethering the head, the complex maintains a high local concentration and a high probability of successful collision. Although the rates achieved are comparable to those expected from a solid-state system with an intermediate electron carrier, we do not believe that the evolution of this mechanism was determined by the need for rapid electron transfer. Rather, the mechanism fits the site for the peculiar needs of the bifurcated reaction, which we discuss at length in a companion paper (25). We see no evidence that protein–protein interactions lead to specific binding to constrain the ISP*ox in particular positions and suggest that catalytic switching is determined more by the liganding with the Qo site occupant in the ES complex than by other protein interactions. However, we have noted that significant conformational changes accompany binding of inhibitors, and these may mimic changes on occupancy of the different domains of the Qo site, by reaction intermediates (12, 13, 25). It seems possible that such changes might hinder movement of the ISP through physical blockage and that linkage to Qo site domain occupancy might provide a switching function.

REFERENCES

Movement of the ISP: Physicochemical Aspects