Ubiquinone at Center N Is Responsible for Triphasic Reduction of Cytochrome b in the Cytochrome bc₁ Complex

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We have examined the pre-steady state reduction kinetics of the Saccharomyces cerevisiae cytochrome bc₁ complex by menaquinol in the presence and absence of endogenous ubiquinone to elucidate the mechanism of triphasic cytochrome b reduction. With cytochrome bc₁ complex from wild type yeast, cytochrome b reduction was triphasic, consisting of a rapid partial reduction phase, an apparent partial reoxidation phase, and a slow rereduction phase. Absorbance spectra taken by rapid scanning spectroscopy at 1-ms intervals before, during, and after the apparent reoxidation phase showed that this was caused by a bona fide reoxidation of cytochrome b and not by any negative spectral contribution from cytochrome c₁. With cytochrome bc₁ complex from a yeast mutant that cannot synthesize ubiquinone, cytochrome b reduction by either menaquinol or ubiquinol was rapid and monophasic. Addition of ubiquinone restored triphasic cytochrome b reduction, and the duration of the reoxidation phase increased as the ubiquinone concentration increased. When reduction of the cytochrome bc₁ complex through center P was blocked, cytochrome b reduction through center N was biphasic and was slowed by the addition of exogenous ubiquinone. These results show that ubiquinone residing at center N in the oxidized cytochrome bc₁ complex is responsible for the triphasic reduction of cytochrome b.

Although the protonmotive Q cycle mechanism of the cytochrome bc₁ complex is generally well understood (1–3), the redox behavior of cytochrome b during pre-steady state reduction of the bc₁ complex is not fully understood. Cytochrome b reduction is triphasic, consisting of a partial reduction phase, a partial reoxidation phase, and a slow rereduction phase. This behavior is puzzling, because the reoxidation phase occurs while reduced substrate is still available, and continued reduction of cytochrome b would be expected.

Previous examinations of the pre-steady state reduction kinetics of the bc₁ complex were limited to single wavelength kinetics, and the spectral data, when collected, extended over time ranges that were long relative to the half-times of the reactions (4–9). The substrates used in these studies, succinate, duroquinol, trimethylquinol, and ubiquinol, have relatively high redox potentials and reduce only a small percentage of cytochrome b. This is of concern because the high redox potential may predispose these substrates to oxidize cytochrome b and thus introduce artifacts into the pre-steady state kinetics in the absence of a low potential reductant.

Several explanations for the triphasic reduction have been put forth. One proposal is that ubiquinone formed at center P is not in rapid equilibration with the quinone pool and oxidizes cytochrome b at center N (5, 10). Crystal structures of the mitochondrial cytochrome bc₁ complexes show a pear-shaped and dimeric integral membrane protein that extends ~80 Å into the matrix and ~30 Å into the intermembrane space (11, 12). There are two large cavities within the bc₁ dimer that link center P of one monomer to center N of the second monomer.

Our results show that triphasic reduction results from a bona fide reoxidation and rereduction of cytochrome b and that endogenous ubiquinone is responsible for triphasic reduction. We propose that ubiquinone at center N within the oxidized bc₁ complex is responsible for the partial reduction and reoxidation phases of the triphasic reduction by rapidly oxidizing cytochrome b that has been reduced through center P. Equilibration of the first electron from quinol oxidation through center P between cytochrome b₁₄ and ubiquinol at center N causes the partial reduction phase. When a second electron from center P reduces ubisemiquinone to ubiquinol there is a partial reoxidation of cytochrome b. After both the iron-sulfur protein and cytochrome c₁ are reduced, menaquinol cannot be oxidized at center P, and menaquinol rereduces cytochrome b through center N. The duration of the reoxidation or lag phase is dependent upon the amount of ubiquinone available to oxidize cytochrome b at center N after it has been reduced by menaquinol. After the ubiquinone pool has been reduced by a transhydrogenase reaction at center N, cytochrome b remains reduced, and this causes the rereduction phase.

EXPERIMENTAL PROCEDURES

Materials—Dodecyl maltoside was obtained from Roche Molecular Biochemicals. DEAE-Biogel A was obtained from Bio-Rad. Antimycin,
disopropyl fluorophosphate, PMSF, 2,3-dimethoxy-5-methyl-6-decyl benzoquinone ("decy CoQ") and menaquinone were purchased from Sigma. Stigmatellin was purchased from Fluka Biochemika. Yeast extract and peptone were from Difco. The yeast Δcoq2 mutant was obtained from Dr. Catherine Clarke (UCLA). The 2,3-dimethoxy-5,6-dimethyl benzoquinone was obtained from Dr. Chang-an Yu (University of Oklahoma).

Preparation of Menaquinone—A 100 ml stock solution of menaquinone was prepared in ethanol. From this stock a 2 mM solution of menaquinone was prepared by dilution into 50 mM potassium phosphate, pH 6.0, 250 mM sucrose, 0.2 mM EDTA, 1 mM NaN3, 0.1% bovine serum albumin. Because menaquinone is insoluble in water the buffers it precipitated from the solution. The menaquinone was reduced with 2-fold molar excess of sodium borohydride. As the menaquinone was reduced it became soluble in the aqueous buffer and was mixed until it was completely solubilized and no more bubbles were released. A fresh menaquinol solution was prepared prior to each kinetic experiment, kept under anaerobic conditions, and diluted into degassed buffer immediately prior to its use. Control experiments established that the reduction of the cytochrome bc1 complex is caused by menaquinol and not by any residual sodium borohydride.

The ubiquinone analogue, 2,3-dimethoxy-5,6-dimethyl benzoquinone, was dissolved directly in 50 mM potassium phosphate, pH 6.0, 250 mM sucrose, 0.2 mM EDTA, 1 mM NaN3, 0.1% bovine serum albumin and reduced with sodium borohydride. This quinol was used as substrate for the experiments in Fig. 4.

Purification of Cytochrome bc1 Complex—Two pounds of Red Star baker's yeast were washed once with distilled water and once with disruption buffer (100 mM Tris, 250 mM sorbitol, 5 mM MgCl2, 150 mM potassium acetate, 1 mM dithiothreitol, pH 8.0). The washed yeast cells were resuspended by adding 40 ml of disruption buffer and frozen by stirring for 45 min at 4 °C. The membrane extract was clarified by sedimentation at 20,000 g. The mitochondrial membranes were washed twice in 50 mM Tris acetate, 0.4 M mannitol, nitrogen was periodically added to prevent thawing of the cells. The washed membranes were resuspended by adding 40 ml of disruption buffer and frozen by stirring for 45 min at 4 °C. The membrane extract was clarified by sedimentation at 3000 g.

Mitochondrial membranes were stored in 150 mM potassium acetate, 50 mM Tris acetate, 2 mM EDTA, 50% glycerol, pH 8.0. Mitochondrial membranes were solubilized in 100 mM Tris-HCl, 1 mM EDTA, 1 mM MgSO4, 1 mM PMSF, 100 mM NaCl, pH 8.0. After loading, the column was washed with two column volumes of the same buffer obtained from the spin pack. Mitochondrial membranes were suspended in 50 mM potassium phosphate, pH 6.0, containing 250 mM sucrose, 0.2 mM EDTA, 1 mM NaCl, 0.1% bovine serum albumin and reduced with sodium borohydride. This quinol was used as substrate for the experiments in Fig. 4.

RESULTS

Kinetics of Reduction of the Cytochrome bc1 Complex by Menaquinol—Menaquinol is a preferable substrate for reduction of the cytochrome bc1 complex because the oxidation-reduction potential (Em2 = −74 mV; Ref. 13) is low enough to reduce all of cytochrome b1 and a portion of cytochrome c1 in the Rieske iron-sulfur cluster and cytochrome c1. We previously established that menaquinol rapidly reduces the bc1 complex through the catalytic centers P and N and that menaquinol reduction via center P and N is not dependent upon endogenous ubiquinone (15). By using menaquinol to reduce the bc1 complex and monitoring the reaction with rapid scanning stopped flow spectroscopy, it is possible to examine the time course of cytochrome b and c reduction in a single reaction and to obtain time resolved optical spectra at 1-ms intervals during the reaction.

Under continuous conditions turnover, where the catalytic reaction is zero order with respect to ubiquinol and cytochrome c, the turnover number of the yeast bc1 complex approaches 200 s−1 (17). From this catalytic activity one can estimate that the half-life for the transit of a single electron through the enzyme from ubiquinol to cytochrome c would occur within approximately 5 ms. It is clearly not possible to monitor pre-steady state reduction of the bc1 complex under conditions where the reaction is zero order with respect to menaquinol, because much of the reaction would occur within the 2-ms mixing time of the instrument. However, by lowering the concentration of menaquinol, the pre-steady state reduction can be monitored on a ms time scale under conditions where the reduction is first order with respect to menaquinol.

The traces in Fig. 1 show the time course of reduction of cytochrome b and c1 when 1 μM bc1 complex is reduced with 6 μM menaquinol. Cytochrome c1 reduction was monophasic and within the low concentration of menaquinol occurred at 4.6 ± 0.2 s−1. In contrast, cytochrome b reduction was triphasic and consisted of a rapid partial reduction phase, a partial reoxidation phase, and slow reoxidation phase. During the partial reduction phase ~30% of the cytochrome b was reduced and reached its maximum value at 70 ms. During the reoxidation phase ~20% of the cytochrome b remained reduced, and the minimum value was reached at 280 ms. The reoxidation phase was monophasic and occurred at a rate of 1.9 ± 0.4 s−1.

Time resolved spectra averaged across 15-ms intervals before (at 70 ms), during (at 280 ms), and after (at 2 s) the triphasic reduction confirm that the apparent reoxidation phase was caused by the net oxidation of cytochrome b and not by spectral overlap of cytochrome c1. From the absorption spectra A and C in the inset of Fig. 1, one can calculate that the expected absorbance for cytochrome b at 563.3 nm in spectrum B would be 0.031–0.043, absent any reoxidation of the cyto-

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1 The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; Q, ubiquinone; QH2, ubiquinol.
Triphasic Reduction of the bc1 Complex

Fig. 1. Pre-steady state reduction of the cytochrome bc1 complex by menaquinol in the absence of inhibitors. The traces show the time course of the reduction of 1 μM cytochrome bc1 complex by 6 μM menaquinol. The traces labeled cyt b and cyt c1 correspond to the absorbance changes at 563.3 and 554.6 nm, respectively. The inset shows the reduced minus oxidized spectra of the bc1 complex at 70 ms, 280 ms, and 2 s during the reaction. Each spectrum is an average of 15 individual spectra collected at 1-ms intervals over the 15-ms intervals indicated by the horizontal bars labeled A, B, and C.

We conclude that our prior interpretation of the triphasic reduction is correct (6). That is, the reaction consists of an initial partial reduction of cytochrome b through center P, followed by and possibly partially coinciding with reoxidation through center N. When the iron-sulfur protein and cytochrome c1 become reduced, further reduction of cytochrome b linked to reduction of the high potential acceptors at center P is no longer possible. At this point in the reaction, corresponding to ~400 ms in Fig. 1, reduction of cytochrome c1 resumes through center N at a slower rate.

At high concentrations of menaquinol (e.g., 200 μM), the rate of b reduction does not appear to be triphasic. We thus examined the effects of increasing the menaquinol concentration on the time course of cytochrome b and c1 reduction as shown in Fig. 2. As the menaquinol concentration increased, the rate of cytochrome c1 reduction increased and remained monophasic, and at 100 μM menaquinol the rate was 28 ± 1 s⁻¹. There was a hyperbolic relationship between menaquinol concentration and the rate of cytochrome c1 reduction, and a double reciprocal plot produced a Vmax for c1 reduction of 40 ± 4 s⁻¹ (data not shown). When the menaquinol concentration is no longer rate-limiting for c1 reduction, electron transfer from menaquinol to the iron-sulfur becomes limiting (18).

For cytochrome b, increasing the menaquinol concentration increased the rate of the partial reduction phase, such that at 100 μM menaquinol this phase occurred during the 2-ms mixing time (Fig. 2). As the concentration of menaquinol increased, there was a gradual elimination of the reoxidation phase and an increase in the rate of the reduction phase. At 100 μM menaquinol, the reoxidation phase was absent, and the trace consisted of a very rapid partial reduction, a brief plateau, and a slower reduction phase, at a rate of 21 ± 2 s⁻¹. The tracings in Fig. 2 demonstrate that the apparent lack of triphasic reduction at high concentrations of menaquinol results from increasingly fast reduction and rereduction phases as the menaquinol concentration is increased, such that the three phases of the reaction coalesce into an apparently biphasic reduction.

The most unusual aspect of these results is that cytochrome b goes partially reoxidized under conditions where the menaquinol pool remains highly reduced. For example, at 50 ms during the triphasic reduction with 12 μM menaquinol (Fig. 2), ~0.3 μM cytochrome b has been reduced, and >90% of the menaquinol remains in the reduced form. Contrary to what would be expected, during the ensuing 100 ms cytochrome b undergoes partial reoxidation while the calculated concentration of menaquinol is >10 μM, and the potential of the menaquinol pool at pH 6 is <−60 mV. As discussed below, the lack of equilibration of cytochrome b with the menaquinol pool suggests that triphasic cytochrome b reduction is caused by the presence of ubiquinone at center N that oxidizes cytochrome b and that this ubiquinone does not rapidly equilibrate with the menaquinol pool.

Kinetics of Reduction of the Cytochrome bc1 Complex by Menaquinol in the Absence of Ubiquinone—To determine whether ubiquinone is responsible for triphasic cytochrome b reduction, we isolated the cytochrome bc1 complex from a yeast mutant (Δcoq2) that lacks ubiquinone because of the deletion of a gene for an enzyme in the ubiquinone biosynthetic pathway (19). The Δcoq2 mutant is unable to respire but can grow on fermentable carbon sources. Using this mutant to obtain bc1 complex lacking ubiquinone avoids any damage to the enzyme that might result from extraction of ubiquinone with organic solvents and eliminates the possibility that any residual
ubiquinone remains in the bc1 complex.

Having previously shown that menaquinol can reduce the b and c1 cytochromes through center N and center P in the bc1 complex isolated from the Δcoq2 mutant (15), we examined the time course of the reduction of cytochrome b and c1 over a range of menaquinol concentrations as shown in Fig. 3. The most obvious difference in the pre-steady state reduction of the bc1 complex from the mutant lacking endogenous ubiquinone is that reduction of cytochrome b is not triphasic but rather a rapid monophasic reaction. A similar lack of triphasic reduction was reported in two previous studies (20, 21). With 6 μM menaquinol the rate of cytochrome b reduction was monophasic and occurred at 9.1 ± 1.9 s⁻¹. The rate increased linearly with menaquinol concentration such that at 50 μM menaquinol the rate was ~90 s⁻¹, with a large portion of the reduction occurring during the 2-ms mixing time. These results are similar to what is seen in the presence of antimycin (15), which blocks electron transfer through center N.

In addition, at each of the menaquinol concentrations tested, the rate of cytochrome c1 reduction was about four times slower than observed with the bc1 complex from wild type yeast. Using 6 μM menaquinol, the rate of cytochrome c1 reduction occurred at 0.9 s⁻¹ and increased with menaquinol concentration such that at 50 μM menaquinol the rate was 4.0 s⁻¹. With the bc1 complex from the wild type yeast, the corresponding rates were 3.3 and 18 s⁻¹. An explanation for the decreased rate of c1 reduction is discussed below.

The lack of triphasic reduction in the Δcoq2 bc1 complex is not dependent on the low potential of the menaquinol substrate, because the same effect is observed with ubiquinol. As shown in Fig. 4, reduction of cytochrome b by 2,3-dimethoxy-5,6-dimethyl benzoquinol, a ubiquinol analogue, in the bc1 complex from the wild type yeast is triphasic but is monophasic in the Δcoq2 bc1 complex. The only apparent difference in the reduction of b in the Δcoq2 bc1 complex by ubiquinol versus menaquinol is that the transient over-reduction and equilibration by reoxidation at the end of the reaction is more pronounced with ubiquinol (Fig. 4, bottom panel) than with menaquinol (Fig. 3).

Restoration of Triphasic Cytochrome b Reduction in the bc1 Complex from the Δcoq2 Mutant by Ubiquinone—To confirm that ubiquinone is responsible for triphasic cytochrome b reduction, we added back ubiquinone to cytochrome bc1 complex isolated from the Δcoq2 mutant. Adding back various amounts of ubiquinone to the ubiquinone-deficient cytochrome bc1 complex alters the reduction by menaquinol as shown in Fig. 5. Over the range of ubiquinone added, the kinetics of cytochrome c1 reduction was monophasic. With 1 μM ubiquinone added, the rate of cytochrome c1 reduction occurred at ~3.3 s⁻¹ and decreased only slightly to 2.5 s⁻¹ with 8 μM ubiquinone. It was surprising that adding ubiquinone did not significantly slow the rate of cytochrome c1 reduction, because it could conceivably act as a competitive oxidant of menaquinol. If reduction of the ubiquinone pool was a prerequisite to reduction of the complex through center P, one would expect a much larger decrease in the rate of cytochrome c1 reduction as the ubiquinone content increased. This also suggests that in the oxidized bc1 complex ubiquinone does not interfere with menaquinol access to center P.

The addition of ubiquinone to the ubiquinone-deficient cytochrome bc1 complex restored triphasic cytochrome b reduction (Fig. 5). With 1 μM ubiquinone added, the time course of cytochrome b reduction consisted of a rapid partial reduction phase, a small lag phase, and a slow reduction phase. The rapid reduction phase comprised ~35% of the total absorbance change and was complete within 50 ms. As the ubiquinone concentration increased, the reduction of cytochrome b clearly became triphasic and consisted of a rapid partial reduction phase, a lag phase, and a slow reduction phase. Also, the portion of the cytochrome b reduced during the rapid reduction
phase decreased, and the lag phase became longer. With 8 μM ubiquinone added, only 25% of the cytochrome b was reduced during the rapid partial reduction phase that was complete within 50 ms. The lag phase extended for over 500 ms and was followed by the slow reduction phase.

It has been previously shown that the reduction of the Q pool occurs in parallel with the rereduction phase of triphasic reduction (22). Thus, the ubiquinone pool is unlikely to be immediately reduced upon the addition of menaquinol. As we increased the ubiquinone concentration we apparently slowed the reduction of the quinone pool, which apparently must occur for cytochrome b to remain reduced. Our results show that the rapid reduction of cytochrome b through center P is not affected by exogenous ubiquinone, although the equilibration of b1H with the quinone pool appears to be shifted, because less cytochrome b is reduced during the initial phase of the triphasic reduction as the ubiquinone concentration is increased.

Fig. 6 shows the effects of increasing menaquinol concentration on the time course of cytochrome b and c1 reduction when 2 μM ubiquinone was added to 1 μM ubiquinone-deficient cytochrome bc1 complex. Over the range of menaquinol concentrations tested, the reduction of cytochrome c1 was monophasic and increased from 1.6 s⁻¹ at 6 μM menaquinol to 8.2 s⁻¹ at 50 μM menaquinol. Because there was a linear relationship between menaquinol and the rate of cytochrome c1 reduction, the reaction is limited by menaquinol concentration, and not by the rate of electron transfer from the iron-sulfur protein to cytochrome c1.

For cytochrome b, the reduction was clearly triphasic at the lower menaquinol concentrations and became increasingly biphasic as the menaquinol concentration increased. The proportion of the cytochrome b reduced during the fast reduction phase remained constant at ~25%, and this change occurred during the 2-ms dead time at the higher menaquinol concentrations. The lag between the two phases decreased as the menaquinol concentration increased, consistent with the more rapid reduction of the quinone pool by menaquinol.
Triphasic Reduction of the $bc_1$ Complex

FIG. 7. Effect of exogenous ubiquinone on the time course of the reduction of the cytochrome $bc_1$ complex. The traces show the reduction of cytochromes ($cyt$) $b$ and $c_1$ when 1 $\mu$M cytochrome $bc_1$ complex containing endogenous ubiquinone is mixed with 2, 4, or 8 $\mu$M decyl ubiquinone and then reduced by 25 $\mu$M menaquinol.

or N will be continually oxidized at center N. As the ubiquinone pool becomes reduced, less is available to oxidize cytochrome $b$, thus allowing cytochrome $b$ to remain reduced.

Effect of Exogenous Ubiquinone on Cytochrome $b$ Reduction through Center N in Wild Type and Ubiquinone-deficient Cytochrome $bc_1$ Complex—To confirm that the rereduction phase requires the reduction of the ubiquinone pool, we examined the effects of exogenous ubiquinone on the time course of cytochrome $b$ reduction through center P. In these experiments stigmatellin was included to block reduction through center P. The top left panel in Fig. 8 shows the effect of exogenous ubiquinone on the time course of cytochrome $b$ reduction in wild type cytochrome $bc_1$ complex through center N. In the absence of exogenous ubiquinone, cytochrome $b$ reduction was biphasic with 80% reduced at 8.7 s$^{-1}$ and 20% reduced at 1.1 s$^{-1}$. When 2 $\mu$M ubiquinone was added the rates decreased to 70% reduced at 5.0 s$^{-1}$ and 30% reduced at 0.4 s$^{-1}$.

The top right panel in Fig. 8 shows a similar experiment with $bc_1$ complex from the yeast mutant lacking endogenous ubiquinone. Without the addition of ubiquinone cytochrome $b$ reduction was biphasic with 66% reduced at 17 s$^{-1}$ and 33% reduced at 1.2 s$^{-1}$. With 2 $\mu$M ubiquinone the fast rate decreased to 50% reduced at 10 s$^{-1}$ and 50% reduced at 0.7 s$^{-1}$. Thus, $b$ reduction by menaquinol through center N is approximately twice as fast in the absence of ubiquinone and addition of two equivalents of ubiquinone to the ubiquinone-deficient complex reduced the rate to approximately that seen with the complex containing endogenous ubiquinone.

Effect of Exogenous Ubiquinone on Cytochrome $b$ and $c_1$ Reduction through Center P in Ubiquinone-deficient Cytochrome $bc_1$ Complex—To confirm that exogenous ubiquinone had little effect upon the reactions at center P, we examined the time course of cytochrome $b$ and $c_1$ reduction in the presence of antimycin. The top left panel in Fig. 8 shows the time course of cytochrome $b$ and $c_1$ reduction in the ubiquinone-deficient cytochrome $bc_1$ complex. The results show that cytochrome $b$ was biphasic with 50% occurring at 22 s$^{-1}$ and 50% occurring at 5.0 s$^{-1}$. Cytochrome $c_1$ reduction was monophasic and occurred at a rate similar to the slow rate of cytochrome $b$ reduction at 4.3 s$^{-1}$.

The bottom right panel in Fig. 8 shows the effects of exogenous ubiquinone on the kinetics of cytochrome $b$ and $c_1$ reduction within the cytochrome $bc_1$ complex from the mutant lacking endogenous ubiquinone. Again, cytochrome $b$ reduction was biphasic and occurred with rates of 21 s$^{-1}$ and 2.7 s$^{-1}$. Cytochrome $c_1$ reduction was monophasic and occurred at 2.6 s$^{-1}$. Thus, two equivalents of ubiquinone had no effect upon the fast phase of $b$ reduction through center P.

Kinetics of Ubiquinone Reduction by Menaquinol in the Absence or Presence of the Cytochrome $bc_1$ Complex—To clarify the role of endogenous ubiquinone on reduction of the enzyme by menaquinol, we measured the rate of ubiquinone reduction by menaquinol in the absence or presence of ubiquinone-deficient cytochrome $bc_1$ complex. In the absence of enzyme, the reduction of 20 $\mu$M ubiquinone by 100 $\mu$M menaquinol occurred with a half-time of 10.0 s (data not shown). This rate is so slow that menaquinol is unlikely to reduce the cytochrome $bc_1$ complex via endogenous ubiquinone but rather reduces the enzyme directly.

Cytochrome $bc_1$ complex catalyzed the transhydrogenation reduction of ubiquinone by menaquinol. With 0.05 $\mu$M enzyme the half-time for reduction of 20 $\mu$M ubiquinone by 100 $\mu$M menaquinol decreased to 7.5 s, and with 0.25 $\mu$M enzyme the half-time decreased to 3.2 s. Extrapolation of the rates to the concentration of enzyme used in our pre-steady state reduction experiments resulted in a half-time of 1.2 s. This rate is fast enough to account for the rereduction phase observed during
Triphasic reduction and for the extension of the reoxidation phase resulting from the addition of exogenous ubiquinone. This activity was measured in the presence of stigmatellin and was blocked by the presence of antimycin, confirming that this transhydrogenase reaction is catalyzed at center N (21).

**DISCUSSION**

We re-investigated the triphasic reduction of cytochrome $b$ to determine whether the apparent triphasic reduction results from a true reoxidation of the $b$ (6) or is an artifact resulting from declining absorption in the $c_1$ spectrum and overlap at the wavelength (563 nm) typically used to monitor $b$ reduction (7, 8). We also wanted to know whether triphasic reduction is uniquely dependent on the relatively high reduction potential of the substrates typically employed to elicit this reaction, and finally, we sought to clarify the role, if any, of endogenous ubiquinone in the triphasic reaction.

Our results clearly establish that in the absence of inhibitors pre-steate state reduction of cytochrome $b$ is a triphasic reaction, even when the potential of the substrate is significantly lower than that of ubiquinol and the $b_{11}$ heme. Time resolved optical spectra during the triphasic reduction demonstrate that cytochrome $b$ undergoes partial reduction, partial reoxidation, and then reoxidation. The reaction pattern changes from triphasic to biphasic and eventually to apparently monophasic as the concentration of menaquinol is increased and the reaction approaches first order.

Our results also show that endogenous ubiquinone is responsible for the triphasic reduction of cytochrome $b$. In the absence of endogenous ubiquinone we no longer observed the triphasic reduction of cytochrome $b$, but upon ubiquinone addition triphasic reduction was restored. These results agree with previous studies, in which extraction of ubiquinone from the cytochrome $bc_1$ complex eliminated triphasic reduction (20), and triphasic reduction was restored upon ubiquinone addition (21). Our finding that the rates of the three phases depend on the ubiquinone content of the $bc_1$ complex explains why in some instances the reaction was reportedly biphasic (20).

We have previously shown that menaquinol can rapidly reduce cytochrome $b$ through center N in the absence of endogenous ubiquinone when center P is blocked (15). Endogenous ubiquinone or ubiquinone added to the ubiquinone-deficient $bc_1$ complex apparently inhibits reduction of cytochrome $b$ through center N. If stigmatellin is present to block reduction of $b$ through center P, the rate of $b$ reduction through center N is slower in $bc_1$ complex in which ubiquinone is present than in the ubiquinone-deficient complex. Addition of one equivalent of ubiquinone to the ubiquinone-deficient $bc_1$ complex slowed the reduction through center N in the presence of stigmatellin but had no effect on the fast phase of $b$ reduction through center P, measured in the presence of antimycin. When antimycin is present ubiquinone would be blocked from binding to center N but could bind to center P. Although we saw no effect on the fast phase of $b$ reduction, there was a 2-fold decrease in the slow phase of $b$ reduction and the rate of cytochrome $c_1$ reduction. This may result from ubiquinone oxidizing the iron-sulfur protein after it has been reduced by menaquinol, thus slowing the apparent rate of cytochrome $c_1$ reduction.

The partial reoxidation of cytochrome $b$ that occurs under conditions where the potential of the substrate is low enough to reduce cytochrome $b$ requires that an oxidant for cytochrome $b$ must be electronically isolated from the ubiquinol or menaquinol pool. To allow for this it was proposed that ubiquinone formed at center P moves to center N and creates a locally high ubiquinone concentration in proximity to $b_{11}$ that is not in rapid equilibrium with the ubiquinol pool (5). The possibility of exchange of ubiquinone between center P and N is supported by the crystal structure, which shows a channel that may connect center P of one monomer with center N of the other monomer (11). However, in our experiments pre-existent ubiquinone residing at center N in the wild type complex would obviate the necessity of any such movement.

We propose that within the oxidized $bc_1$ complex ubiquinone occupying center N prevents the rapid reduction of cytochrome $b$ through center N and oxidizes cytochrome $b$ that has been reduced via center P. This occupancy creates the partial reduction and reoxidation phases. As long as ubiquinone is available cytochrome $b$ will remain oxidized, and as the ubiquinone pool becomes reduced the reoxidation phase proceeds. The transhydrogenase catalyzed equilibration of ubiquinone with menaquinol at center N is slow relative to $b_{11}$ oxidation by ubiquinone, and this rate difference allows cytochrome $b_{11}$ to be oxidized and then slowly reduced during the reoxidation phase as it equilibrates with the menaquinol pool.

The direct reduction of ubiquinone by menaquinol is slow relative to the transhydrogenase reaction at center N. This allows ubiquinone to oxidize cytochrome $b_{11}$ during triphasic reduction that has been reduced through center P and to oxidize $b_{11}$ that has been reduced by menaquinol through center N. Thus, the addition of endogenous ubiquinone extends the reoxidation phase and slows the reoxidation phase by reoxidizing cytochrome $b$ faster than it can be reduced through either center P or N.

In the ubiquinone-deficient complex, menaquinol is apparently unable to rapidly reoxidize cytochrome $b$ through center N, because either menaquinol formed at center P is unable to move within the enzyme to center N for steric reasons or, if it moves, it cannot oxidize $b_{11}$ in the presence of excess menaquinol, because it is a poor oxidant ($E_{m} = -74$ mV). This results in the lack of triphasic reduction. This limitation only manifests under conditions of the pre-steate state experiments. The cytochrome c reductase activity of the ubiquinone-deficient complex was $100 \text{ s}^{-1}$ with menaquinol as substrate, compared with $70 \text{ s}^{-1}$ with ubiquinol as substrate, indicating that reoxidation of $b_{11}$ by menaquinol is not thermodynamically limited under conditions of catalytic turnover. This is consistent with the oxidation of substrate at center P by the iron-sulfur protein being the rate-limiting step within the catalytic cycle (18) and not the oxidation of cytochrome $b_{11}$ by either ubiquinone or menaquinone.

When ubiquinol was used as a substrate we only observed triphasic reduction when endogenous ubiquinone was present. This shows that endogenous ubiquinone occupying center N is responsible for the oxidation of cytochrome $b$ during triphasic reduction. This does not exclude the possibility that ubiquinone formed at center P can move directly to center N. However, the water-soluble ubiquinone analogue used as substrate for reduction of the quinone-deficient complex would not be expected to cycle effectively from one center to the other within the hydrophobic interior of the enzyme.

In the ubiquinone-deficient complex cytochrome $b$ can be rapidly reduced under pre-steate state conditions through both center N and center P, and triphasic reduction is not observed. In those complexes in which reduction occurs through center N, the reduction is uncoupled from $c_1$ reduction. Consequently, the extent to which cytochrome $b$ is reduced through center N can be estimated by the shortfall in $c_1$ reduction in the ubiquinone-deficient complex compared with the wild type complex. The extent of $c_1$ reduction is only slightly less in the absence of endogenous ubiquinone (Fig. 3 versus Fig. 2), indicating that most of the complexes are reduced through the thermodynamically preferred center P pathway.

The decreased rate of $c_1$ reduction seen in the ubiquinone-
Triphasic Reduction of the \(bc_1\) Complex

deficient complex (Fig. 3 versus Fig. 2) is comparable with the decreased rate of \(c_1\) reduction that is observed in the presence of antimycin. This results from the fact that reduction of the high potential centers of the \(bc_1\) complex, the iron-sulfur protein and cytochrome \(c_1\), is dependent upon the availability of oxidized low potential centers, which in turn is affected by the redox equilibration between \(b_H\) and ubiquinone. This aspect of the Q cycle mechanism is discussed in more detail elsewhere.\(^2\)

In our mechanism, within the native oxidized complex, ubiquinone would occupy center N and be capable of rapidly oxidizing cytochrome \(b\) reduced through center P. Thus, when menaquinol is oxidized at center P one electron reduces the iron-sulfur protein, and the second electron reduces cytochrome \(b\). Because ubiquinone occupies center N, a single electron in cytochrome \(b\) will equilibrate between \(b_H\) and Q, forming the species \((b_H^+Q)^-\). The equilibration of the first electron between \(b_H\) and Q causes the partial reduction of cytochrome \(b\). When a second menaquinol molecule is oxidized at center P, both the iron-sulfur protein and cytochrome \(c_1\) become reduced, which prevents any subsequent reactions at center P. A second electron enters cytochrome \(b\) and reduces \((b_H^+Q)^-\) to \(b_H^+\)QH\(_2\), which causes the partial reoxidation phase.

Equilibration of the first electron introduced through center P between \(b_H\) and Q at center N is consistent with an electronically coupled complex between \(b_H\) and \(Q^+\) (23, 24) and with their relative midpoint potentials. At room temperature, the midpoint potential of \(b_H\) in yeast was reported to be +60 mV, and the potentials for the two half-reactions converting ubiquinone to ubinonyl at center N were calculated to be 110 mV (\(Q/Q^+\)) and 200 mV (\(Q^+/QH_2\)), respectively (25). Assuming \(E_{m7}\) for the \(Q/QH_2\) couple to be +90 mV (14) in the absence of any preferential binding, these potentials reflect approximately 100 times tighter binding of Q than QH\(_2\) at center N. It would be expected that a single electron would equilibrate between \(b_H\) and the Q, but when a second electron enters cytochrome \(b\) via center P cytochrome \(b_H\) would remain oxidized and QH\(_2\) would be formed and displaced from center N by Q. The semiquinone at center N cannot rapidly exchange with the ubinonyl pool, which accounts for the existence of a stable semiquinone at center N (26).

Our model is consistent with the nonequilibrium experiments where the formation of a semiquinone at center N paralled the rapid reduction phase of cytochrome \(b\) (4). The semiquinone concentration decreased in parallel with the reoxidation phase and increased in parallel with the reoxidation phase. In the presence of antimycin or upon ubiquinone extraction, the kinetics of cytochrome \(b\) reduction was biphasic, and no semiquinone was formed (20). These results agree with our interpretation that ubiquinone at center N, and the stabilization of the semiquinone creates the first phase of triphasic cytochrome \(b\) reduction.

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\(^2\) C. Snyder and B. Trumpower, manuscript in preparation.