

Ubiquinone at Center N Is Responsible for Triphasic Reduction of Cytochrome *b* in the Cytochrome *bc*₁ Complex*

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We have examined the pre-steady state reduction kinetics of the *Saccharomyces cerevisiae* cytochrome *bc*₁ complex by menaquinol in the presence and absence of endogenous ubiquinone to elucidate the mechanism of triphasic cytochrome *b* reduction. With cytochrome *bc*₁ 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 *c*₁. With cytochrome *bc*₁ 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 *bc*₁ 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 *bc*₁ complex is responsible for the triphasic reduction of cytochrome *b*.

Although the protonmotive Q cycle mechanism of the cytochrome *bc*₁ complex is generally well understood (1–3), the redox behavior of cytochrome *b* during pre-steady state reduction of the *bc*₁ complex is not fully understood. Cytochrome *b* reduction is triphasic, consisting of a rapid 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 *bc*₁ 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 *bc*₁ 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 *bc*₁ dimer that link center P of one monomer to center N of the second monomer. The presence of these cavities may allow ubiquinol or ubiquinone to exchange between these two sites without having to diffuse into the membrane (11). It has also been suggested that the decreased absorbance at the cytochrome *b* wavelength is not true oxidation but rather spectral overlap of cytochrome *c*₁ that gives the appearance of cytochrome *b* oxidation (8).

We have examined the pre-steady state reduction kinetics of the *Saccharomyces cerevisiae* *bc*₁ complex by menaquinol using rapid scanning stopped flow spectroscopy. Menaquinol ($E_{m7} = -74$ mV; Ref. 13) reduces a larger percentage of cytochrome *b* than does ubiquinol ($E_{m7} = +90$ mV; Ref. 14) and rapidly reduces cytochrome *b* through center P or center N (15). Rapid scanning stopped flow spectroscopy allows the simultaneous monitoring of the time course of reduction of cytochrome *b* and *c*₁ in a single reaction and the examination of absorbance spectra at any time point during the reaction.

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 *bc*₁ 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 *b*_H and ubiquinone at center N causes the partial reduction phase. When a second electron from center P reduces ubiquinone to ubiquinol there is a partial reoxidation of cytochrome *b*. After both the iron-sulfur protein and cytochrome *c*₁ 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,

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diisopropyl fluorophosphate, PMSF,¹ 2,3-dimethoxy-5-methyl-6-decyl benzoquinone ("decyl CoQ") and menaquinone were purchased from Sigma. Stigmatellin was purchased from Fluka Biochemika. Yeast extract and peptone were from Difco. The yeast $\Delta coq2$ 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 Menaquinol—A 100 mM 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 NaN_3 , 0.1% bovine serum albumin. Because menaquinone is insoluble in aqueous buffers it precipitated from the solution. The menaquinone was reduced with a 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 bc_1 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 NaN_3 , 0.1% bovine serum albumin and reduced with borohydride. This quinol was used as substrate for the experiments in Fig. 4

Purification of Cytochrome bc_1 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 MgCl_2 , 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 slowing pouring the suspension as a thin stream into liquid nitrogen. The frozen yeast were blended in liquid nitrogen in a stainless steel Waring blender for a total of 5 min at 1-min intervals. Additional liquid nitrogen was periodically added to prevent the thawing of the cells.

The lysed cell powder was thawed under warm water with the addition of 1 mM diisopropyl fluorophosphate and 1 mM PMSF. The cell debris was sedimented at $3000 \times g$ for 10 min, and the pellet was washed once in disruption buffer and sedimented at $3000 \times g$ for 10 min. The supernatants were combined, and the mitochondrial membranes were sedimented at $20,000 \times g$ for 30 min. The mitochondrial membranes were washed twice in 50 mM Tris acetate, 0.4 M mannitol, 2 mM EDTA, pH 8.0, containing 1 mM diisopropyl fluorophosphate and once in 150 mM potassium acetate, 50 mM Tris acetate, 2 mM EDTA, pH 8.0. Mitochondrial membranes were stored in 150 mM potassium acetate, 50 mM Tris acetate, 2 mM EDTA, 50% glycerol, pH 8.0, at -20°C . Membrane protein concentrations were determined by a modified Lowry method (16)

To purify the bc_1 complex mitochondrial membranes were suspended at 10 mg/ml in 50 mM Tris-HCl, 1 mM MgSO_4 , 1 mM PMSF, pH 8.0, and 0.8 g of dodecyl maltoside/g of membrane protein was added and slowly stirred for 45 min at 4°C . The membrane extract was clarified by centrifugation at $100,000 \times g$ for 90 min. After the addition of 100 mM NaCl and stirring for 60 min, the extract was loaded onto a 1.5×20 cm DEAE-Biogel A chromatography column equilibrated with 50 mM Tris-HCl, 1 mM MgSO_4 , 1 mM PMSF, 100 mM NaCl, pH 8.0. After loading, the column was washed with two column volumes of the same buffer and eluted with six column volumes of a linear gradient of 100–400 mM NaCl in 50 mM Tris-HCl, 1 mM MgSO_4 , 1 mM PMSF, pH 8.0. The bc_1 complex eluted at approximately 280 mM NaCl. The combined bc_1 fractions were concentrated to $\sim 50 \mu\text{M}$ cytochrome bc_1 complex (17) using Amicon Centriprep 30 tubes.

The yeast mutant $\Delta coq2$ was grown in 80 liters of YPD and was harvested by centrifugation. The cytochrome bc_1 complex from $\Delta coq2$ was isolated as described above.

Kinetic Measurements—Kinetic measurements were performed at room temperature by rapid scanning stopped flow spectroscopy, using an OLIS Rapid Scanning Monochromator (On-Line Instrument Systems, Inc., Bogart, GA) equipped with a 1200 lines/mm grating blazed at 500 nm. This produced a spectrum of 75 nm width, centered at 555 nm, with a resolution of 0.4 nm. The dead time of the instrument was ~ 2 ms, and the end of this period was chosen as time 0, after which data were collected at 1000 scans/s.

Reactions were started by mixing $2 \mu\text{M}$ bc_1 complex in 50 mM potas-

sium phosphate, pH 6.0, containing 250 mM sucrose, 0.2 mM EDTA, 1 mM NaN_3 , and 1.0 mg/ml bovine serum albumin against an equal volume of buffer containing menaquinol. An oxidized spectrum was obtained by mixing the oxidized bc_1 complex against buffer and averaging the data set to a single scan. For each experiment three data sets were averaged, and the oxidized spectrum was subtracted from each scan. From the three-dimensional data set, which is comprised of wavelength, absorbance, and time, we examined the time course of cytochrome b and c_1 reduction at 563.3 and 554.6 nm, respectively.

The rates of ubiquinone reduction by menaquinol were measured in an Aminco DW2A spectrophotometer. The instrument was in split beam mode, and equal concentrations of decyl ubiquinone were in each cuvette. Menaquinol was added to one cuvette, and the rate of ubiquinone reduction was monitored by the decrease in absorbance at 281 nm. This wavelength was chosen because it is an isosbestic point for menaquinol/menaquinone. These experiments were performed using the same buffer used in the stopped flow experiments, with the addition of 0.05% dodecylmaltoside to keep decyl ubiquinone in solution.

RESULTS

Kinetics of Reduction of the Cytochrome bc_1 Complex by Menaquinol—Menaquinol is a preferable substrate for reduction of the cytochrome bc_1 complex because the oxidation-reduction potential ($E_{m7} = -74$ mV; Ref. 13) is low enough to reduce all of cytochrome b_H and a portion of cytochrome b_L , in addition to the Rieske iron-sulfur cluster and cytochrome c_1 . We previously established that menaquinol rapidly reduces the bc_1 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 bc_1 complex and monitoring the reaction with rapid scanning stopped flow spectroscopy, it is possible to examine the time course of cytochrome b and c_1 reduction in a single reaction and to obtain time resolved optical spectra at 1-ms intervals during the reaction.

Under conditions of continuous turnover, where the catalytic reaction is zero order with respect to ubiquinol and cytochrome c , the turnover number of the yeast bc_1 complex approaches 200 s^{-1} (17). From this catalytic activity one can estimate that the half-time 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 bc_1 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 c_1 when $1 \mu\text{M}$ bc_1 complex is reduced with $6 \mu\text{M}$ menaquinol. Cytochrome c_1 reduction was monophasic and with this low concentration of menaquinol occurred at $4.6 \pm 0.2 \text{ s}^{-1}$. In contrast, cytochrome b reduction was triphasic and consisted of a rapid partial reduction phase, a partial reoxidation phase, and slow rereduction phase. During the partial reduction phase $\sim 30\%$ of the cytochrome b was reduced and reached its maximum value at 70 ms. During the reoxidation phase $\sim 20\%$ of the cytochrome b remained reduced, and the minimum value was reached at 280 ms. The rereduction phase was monophasic and occurred at a rate of $1.9 \pm 0.4 \text{ s}^{-1}$.

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 c_1 . 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-

¹ The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; Q, ubiquinone; QH₂, ubiquinol.

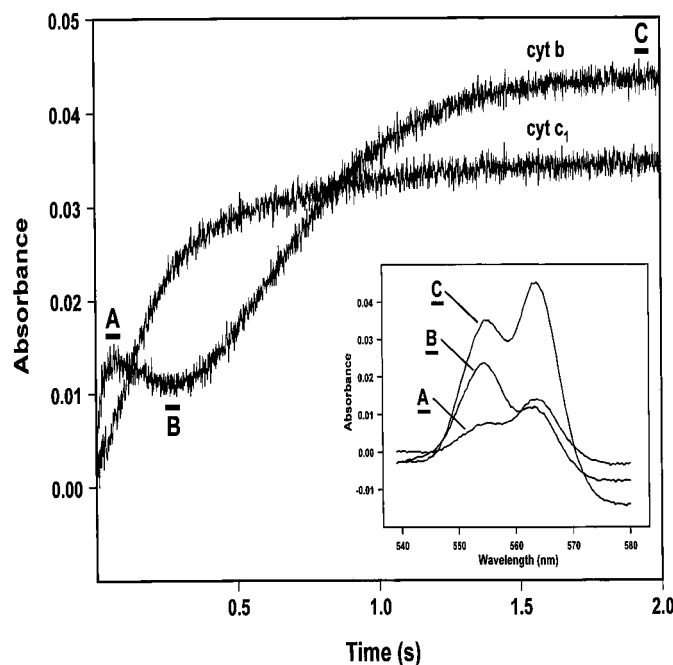


FIG. 1. Pre-steady state reduction of the cytochrome bc_1 complex by menaquinol in the absence of inhibitors. The traces show the time course of the reduction of $1 \mu\text{M}$ cytochrome bc_1 complex by $6 \mu\text{M}$ menaquinol. The traces labeled *cyt b* and *cyt c₁* correspond to the absorbance changes at 563.3 and 554.6 nm, respectively. The inset shows the reduced minus oxidized spectra of the bc_1 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.

chrome *b*. Instead, in the time interval between 70 and 280 ms, the absorbance at 563.3 nm decreased to 0.012 in spectrum B. This decrease cannot be accounted for by spectral overlap from cytochrome c_1 , because this would require a >0.02 decrement in the c_1 spectrum at 563.3 nm, which is greater than the absorbance increase (~ 0.016) because of c_1 reduction at the 554.6 nm absorbance maximum of reduced c_1 . Furthermore, any small decrease in absorbance at 563.3 nm because of c_1 reduction must also be included in spectra A 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 c_1 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, rereduction of cytochrome *b* resumes through center N at a slower rate.

At high concentrations of menaquinol (e.g. $200 \mu\text{M}$), 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 c_1 reduction as shown in Fig. 2. As the menaquinol concentration increased, the rate of cytochrome c_1 reduction increased and remained monophasic, and at $100 \mu\text{M}$ menaquinol the rate was $28 \pm 1 \text{ s}^{-1}$. There was a hyperbolic relationship between menaquinol concentration and the rate of cytochrome c_1 reduction, and a double reciprocal plot produced a V_{max} for c_1 reduction of $40 \pm 4 \text{ s}^{-1}$ (data not shown). When the menaquinol concentration is no longer rate-limiting for c_1 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

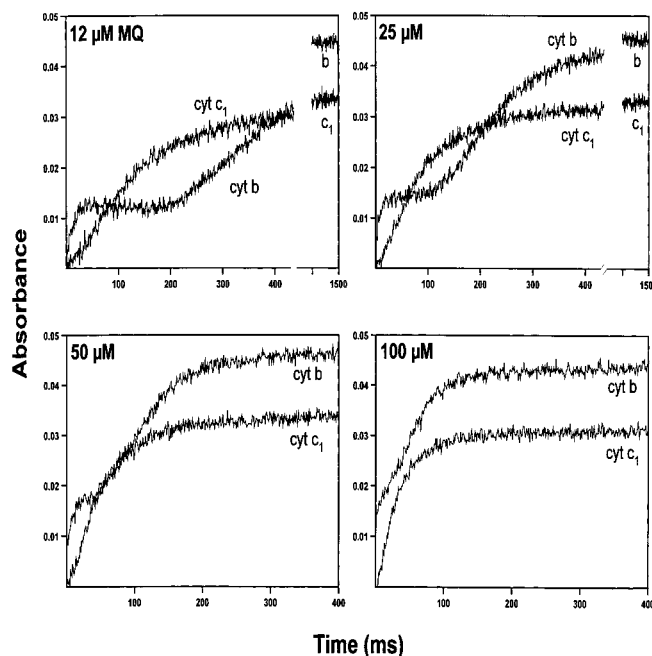


FIG. 2. Effect of increasing menaquinol concentration on the pre-steady state reduction of the cytochrome bc_1 complex. The traces show the time course of reduction of cytochromes (*cyt*) *b* and c_1 when $1 \mu\text{M}$ cytochrome bc_1 complex is reduced by 12, 25, 50, or $100 \mu\text{M}$ menaquinol.

$100 \mu\text{M}$ 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 rereduction phase. At $100 \mu\text{M}$ 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 $21 \pm 2 \text{ s}^{-1}$. The tracings in Fig. 2 demonstrate that the apparent lack of triphasic reduction at high concentrations of menaquinol results from increasingly fast reduction and reoxidation 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 $12 \mu\text{M}$ menaquinol (Fig. 2), $\sim 0.3 \mu\text{M}$ 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 $>10 \mu\text{M}$, 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 bc_1 Complex by Menaquinol in the Absence of Ubiquinone—To determine whether ubiquinone is responsible for triphasic cytochrome *b* reduction, we isolated the cytochrome bc_1 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 bc_1 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

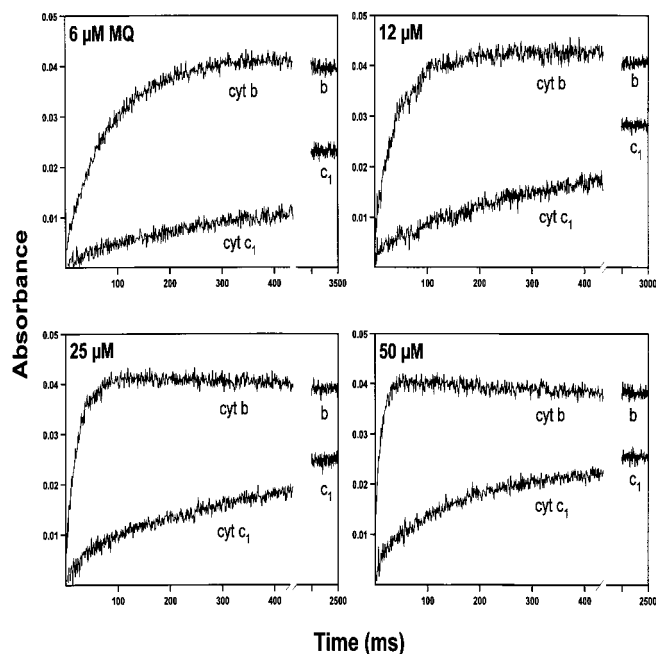


FIG. 3. Absence of triphasic cytochrome b reduction in cytochrome bc_1 complex lacking endogenous ubiquinone. The traces show the time course of reduction of cytochromes (cyt) b and c_1 when $1 \mu\text{M}$ cytochrome bc_1 complex isolated from the $\Delta coq2$ yeast mutant is reduced by 6, 12, 25, or $50 \mu\text{M}$ menaquinol.

ubiquinone remains in the bc_1 complex.

Having previously shown that menaquinol can reduce the b and c_1 cytochromes through center N and center P in the bc_1 complex isolated from the $\Delta coq2$ mutant (15), we examined the time course of the reduction of cytochrome b and c_1 over a range of menaquinol concentrations as shown in Fig. 3. The most obvious difference in the pre-steady state reduction of the bc_1 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 $6 \mu\text{M}$ menaquinol the rate of cytochrome b reduction was monophasic and occurred at $9.1 \pm 1.9 \text{ s}^{-1}$. The rate increased linearly with menaquinol concentration such that at $50 \mu\text{M}$ menaquinol the rate was $\sim 90 \text{ s}^{-1}$, 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 c_1 reduction was about four times slower than observed with the bc_1 complex from wild type yeast. Using $6 \mu\text{M}$ menaquinol, the rate of cytochrome c_1 reduction occurred at 0.9 s^{-1} and increased with menaquinol concentration such that at $50 \mu\text{M}$ menaquinol the rate was 4.0 s^{-1} . With the bc_1 complex from the wild type yeast, the corresponding rates were 3.3 and 18 s^{-1} . An explanation for the decreased rate of c_1 reduction is discussed below.

The lack of triphasic reduction in the $\Delta coq2$ bc_1 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 bc_1 complex from the wild type yeast is triphasic but is monophasic in the $\Delta coq2$ bc_1 complex. The only apparent difference in the reduction of b in the $\Delta coq2$ bc_1 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 mena-

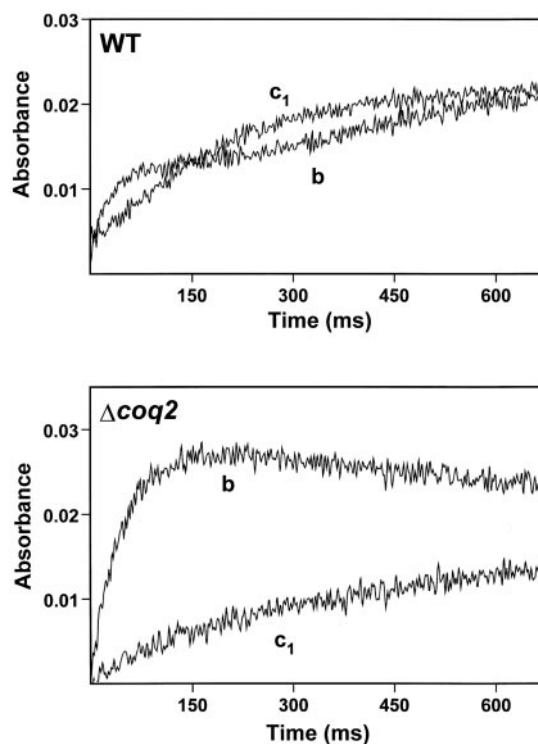


FIG. 4. Pre-steady state reduction of the cytochrome bc_1 complex by ubiquinol in the presence or absence of endogenous ubiquinone. The top panel shows the time course of cytochrome b and c_1 reduction when $1 \mu\text{M}$ native cytochrome bc_1 complex is reduced by $150 \mu\text{M}$ 2,3-dimethoxy-5,6-dimethyl benzoquinol, a ubiquinol analogue. The bottom panel shows the time course of cytochrome b and c_1 reduction when $1 \mu\text{M}$ ubiquinone-deficient cytochrome bc_1 complex is reduced by $150 \mu\text{M}$ ubiquinol analogue.

quinol (Fig. 3).

Restoration of Triphasic Cytochrome b Reduction in the bc_1 Complex from the $\Delta coq2$ Mutant by Ubiquinone—To confirm that ubiquinone is responsible for triphasic cytochrome b reduction, we added back ubiquinone to cytochrome bc_1 complex isolated from the $\Delta coq2$ mutant. Adding back various amounts of ubiquinone to the ubiquinone-deficient cytochrome bc_1 complex alters the reduction by menaquinol as shown in Fig. 5. Over the range of ubiquinone added, the kinetics of cytochrome c_1 reduction was monophasic. With $1 \mu\text{M}$ ubiquinone added, the rate of cytochrome c_1 reduction occurred at $\sim 3.3 \text{ s}^{-1}$ and decreased only slightly to 2.5 s^{-1} with $8 \mu\text{M}$ ubiquinone. It was surprising that adding ubiquinone did not significantly slow the rate of cytochrome c_1 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 c_1 reduction as the ubiquinone content increased. This also suggests that in the oxidized bc_1 complex ubiquinone does not interfere with menaquinol access to center P.

The addition of ubiquinone to the ubiquinone-deficient cytochrome bc_1 complex restored triphasic cytochrome b reduction (Fig. 5). With $1 \mu\text{M}$ ubiquinone added, the time course of cytochrome b reduction consisted of a rapid partial reduction phase, a small lag phase, and a slower reduction phase. The rapid reduction phase comprised $\sim 35\%$ 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

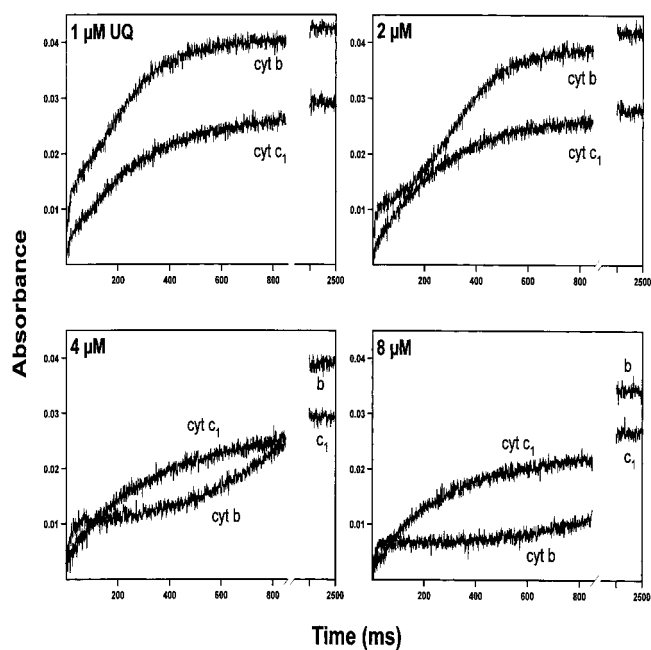


FIG. 5. Restoration of triphasic cytochrome b reduction by addition of ubiquinone to the cytochrome bc_1 complex isolated from a mutant lacking ubiquinone. The traces show the time course of reduction of cytochromes (cyt) b and c_1 when $1 \mu\text{M}$ bc_1 complex isolated from the Δcoq2 yeast mutant is reconstituted with 1, 2, 4, or $8 \mu\text{M}$ decyl ubiquinone and then reduced by $50 \mu\text{M}$ menaquinol.

phase decreased, and the lag phase became longer. With $8 \mu\text{M}$ 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 b_H 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 c_1 reduction when $2 \mu\text{M}$ ubiquinone was added to $1 \mu\text{M}$ ubiquinone-deficient cytochrome bc_1 complex. Over the range of menaquinol concentrations tested, the reduction of cytochrome c_1 was monophasic and increased from 1.6 s^{-1} at $6 \mu\text{M}$ menaquinol to 8.2 s^{-1} at $50 \mu\text{M}$ menaquinol. Because there was a linear relationship between menaquinol and the rate of cytochrome c_1 reduction, the reaction is limited by menaquinol concentration, and not by the rate of electron transfer from the iron-sulfur protein to cytochrome c_1 .

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 $\sim 25\%$, 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.

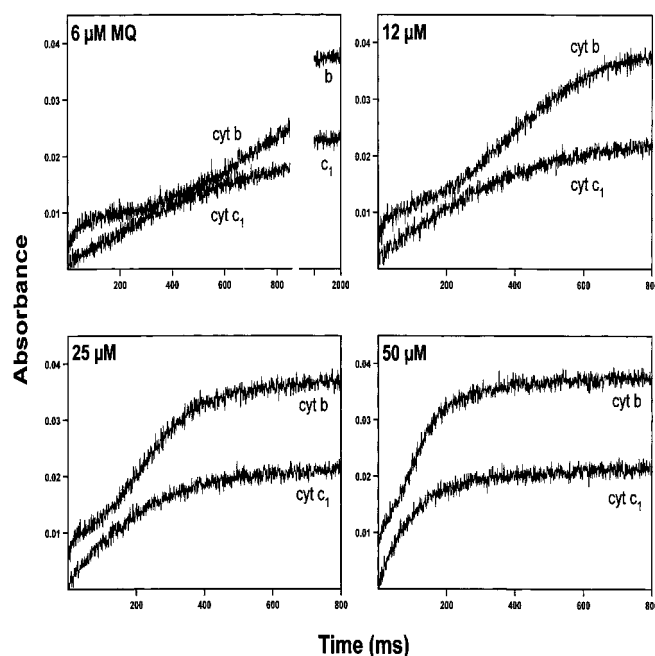


FIG. 6. Effect of increasing menaquinol concentration on the time course of cytochrome bc_1 complex reduction when ubiquinone is added to isolated cytochrome bc_1 complex from a yeast mutant lacking ubiquinone. The traces show the reduction of cytochromes (cyt) b and c_1 when $1 \mu\text{M}$ cytochrome bc_1 complex isolated from the Δcoq2 yeast mutant is reconstituted with $2 \mu\text{M}$ decyl ubiquinone and then reduced by 6, 12, 25, or $50 \mu\text{M}$ menaquinol.

Effect of Exogenous Ubiquinone on Triphasic Cytochrome b Reduction in Wild Type Complex—We also examined the effects of increasing the ubiquinone concentration on the reduction of cytochrome b in cytochrome bc_1 complex containing endogenous ubiquinone. The bc_1 complex from the wild type yeast contains approximately one ubiquinone per enzyme monomer, as determined by extraction of the purified enzyme. Fig. 7 shows the effects of exogenous ubiquinone on the time course of cytochrome b and c_1 reduction by $25 \mu\text{M}$ menaquinol. In the absence of exogenous ubiquinone, cytochrome c_1 reduction was monophasic and occurred at 11 s^{-1} . As the ubiquinone concentration was increased, the kinetics of cytochrome c_1 reduction remained monophasic, and with $8 \mu\text{M}$ added ubiquinone the rate decreased to 6 s^{-1} . Because increasing the total ubiquinone concentration by 8-fold only decreased the rate of cytochrome c_1 reduction by one-half, these results suggest that menaquinol reacts directly with center P and not via the ubiquinone, but that excess ubiquinone competes weakly with menaquinol for center P.

In contrast to cytochrome c_1 , the addition of ubiquinone had a much more dramatic effect upon the kinetics of cytochrome b reduction. As the ubiquinone concentration was increased cytochrome b reduction remained triphasic and became progressively more obvious. The percentage of the cytochrome b reduced during the rapid partial reduction phase decreased from 45% in the absence of exogenous ubiquinone to 30% in the presence of $8 \mu\text{M}$. This suggests that additional ubiquinone alters the distribution of an electron between b_H and ubiquinone at center N. In addition, the duration of the reoxidation phase dramatically increased with increasing ubiquinone concentrations. In the absence of additional ubiquinone the lag phase was only ~ 25 ms, but with $8 \mu\text{M}$ exogenous ubiquinone the lag extended for over 200 ms. These results are consistent with the interpretation that the ubiquinone pool must be reduced for the rereduction phase to proceed. If ubiquinone is available, cytochrome b that has been reduced through center P

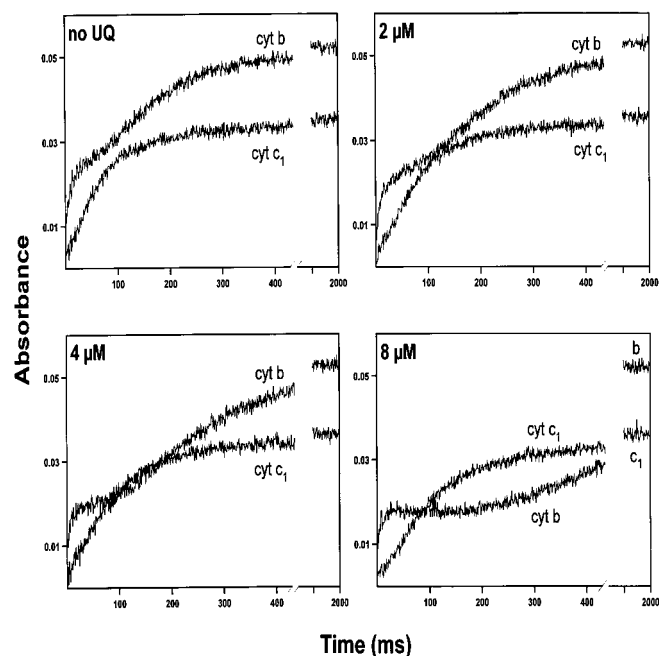


FIG. 7. Effect of exogenous ubiquinone on the time course of the reduction of the cytochrome *bc*₁ complex. The traces show the reduction of cytochromes (*cyt*) *b* and *c*₁ when 1 μM cytochrome *bc*₁ complex containing endogenous ubiquinone is mixed with 2, 4, or 8 μM decyl ubiquinone and then reduced by 25 μM 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 N. 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 μM 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 μM 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 in the absence or presence of exogenous ubiquinone. The bottom 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

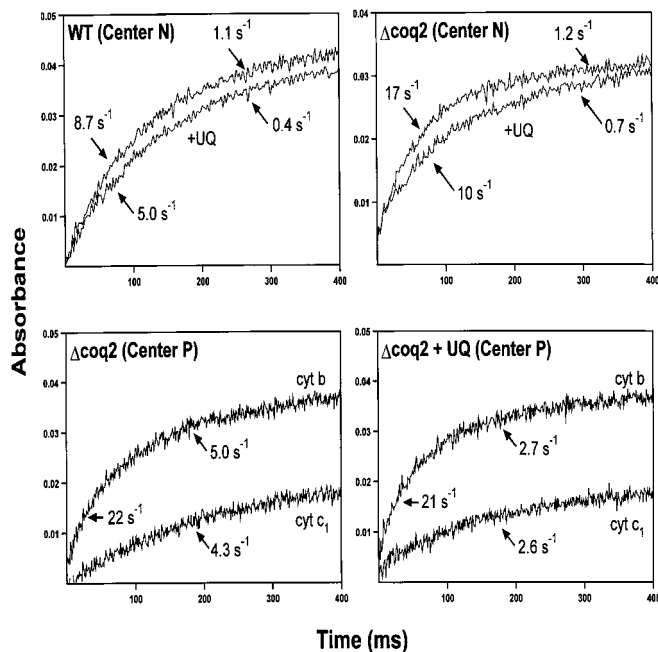


FIG. 8. Effect of exogenous ubiquinone on the time course of cytochrome *b* reduction in native and ubiquinone-deficient cytochrome *bc*₁ complex through center N or center P. The top left panel shows cytochrome *b* reduction when 1 μM native cytochrome *bc*₁ complex is mixed with 2 μM decyl ubiquinone and then reduced by 25 μM menaquinol in the presence of stigmatellin. The top right panel shows cytochrome *b* reduction when 1 μM ubiquinone-deficient cytochrome *bc*₁ complex is mixed with 2 μM decyl ubiquinone and then reduced by 25 μM menaquinol in the presence of stigmatellin. The bottom left panel shows cytochrome *b* and *c*₁ reduction when 1 μM ubiquinone-deficient cytochrome *bc*₁ complex is reduced by 25 μM menaquinol in the presence of antimycin. The bottom right panel shows cytochrome *b* and *c*₁ reduction when 1 μM ubiquinone-deficient *bc*₁ complex mixed with 2 μM decyl ubiquinone is reduced by 25 μM menaquinol in the presence of antimycin.

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 μM ubiquinone by 100 μM 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 μM enzyme the half-time for reduction of 20 μM ubiquinone by 100 μM menaquinol decreased to 7.5 s, and with 0.25 μM 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-steady 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_H heme. Time resolved optical spectra during the triphasic reduction demonstrate that cytochrome *b* undergoes partial reduction, partial reoxidation, and then rereduction. 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_H 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 rereduction phase proceeds. The transhydrogenase catalyzed equilibration of ubiquinone with menaquinol at center N is slow relative to b_H oxidation by ubiquinone, and this rate difference allows cytochrome b_H to be oxidized and then slowly reduced during the rereduction 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_H during triphasic reduction that has been reduced through center P and to oxidize b_H that has been reduced by menaquinol through center N. Thus, the addition of endogenous ubiquinone extends the reoxidation phase and slows the rereduction phase by reoxidizing cytochrome *b* faster than it can be reduced through either center P or N.

In the ubiquinone-deficient complex, menaquinone is apparently unable to rapidly reoxidize cytochrome *b* through center N, because either menaquinone 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_H in the presence of excess menaquinol, because it is a poor oxidant ($E_{m7} = -74$ mV). This results in the lack of triphasic reduction. This limitation only manifests under conditions of the pre-steady state experiments. The cytochrome *c* reductase activity of the ubiquinone-deficient complex was 100 s^{-1} with menaquinol as substrate, compared with 70 s^{-1} with ubiquinol as substrate, indicating that reoxidation of b_H by menaquinone 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_H 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-steady 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-

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 $(b_H \cdot Q)^-$. 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 $(b_H \cdot Q)^-$ to $b_H \cdot QH_2$, 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 ubiquinol 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 ubiquinol 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 par-

alleled the rapid reduction phase of cytochrome b (4). The semiquinone concentration decreased in parallel with the reoxidation phase and increased in parallel with the rereduction 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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