Hydroxyquinone binding to cytochrome bc$_1$ complex

Structure of the Yeast Cytochrome bc$_1$ Complex with a Hydroxyquinone Anion Q$_0$ Site Inhibitor bound

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This work was supported by Deutsche Forschungsgemeinschaft (SFB 472), the Max-Planck Gesellschaft and by National Institutes of Health Research Grant GM 20379. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C> Section 1734 solely to indicate this fact.

Running Title: Hydroxyquinone binding to cytochrome bc$_1$ complex
Abbreviations: UHDBT, 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole; HHDBT, 5-n-heptyl-6-hydroxy-4,7-dioxobenzothiazole; THDBT, 5-n-tridecyl-6-hydroxy-4,7-dioxobenzothiazole; UM, n-undecyl-β-D-maltopyranoside; UQ6, coenzyme Q6; rmsd, root mean square deviation; DBH$_2$, decyl-ubiquinol; PC, phosphatidyl choline.
SUMMARY

Bifurcated electron transfer during ubiquinol oxidation is the key reaction of cytochrome bc₁ complex catalysis. Binding of the competitive inhibitor 5-n-heptyl-6-hydroxy-4,7-dioxobenzothiazol to the Q₀ site of the cytochrome bc₁ complex from Saccharomyces cerevisiae was analysed by X-ray crystallography. This alkyl-hydroxy-dioxobenzothiazol is bound in its ionized form as evident from the crystal structure and confirmed by spectroscopic analysis, consistent with a measured pKₐ = 6.1 of the hydroxy group in detergent micelles. Stabilizing forces for the hydroxyquinone anion inhibitor include a polarized hydrogen bond to the iron-sulfur cluster ligand His¹⁸¹ and on-edge interactions via weak hydrogen bonds with cytochrome b residue Tyr²⁷⁹. The hydroxy group of the latter contributes to stabilization of the Rieske protein in the b-position by donating a hydrogen bond. The reported pH dependence of inhibition with lower efficacy at alkaline pH is attributed to the protonation state of His¹⁸¹ with a pKₐ of 7.5. Glu²⁷², a proposed primary ligand and proton acceptor of ubiquinol, is not bound to the carbonyl group of the hydroxy-dioxobenzothiazol ring, but is rotated out of the binding pocket toward the heme b₅ propionate A, to which it is hydrogen bonded via a single water molecule. The observed hydrogen bonding pattern provides experimental evidence for the previously proposed proton exit pathway involving the heme propionate and a chain of water molecules. Binding of the alkyl-6-hydroxy-4,7-dioxobenzothiazol is discussed as resembling an intermediate step of ubiquinol oxidation, supporting a single occupancy model at the Q₀ site.
INTRODUCTION

Ubiquinol:cytochrome c oxidoreductase [cytochrome bc\(_1\) complex, E.C.1.10.2.2 (bc\(_1\) complex)] is a multi-subunit membrane protein complex, which is one of the fundamental components of respiratory and photosynthetic electron transfer chains. The enzyme catalyses electron transfer from ubiquinol to cytochrome c and couples this process to electrogenic translocation of protons across the membrane (1, 2). Each functional unit of the homodimeric complex consists of 3 catalytic subunits: cytochrome b with two b type hemes, cytochrome c\(_1\) with one c type heme and the Rieske protein containing a [2Fe-2S] cluster. Mitochondrial bc\(_1\) complexes contain up to 8 additional subunits. Structures of vertebrate and yeast bc\(_1\) complexes were determined, providing a breakthrough in understanding the enzyme mechanism and structure-function relationships within the enzyme (3-7). The 2.3 Å resolution structure from the yeast Saccharomyces cerevisiae has the highest resolution available so far. It allowed a detailed description of substrate and inhibitor binding sites elucidating parts of the enzyme mechanism and suggesting pathways for proton transfer.

The mechanism of the enzyme known as the protonmotive Q cycle (8) involves separate catalytic sites for ubiquinol oxidation (Q\(_o\) site) and ubiquinone reduction (Q\(_i\) site). Protons are taken up from the matrix side when ubiquinone is reduced and released to the inter-membrane side when ubiquinol is oxidized. The key reaction, ubiquinol oxidation, involves a bifurcated electron transfer. One electron is passed via the [2Fe-2S] cluster to heme c\(_1\), subsequently reducing the substrate cytochrome c. The electron transfer to cytochrome c\(_1\) involves a large-scale domain movement of the extrinsic part of the Rieske protein (4). The second electron from ubiquinol is transferred
via the low and the high potential $b$ type hemes to ubiquinone. The resulting stable semiquinone is fully reduced after a second ubiquinol molecule is oxidized at the $Q_o$ site.

While the main features of catalysis are understood, the molecular mechanism of ubiquinol oxidation is not clear. Also, pathways for proton uptake and release are hypothetical (7, 9, 10). Several hypotheses have been proposed to explain the divergent transfer of electrons into thermodynamically different pathways (see Ref. 2). The double occupancy model suggests synergistic interaction between two quinone molecules which occupy the $Q_o$ site simultaneously (11-13). The proton-gated charge transfer mechanism proposes that the activation barrier is a function of the deprotonation of ubiquinol (14), but this mechanism is not supported by other kinetic studies (15). Single occupancy models include simultaneous as well as sequential electron transfer to the acceptors. For the latter a proton-gated affinity change mechanism claims the presence of a relatively stable intermediate in the transition state with the rate-limiting step at the second electron transfer (16). Since a semiquinone radical has not been detected at the $Q_o$ site, this has been explained by an EPR silent anti-ferromagnetically coupled semiquinone-$[2Fe-2S]^{\text{reduced}}$ pair (16, 17). Another explanation for the undetectable semiquinone is provided by Crofts and colleagues, who suggest rapid dissociation of the product after the first electron transfer and movement of the semiquinone within the bilobal $Q_o$ binding pocket to allow rapid reduction of heme $b_L$ (10, 18). Recent kinetic data show that the midpoint potentials of $b$ type hemes control the rate of cytochrome $c_1$ reduction. This is consistent with the view that ubiquinol oxidation is a concerted reaction (19).
Inhibitors are important tools to analyze the molecular mechanism of Qₐ site catalysis. Three types of inhibitors can be distinguished: ligands binding at the proximal domain and therefore perturbing the spectroscopic properties of heme \( b_L \) (Qo-I e.g. myxothiazol, MOA-stilbene), those binding to the distal domain and affecting the Rieske \([2Fe-2S]\) EPR lineshape (Qo-II e.g. UHDBT), or compounds exhibiting both effects (Qo-III e.g. stigmatellin) (20). Kinetic data indicates that occupation of these inhibitors at the Qₐ site is mutually exclusive, suggesting overlapping binding sites. This was observed in crystal structures where these inhibitors are found to bind in different but overlapping domains of the bilobal Qₐ site, termed distal and proximal to heme \( b_L \) (20).

Analysis of anomalous scattering data indicated a high occupancy of the catalytic Rieske domain in the b-position in the presence of inhibitors that bind to the distal domain, such as stigmatellin and UHDBT (20). However, in previous crystallographic studies on UHDBT binding (18, 20) the inhibitor could not be refined and high resolution structural information about UHDBT binding at the active site has not been available up to now. The substrate ubiquinol has not been detected in the Qₐ site by X-ray structural analysis. Therefore, the analysis of structural analogs of the substrate which function as competitive inhibitors is important.

Here, we present the three-dimensional structure of a UHDBT analog, HHDBT, inhibited \( bc_1 \) complex from the yeast \textit{Saccharomyces cerevisiae} at 2.5 Å resolution. This hydroxyquinone binds in its ionized form and its binding is discussed as resembling an intermediate step of ubiquinol oxidation. Conformational changes at the binding site confirm the previously postulated proton transfer pathway and reveal plasticity at the active site.
EXPERIMENTAL PROCEDURES

*Protein Purification and Crystallization* - The \( bc_1 \) complex from the yeast *S. cerevisiae* was purified and a co-complex with the antibody fragment Fv\(_{18E11}\) was formed and crystallized as previously described with the following minor modifications (6, 21). The buffer volume for detergent exchange in the second DEAE anion exchange chromatographic step was reduced by 5-fold. HHDBT was added at a final concentration of 100 µM to the purified \( bc_1 \) complex-Fv co-complex after size exclusion chromatography (TSK4000, *TosoHaas*) prior to crystallization. The final purification step was performed at pH 7.5. The crystals were obtained using micro seeding and vapor diffusion technique against PEG4000 at 4°C. The protein solution (50 mg/ml) was mixed with precipitation agent [5 % PEG4000, Tris-HCl pH 7.5 (adjusted at room temperature), 0.05% \( n \)-undecyl-\( \beta \)-D-maltopyranoside, 10 µM HHDBT] resulting in a pH of 8.0 at 4 °C, that is 0.5 pH unit lower than the structure of the stigmatellin inhibited enzyme (6). Crystals grew within a few days to a size suitable for X-ray analysis (~ 0.5 x 0.5 x 1.0 mm).

Total protein determination was performed with a modified Lowry procedure, using the BC Assay protein quantitation kit (*Uptima*) (22). The \( bc_1 \) complex content was estimated as half the amount from spectroscopic quantification of the two \( b \)-type hemes using an extinction coefficient of 28.5 mM\(^{-1}\)cm\(^{-1}\) for the dithionite-reduced minus ferricyanide-oxidized difference spectra (262-275 nm). Enzyme activity was determined by monitoring cytochrome \( c \) reduction in a spectrophotometric assay at 550 nm using an extinction coefficient of 18.5 mM\(^{-1}\)cm\(^{-1}\) for cytochrome \( c \). Turnover numbers refer to mol cytochrome \( c \) reduced per mol \( bc_1 \) complex per second under conditions of continuous
turnover where the catalytic reaction is zero order with respect to decylubiquinol and cytochrome c. A detailed description is reported elsewhere (21).

\[ pK_a \]

Determination of Hydroxy-Dioxobenzothiazoles - For determination of the pK\(_a\) in detergent micelles the longer tridecyl sidechain analog was used to retain partitioning of the inhibitor into the micelle. THDBT was dissolved at 5 µM in a buffered mixture containing 20 mM MES, 20 mM MOPS, 20 mM TAPS, 100 mM NaCl and 400 µM \( n\)-dodecyl-\( \beta\)-D-maltopyranoside, pH 3.5. The pH was adjusted by increments of 0.2-0.5 pH units from pH 3.5 to 8.5 by adding 5 M KOH. Optical spectra were recorded from 250-350 nm with a slit width of 1.5 nm on a computerized DW2a dual wavelength spectrophotometer controlled by OLIS software (On-Line Instruments Systems, Bogart, GA).

Determination of The Apparent \( K_m \) of Yeast \( bc_1 \) Complex For Ubiquinol in The Absence and Presence of UHDBT - The reaction mixture consisted of 25 µM cytochrome c in 50 mM potassium phosphate pH 7, 250 mM sucrose, 1 mM sodium azide, 200 µM EDTA and 0.01% Tween 20. Prior to the reaction the complex was diluted to 15 µM in the same buffer and incubated for approximately 45 minutes on ice. The concentration of decyl-ubiquinol was varied and the activity measured in the absence or presence of UHDBT.

Determination of the Ionization State of Bound THDBT - The \( bc_1 \) complex was diluted to a concentration of 2.84 µM in a buffer at pH 6.0 [50 mM MES, 50 mM MOPS, 50 mM TAPS, 250 mM sucrose, 200 µM EDTA, 2 mM NaN\(_3\), 0.1% Tween 20]. The complex had an absorbance at 280 nm = 2.7 and a 280:414 absorbance ratio = 3. Spectra were recorded from 250-350 nm on the DW2a dual wavelength
spectrophotometer. In order to obtain maximum illumination the slit was set at 6 nm and
the UV filter, quartz diffuser and beam scrambler were removed from the
spectrophotometer.

**Data Collection and Refinement** - X-ray diffraction data were collected at 4°C at
the synchrotron beamline ID14EH3 at the European Synchrotron Radiation Facility,
Grenoble (France), using a charge-coupled device detector (marCCD, mar USA,
Evanston, IL). Data were processed with the program DENZO and merged using
SCALEPACK from the HKL package (HKL Research, Charlottesville, NC) (23). The
crystals belong to the space group C2, with unit cell parameters, a=215.0 Å, b=165.1 Å,
c=147.5 Å, and β=117.3°. The structure was refined using the coordinates of the
stigmatellin inhibited enzyme as a model (PDB code 1KB9) after excluding all non-
protein molecules (7). Energy minimization and B-factor refinement were performed
using the CNS program package (V.1.0) (24). Maximum likelihood function was used as
target for refinement. The model was improved based on Fo-Fc and 3Fo-2Fc electron
density maps, using program O (V.8.0.4) (25). Amino acid displacements were manually
adjusted, followed by stepwise inclusion of the energy minimized structure of the
inhibitor HHDBT, UQ6, phospholipids, and UM and finally manually repositioning a
displaced loop. Each step of model building was followed by a refinement cycle.
Topology and parameter files were generated using the program Xplo2d and torsion
data-blocks prepared with program Moleman2 (X-UTIL package,
http://xray.bmc.uu.se/usfl/) (26). The difference electron density map (Fo-Fc) indicated
the presence of several additional phospholipids, manifested as elongated hairpin-like
features. One phospholipid bound close to the Qₐ site was identified and refined in
addition to the previously assigned phospholipids (7). Finally, water molecules were included according to peaks observed in the Fo-Fc electron density map contoured at 3\(\sigma\). Their positions were refined yielding 326 molecules of which 203 are the same as in the original model (1KB9) and their numbering was kept. New water molecules were numbered as starting from Wat\(^{500}\). Refinement resulted in final R factor and free R factor of 22.8\% and 25.2\%, respectively (Table 1). Coordinates of the HHDBT inhibited enzyme have been deposited in the database (PDB entry 1P84).

For comparison stigmatellin-inhibited bc\(_1\) complex was crystallized at the same pH as the HHDBT containing enzyme. The control data-set was collected with 2.8 Å resolution, 93\% completeness and R\(_{sym}\) 5.8\%. Refinement resulted in final R factor of 20.8 \% and R\(_{free}\) 24.5 \%. Lowering the pH by 0.5 unit does not affect the structure of the catalytic subunits of the stigmatellin inhibited enzyme, as judged by positional root mean square deviation (rmsd, Å) of superimposed atoms with LSQMAN yielding rmsd\(_{all}/rmsd_{C\alpha}\) of 0.142/0.098, 0.175/0.118 and 0.204/0.133 for cytochrome b, cytochrome c\(_1\) and the Rieske protein respectively (Dejavuu package, http://xray.bmc.uu.se/usf/). Comparison between the HHDBT and the stigmatellin inhibited enzyme was therefore based on the recently published 2.3 Å resolution structure (1KB9) (6). The structures were superimposed using explicit least squares option in LSQMAN and inspected in Program O. By-residue analysis of RMS deviation in C\(_\alpha\) trace position and orientation was performed using the McLachlan algorithm as implemented in the program ProFit V2.2 (http://www.bioinf.org.uk/software/profit).

Analysis of neighbouring atoms and hydrogen bond interactions was performed using the programs HBPlus (27) and contact analysis from CNS. Accessibility was
estimated and buried surface calculations performed using the program NACCESS (28). PROCHECK (V.3.2) analysis verified the stereochemical quality of the coordinates (Table 1) (29). Hydrogen bonds were assigned according to appropriate distance and geometry. For analysis of weak hydrogen bonds an estimation of hydrogen atom position was made by generating a structural model with hydrogens added using CNS (V.1.0). Criteria for identifying weak hydrogen bonds were extracted from (30). Hydrogen bond angle is denoted as $\theta$ (X-H···A) and the bending angle at acceptor atom $\phi$ (H···A-C). Illustrations were prepared using program O (25), LIGPLOT V.4.0 (31), MolScript V.1.4 (32), BobScript (33) and Raster3D (34).
RESULTS

Crystallization of HHDBT Inhibited bc₁ Complex - The optimized purification of yeast bc₁ complex resulted in a pure and more active membrane protein complex with a higher turnover number of 82 s⁻¹ compared with the previously reported activity of 64 s⁻¹ (21). The increase is most likely due to higher phospholipid content of the modified protein preparation (Fig.1) (35, 36). Effective inhibition of the complex has been shown for 5-n-alkyl-6-hydroxy-4,7-dioxobenzothiazoles containing 7-15 carbon alkyl side-chains (37). Here, the shorter heptyl side-chain analog of UHDBT was used for crystallization, in order to avoid non-specific binding that might occur with the longer alkyl side-chains at the high concentrations used.

The inhibitory efficacy of UHDBT was shown to depend on the oxidation-reduction poise of the catalytic subunits, demonstrated by enhanced binding when the Rieske protein is reduced (37). The purified bc₁ complex used in this study has a partially reduced Rieske and is fully inhibited by the applied amount of HHDBT (results not shown). A pKₐ of 6.5 has been determined for the weakly acidic hydroxy group of UHDBT, which was measured in phosphate buffer containing 1% ethanol, and deprotonation of the hydroxy group is manifested by a color change from yellow to rose-violet (38). Here, the complex was crystallized at pH 8.0 as a protein-detergent complex, therefore the acidity of the hydroxy group was measured in detergent micelles by monitoring the blue shift in the optical spectrum upon ionization. The pKₐ determined by spectrophotometric titration in detergent micelles was 6.1 (results not shown). This suggests that 98% of the inhibitor is ionized in the crystallization mixture, demonstrated by the violet color of the solution, and finally a purple tint of the crystals.
A difference spectrum of $bc_1$ complex with bound inhibitor versus the complex alone shows that the inhibitor is ionized when bound to the enzyme while the pH of the buffer is close to the pK$_a$ of the unbound inhibitor (Fig.2). The difference spectrum of the complex at pH 6.0 with inhibitor bound at substoichiometric amount is similar to that of the inhibitor alone at pH 8.7. As inhibitor is added in molar excess, the mixture consists of bound and unbound inhibitor and the absorbance maximum shifts to longer wavelengths.

**Analysis of HHDBT Binding at the Q$_o$ Site** - The difference electron density map (Fo-Fc) calculated prior to inclusion of HHDBT clearly showed the localization of the bound inhibitor at the Q$_o$ site (Fig.1, 3). The clear-cut and asymmetric form of the difference density for the head moiety allowed unambiguous orientation of the hydroxy-dioxobenzothiazole ring. Furthermore, the position of the alkyl side chain was defined in full length.

The inhibitor binds in the distal domain of the Q$_o$ site, located between the two electron acceptors of ubiquinol oxidation, namely heme $b_L$ and the [2Fe-2S] cluster of the Rieske protein. The catalytic domain of the latter is docked onto cytochrome $b$ (i.e. in the b-position). The hydroxy-dioxobenzothiazole head group is stabilized by a network of weak and strong hydrogen bonds, and numerous van der Waals interactions with neighbouring residues (Table 2). Importantly, the oxygen atom of the ionized 6-hydroxy group (O6) is in close contact with the nitrogen atom N$_{ε2}$ of His$^{181}$, of which the second imidazole nitrogen coordinates the [2Fe-2S] cluster of the Rieske protein. The distance and geometry of the interaction ($d_{N_{ε2}-O6} = 2.8$ Å; $θ = 148°$) are in good agreement with a conventional hydrogen bond.
Crystallographic analysis of stigmate binding clearly showed that Nε2 is protonated at pH 8.5, as the fixation of the Rieske protein in the b-position is stabilized by a hydrogen bond between this atom and the carbonyl group of stigmate (6). For crystallization of the bc1 complex in the presence of HHDBT the pH was lowered by half a unit, therefore, Nε2 is expected to be protonated under these conditions (see Discussion). With both donor and acceptor of the hydrogen bond being ionized, this type of hydrogen-bonded-ion bridge provides a strong interaction for stabilizing ligand binding. Furthermore, this is the main interaction, which locks the catalytic Rieske domain in the b-position. Also, a weak hydrogen bond between the slightly acidic Cε1 atom of His181 and the carbonyl O7 atom of HHDBT adds to this stabilizing effect.

Interestingly, the ionized hydroxy group of the inhibitor interacts with the phenyl ring of Tyr279 of cytochrome b. The ring plane of the inhibitor is at a 90 degree angle on-edge to the aromatic side chain of Tyr279 (Fig. 3). The distances between the O6 atom of HHDBT and the Tyr279 side chain atoms, Cδ1 and Cε1, respectively, are well below the sum of their van der Waals radii (dCδ1-O6=3.2 Å and dCε1-O6=3.3 Å), indicating the presence of weak, non-conventional hydrogen bonds (C-H-O) with the aromatic C-H groups as donors. Hydrogen bond angles and bending angles at the acceptor atom (θCδ1-H-O6 =125 °, θCε1-H-O6 =113 °, φHδ1-O6-C6 =107 °, φHε1-O6-C6 =94 °) are slightly below the optimal range for non-conventional hydrogen bonds, a feature which has been observed for this type of hydrogen bond with a bifurcated acceptor (30). The bifurcation is not fully symmetrical as conditions for the bond involving Cδ1 are more favorable. The acidity of the aromatic C-H groups is increased, because the hydroxy group of Tyr279 of cytochrome b donates a hydrogen bond to the backbone oxygen of Cys180 of the Rieske
Hydroxyquinone binding to cytochrome bc\(_1\) complex protein (d\(_{\text{OH-O}}\) = 2.7 Å; \(\varphi_{\text{Cz-OH-O}} = 112^\circ\)). The latter provides additional stabilization of the Rieske domain in the b-position.

Furthermore, the oxygen atom of the carbonyl group O\(_4\), which is oriented towards heme \(b_L\), is within hydrogen bonding distance to a water molecule Wat\(^{518}\) that is stabilized by a hydrogen bond with the backbone nitrogen atom of Glu\(^{272}\) of cytochrome \(b\). Several non-polar interactions contribute to stabilization of the hydroxydioxobenzothiazole ring, involving the following residues of cytochrome \(b\): Met\(^{139}\), Trp\(^{142}\), Gly\(^{143}\), Val\(^{146}\), Ile\(^{269}\) and Pro\(^{271}\). Additionally, a few van der Waals interactions with Rieske protein residues His\(^{181}\), ligand of the [2Fe-2S], and Cys\(^{180}\) are present (Table 2), demonstrating the tight interaction of the Rieske cluster bearing domain with cytochrome \(b\) at the Q\(_o\) site. Notably, all of the residues involved in binding of the hydroxydioxobenzothiazole head group are fully conserved among mitochondrial bc\(_1\) complexes (39). Mutations in the position of residues that are interacting with the head group, Trp\(^{142}\), Gly\(^{143}\), Ile\(^{269}\), Pro\(^{271}\) and Tyr\(^{279}\), or their homologs, result in disturbed ubiquinol occupancy and/or oxidation (40, 41). The short saturated heptyl side-chain is stabilized by van der Waals interactions with cytochrome \(b\) residues Ile\(^{147}\), Leu\(^{275}\), Phe\(^{278}\) and Met\(^{295}\).

**UHDBT is a Competitive Inhibitor** – To analyze if hydroxy-dioxobenzothiazoles compete with substrate at the Q\(_o\) site, the apparent K\(_m\) for decyl-ubiquinol binding to yeast bc\(_1\) complex was determined at varying concentrations of UHDBT. The K\(_m\) varied with inhibitor concentration, whereas V\(_{\text{max}}\) remained constant, clearly demonstrating that UHDBT is a competitive inhibitor (Fig.4). Therefore, hydroxy-dioxobenzothiazoles may be regarded as substrate analogs. High structural similarity to the substrate and
competitive inhibition by HHDBT suggest that it is a substrate analogue in which the ring methyl group is replaced by a deprotonated hydroxy group, the two methoxy groups are replaced by a fused thiazole ring and the isoprenoid tail is replaced by a short saturated side chain (see insert, Fig.3).

**Comparative Analysis of Substrate Analogs Binding at the Active Site** - Stigmatellin binding to $bc_1$ complex was proposed to mimic the enzyme-substrate complex at an intermediate step of ubiquinol oxidation (6). The binding sites for stigmatellin and HHDBT are overlapping with the head groups positioned in a tight binding pocket, whereas the side chains extend into a gradually opening hydrophobic cleft. B factor analysis indicates very tight binding of stigmatellin with average B factors of 37.4 Å$^2$ and 35.6 Å$^2$ for stigmatellin and cytochrome $b$, respectively. HHDBT appears less tightly bound with average B factor $HHDBT$ 50.9 Å$^2$ and average B factor $cytochrome b$ of 40.2 Å$^2$.

Comparison of the structures of HHDBT and stigmatellin inhibited $bc_1$ complexes revealed that the major difference in C$\alpha$ trace position and side chain orientation is confined to the direct environment of the $Q_o$ site, notably including residues suggested by mutagenesis studies to be actively involved in $Q_o$ site catalysis (39, 40) (Fig.5).

The conformational changes upon HHDBT binding reveal plasticity at the $Q_o$ site. An expansion of the binding pocket is marked by a local displacement of the C$\alpha$ trace accompanied by altered side chain orientation of residues Ala$^{267}$, Ser$^{268}$, Ile$^{269}$ and Val$^{270}$ in a loop region close to the conserved PEWY loop (Pro$^{271}$-Tyr$^{274}$) with maximal displacement of 1.7 Å at Ser$^{268}$. 
The HHDBT head group extends deeper into the distal end of the Qo site than the chromone ring of stigmatellin, with the Ile\textsuperscript{269} side chain bent away from the binding pocket. Additionally, major side chain displacements are observed for His\textsuperscript{253} and Glu\textsuperscript{272}. The side chains of Phe\textsuperscript{129} and Tyr\textsuperscript{132} are not stabilized by interactions with HHDBT and are present in multiple conformations. Remarkably, the oxygen atom O6 of the ionized hydroxy group of HHDBT is at the same position as the carbonyl oxygen atom O8 of stigmatellin, allowing in both cases for a hydrogen bond to the N\textsubscript{ε}2 atom of His\textsuperscript{181} of the Rieske protein. This supports the proposal that ubiquinol binds in the same position with His\textsuperscript{181} as the primary ligand of the enzyme-substrate complex (6, 18, 42).

**Structural Analysis of Proton Transfer Pathways** - Glu\textsuperscript{272} is a primary ligand of stigmatellin and was proposed to be a direct ligand of ubiquinol (6, 10). It has been suggested that Glu\textsuperscript{272} releases the second proton from ubiquinol oxidation upon rotational displacement towards the heme b\textsubscript{L} via an apparent hydrogen-bonding network established by residues Arg\textsuperscript{79}, Tyr\textsuperscript{132}, Asn\textsuperscript{256}, Glu\textsuperscript{272}, Tyr\textsuperscript{274}, the heme b\textsubscript{L} propionate A and several water molecules (6). Here, we find that the carboxylate group of Glu\textsuperscript{272} is not bound to HHDBT, but is dramatically rotated out of the Qo site, and that there is a hydrogen bond connecting the carboxylate to the heme propionate A (L1O2A) via a structural water molecule (Wat\textsuperscript{42}) (Fig.6). The carboxylate atoms (O1\textsubscript{ε} and O2\textsubscript{ε}) of Glu\textsuperscript{272} occupy the exact location of two water molecules (Wat\textsuperscript{428} and Wat\textsuperscript{50}) present in the stigmatellin inhibited complex. The position of the Glu\textsuperscript{272} carboxylate group in the stigmatellin structure is now occupied by a water molecule Wat\textsuperscript{518} which is hydrogen bonded to the carbonyl O4 atom of HHDBT and the backbone nitrogen atom of Glu\textsuperscript{272} (Fig.6). The reorientation of Glu\textsuperscript{272} creates a rearrangement in the hydrogen bond
Hydroxyquinone binding to cytochrome $bc_1$ complex

Tyr$^{274}$ remains in position and stabilizes Glu$^{272}$ via a hydrogen bond to the carboxylate group. His$^{253}$ is rotationally displaced and forms a second hydrogen bond to the Glu$^{272}$ carboxylate. In this position the Glu$^{272}$ protonated upon ubiquinol oxidation can deliver the proton directly to heme propionate A via the water molecule Wat$^{42}$. Arg$^{79}$ is hydrogen bonded to the heme propionate and provides a proton exit pathway to the bulk solvent mediated by a chain of hydrogen bonded water molecules. The residues Arg$^{79}$, Glu$^{272}$, and Tyr$^{274}$ are fully conserved in mitochondrial cytochrome b (39) supporting their importance for the catalytic mechanism.

Assignment of a Tightly Bound Phospholipid at the Q$_o$ Site – Phospholipids are essential for $bc_1$ complex activity (35, 36). Distinct binding sites for five phospholipid molecules have been described for the yeast complex suggesting a specific role for the structural and functional integrity of the enzyme (7). The higher activity of the enzyme preparation used in this study was linked to less delipidation. In line with this assumption, additional tightly bound phospholipid molecules were visible in the crystal structure. Close to the Q$_o$ site a phospholipid molecule, tentatively assigned as a phosphatidyl choline, is bound at the intermembrane leaflet of the phospholipid bilayer. It binds in a hydrophobic cleft at the interface between cytochrome b and c$_1$. Binding of the head group is stabilized by interactions with the highly conserved His$^{185}$ of cytochrome c$_1$ and by cytochrome b residue Ser$^{268}$. PC covers the protein surface at the Q$_o$ site. The acyl chains are in contact with Trp$^{273}$ of the conserved PEWY loop and attached to the displaced loop region (Ala$^{267}$-Val$^{270}$), pointing out that the conformational rearrangement takes place in the transmembrane region.
This is the first phospholipid characterized which binds to the protein surface in the position of the intermembrane bilayer leaflet (Fig. 1). The acyl chains extend to be in contact with the acyl chains of the oppositely oriented matrix leaflet. The distance between the phosphodiester groups of PC and the oppositely oriented phospholipids, cardiolipin and phosphatidyl ethanolamine, is 36 Å. This is in good agreement with the experimentally determined thickness of pure PC bilayers with 18:1 acyl chains, where 38 Å were measured between the phosphodiester groups and 27 Å for the hydrophobic core (43). These lipids form a mediating layer to the membrane spanning part of the protein and help to position the complex vertically in the lipid bilayer. As HHDBT and stigmatellin resemble substrate analogs, the position of the ubiquinol binding site in the phospholipid bilayer can now be determined. The position of the functional groups of the ligand are 6 Å below the phosphodiester group, thereby positioning the ligand at the border between the hydrophobic core and the polar zone of the head group region.
Hydroxyquinone binding to cytochrome bc$_1$ complex

DISCUSSION

Hydroxy-dioxobenzothiazoles efficiently inhibit the bc$_1$ complex in a pH dependent manner (38). It was argued that low efficacy at alkaline pH could be attributed to ionization of the inhibitor due to restricted access to the binding site or to protonation of a functional group within the enzyme (38, 44). Notably, the apparent pK$_a$ of 7.5 for inhibitor efficacy is more alkaline than the pK$_a$ of UHDBT but closely matches the pK$_a$ suggested for the imidazole nitrogen of His$_{181}$ of the oxidized Rieske protein (7.6, Ref. 45). The structural and spectroscopic analysis presented here clearly show that the alkyl hydroxy-dioxobenzothiazole is bound in its ionized form and is stabilized by a hydrogen bond to the protonated imidazole nitrogen of His$_{181}$, as supported by the following evidence. HHDBT is bound in the distal domain of the binding pocket with the 6-hydroxy group facing the aqueous solution when the Rieske protein is not closing the binding site. The pK$_a$ of the hydroxy group, determined for THDBT in detergent micelles (6.1) is below the pH (8.0) used for crystallization. It is unlikely that the pK$_a$ of HHDBT is significantly different from the measured value, the inhibitor is therefore ionized in the open conformation.

His$_{181}$ is expected to be protonated, because it was shown to donate a hydrogen bond to the carbonyl group of stigmatellin at half a pH unit above the one used for HHDBT crystallization. In addition, crystallization is performed close to the pK$_a$ of His$_{181}$. Furthermore, the enzyme preparation is partially reduced and the pK$_a$ of the reduced Rieske imidazole nitrogen is far above (11.5) the crystallization conditions (45). The presence of a hydrogen bond from His$_{181}$ to HHDBT is shown by the fact that ligand binding fixes the mobile Rieske domain in the b-position. Spectroscopic analysis shows
that hydroxy-dioxobenzothiazole is deprotonated upon binding when added in substoichiometric amounts to the complex at pH > pK_a (Fig. 2). The hydrogen bond to the protonated His^{181} in combination with the bifurcated weak hydrogen bond on-edge with Tyr^{279} stabilizes the charge on oxygen atom O6.

In the nondissociated form HHDBT can exist as ortho- and para-quinone tautomers (38). It can be excluded that HHDBT is bound as ortho-quinone with its functional groups in the same orientation as stigmatellin, because the hydroxy group would be facing Glu^{272}, but in the HHDBT structure this residue is rotated out of the binding pocket. There is also no indication that the binding site is occupied with a mixed population of tautomers, because all interacting residues show a defined orientation as judged from the clear cut electron density and B-factor distribution.

Stigmatellin (46) and HHDBT (Fig.4) are both competitive inhibitors. Kinetic studies show that stigmatellin is more tightly bound than UHDBT (44, 47). Furthermore, stigmatellin binding raises the midpoint potential (E_m) of the Rieske protein by 250 mV (48), whereas for UHDBT an increase of only 70 mV was determined for the bovine enzyme (37) and for the yeast enzyme (T. Merbitz-Zahradnik and B. Trumpower, unpublished results).

The position of the Rieske protein in the HHDBT inhibited bc_1 complex is the same as in the stigmatellin inhibited enzyme. Almost identical stabilizing interactions between the surfaces of the Rieske protein and cytochrome b, including positions of hydrogen bonded water molecules at the interface, are observed. The different effects on midpoint potentials must be directly related to differences in inhibitor binding. B factor analysis indicates tighter binding of stigmatellin compared to HHDBT and this is
supported by the relative $K_i$ values for the two inhibitors. Stigmatellin is stabilized by strong hydrogen bonds to direct ligands on both sides of the chromone ring. In contrast, direct ligands to the head group of HHDBT are confined to the ionized hydroxy group, whereas only a water molecule is hydrogen bonded to the carbonyl group on the other side. In addition, the more rigid side chain of stigmatellin is bent along the surface and stably held by numerous contacts to cytochrome $b$. The short alkyl chain of HHDBT extends in the same direction but terminates where the stigmatellin side chain bends. Interestingly, the side chain of stigmatellin replaces a phospholipid molecule visible in the electron density of the HHDBT structure, indicating its strong interactions with the surface of the binding pocket (results not shown).

The difference in polarization of the hydrogen bond to His$^{181}$ will add to the effect on the midpoint potential. The hydrogen bonding pattern to the negatively charged hydroxy group creates a lower electron withdrawing effect. Obviously, the tight binding interactions of stigmatellin involve the whole ligand, thus explaining why its binding is not pH dependent. In contrast, stabilization of the charged HHDBT is strongly dependent on the hydrogen bond from the protonated His$^{181}$. This is in agreement with the higher affinity of hydroxy-dioxobenzothiazoles to the reduced Rieske protein independent of chain-length (7-15 carbon alkyl side chains), binding 15 times more strongly to the reduced Rieske protein (37). Experimental evidence and theoretical calculations suggest that reduction of the [2Fe-2S] cluster shifts the pK$_a$ of the cluster ligands, His$^{161}$ and His$^{181}$, to values above 10, obviously favoring protonation of the histidines (49, 50). Consequently, pH dependence of HHDBT binding is related to the protonation state of a functional group within the protein, namely His$^{181}$. 
From structural analysis of the hydroxyquinone anion inhibitor HHDBT and stigmatellin binding at the Q_o site, the following events for electron and proton transfer are deduced (Fig.7). Upon entry into the binding pocket the substrate ubiquinol is stabilized with its functional groups pointing towards the primary acceptors of the low and high potential electron transfer chains, heme b_L and the [2Fe-2S] cluster, respectively. Glu^{272} rotates into the binding pocket and forms a hydrogen bond to the hydroxy group facing the heme b_L as visible when stigmatellin is bound at the Q_o site (6). Since the extrinsic catalytic Rieske domain is mobile and His^{181} has a pK_a of 7.5 when the cluster is oxidized (45, 49), a considerable fraction of the latter is not protonated under physiological conditions. Thus, the Rieske domain can be stabilized in the b-position by forming a hydrogen bond between deprotonated His^{181} and the hydroxy group of ubiquinol. This brings the [2Fe-2S] cluster into a suitable distance for electron transfer. The initial enzyme-substrate complex is stabilized by hydrogen bonds to the primary ligands, His^{181} and Glu^{272}, which additionally serve as primary proton acceptors as previously proposed (6, 10).

Ubiquinol is oxidized in a bifurcated manner transferring two electrons to the [2Fe-2S] cluster and heme b_L. The mechanism and the order of events is heavily debated. Some mechanisms assume a sequential reaction in which the first electron reduces the [2Fe-2S] cluster and a relatively unstable semiquinone intermediate reduces heme b_L (10). Link has proposed a sequential mechanism in which a stable semiquinone is formed and is anti-ferromagnetically coupled to the Rieske center until it is oxidized by heme b_L. The concerted mechanism assumes that neither electron is transferred independently, but rather the semiquinone is so unstable that ubiquinol can...
not reduce the Rieske center unless the semiquinone reduces heme $b_L$ (17, 19). In such a mechanism the concentration of ubisemiquinone is so low as to be almost non-existent.

We suggest that stigmatellin and alkyl-hydroxy-dioxobenzothiazoles mimic either intermediates during ubiquinol oxidation or transition state intermediates. Allowing for the possibility that a stable, anti-ferromagnetically coupled ubisemiquinone might be formed under some conditions, stigmatellin binding would mimic binding of a protonated ubisemiquinone, whereas HHDBT resembles the deprotonated form, i.e. the ubisemiquinone anion as illustrated in Fig. 7, panel 5. In both cases the position of the reduced Rieske protein is stabilized by the hydrogen bond to His$^{181}$. This is in agreement with EPR analysis of oriented membranes which indicated that the reduced Rieske is preferentially located in the b-position (51).

Glu$^{272}$ will accept a proton from ubiquinol or ubisemiquinone and consequently rotate towards heme $b_L$ as seen for HHDBT where it is not liganded to the carbonyl group. If a stable ubisemiquinone anion is formed, it is stabilized by localization of its negative charge on the oxygen atom interacting with protonated His$^{181}$. After transfer of the second electron the product is no longer stabilized and will leave the binding pocket and give more mobility to Tyr$^{279}$, thus breaking its hydrogen bond to the Rieske protein (Fig. 7, Panel 6). Accordingly, the latter is found to be rotated into the binding pocket in the chicken and bovine structures with an empty Q$_o$ site (4, 5).

The importance of Tyr$^{279}$ is supported by its full conservation in mitochondrial cytochrome $b$, and it is only replaced with a phenylalanine in a few chloroplast homologs (39). We propose that the on-edge weak hydrogen bonds from the aromatic side-chain
of Tyr<sup>279</sup> are crucial for positioning ubiquinol in the active site. This is supported by recent mutagenesis studies in <i>Rhodobacter sphaeroides</i> where the conservative mutation of the homologous residue Tyr<sup>303</sup> to phenylalanine has no effect on enzyme activity, whereas exchange to Leu, Gly and Gln resulted in 3, 40, and 50 fold decrease, respectively (41).

There are two options for the final steps of substrate oxidation and product release. Either electron transfer to heme <i>b</i><sub>L</sub> occurs in parallel with proton transfer mediated by Glu<sup>272</sup>, in which case the rotational displacement of the latter would destabilize the position of the quinone resulting in its release. Or, under conditions where a stably bound ubisemiquinone is formed, Glu<sup>272</sup> protonation and rotation occurs as a first step and the negative charge of the ubisemiquinone anion is stabilized as seen for HHDBT. Rieske release subsequently destabilizes the charge and initiates the second electron transfer. In both cases full oxidation of ubiquinol can take place without major repositioning of the substrate head group, as opposed to the proposed mechanism based on movement of the semiquinone in the binding pocket (10).

The two protons are released via two pathways. His<sup>181</sup> releases its proton to the bulk solvent after leaving the b-position, presumably after oxidation by cytochrome <i>c</i><sub>1</sub>. Our studies strongly support that the rotation of Glu<sup>272</sup> is an initial step for the release of the second proton as previously proposed (6, 10, 18). Glu<sup>272</sup> is completely conserved in mitochondrial cytochrome <i>b</i> (39) and the importance of the residue for proton transfer is indicated by mutagenesis studies as alteration to glutamine abolishes ubiquinol oxidation in <i>Rhodobacter sphaeroides</i> (40). Also, kinetic studies recently showed that protonation of a group with pK<sub>a</sub> of 5.7 blocked catalysis and this effect was assigned to
Hydroxyquinone binding to cytochrome bc₁ complex

Here, the hydrogen bond rearrangements which accompany the dramatic displacement of Glu²⁷² upon HHDBT binding provide experimental evidence for the previously postulated proton transfer pathway (6). Glu²⁷² can deliver the proton directly to the heme propionate A via a single water molecule (Fig.6). The subsequent proton release is mediated by a hydrogen bonded water chain stabilised by cytochrome b residues: Arg⁷⁹, Asn⁶⁶, Glu⁶⁶ and Arg⁷⁰.

Finally, the observed binding mode of the hydroxyquinone anion and the deduced proton transfer pathway as well as the demonstration of competitive inhibition by HDBT suggests a feasible catalytic mechanism, which is in line with a single occupancy model for ubiquinol oxidation.

Acknowledgements

We acknowledge support by the staff of beamline ID14-EH3, ESRF, Grenoble, France.
Hydroxyquinone binding to cytochrome $bc_1$ complex

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Figure legends

Fig. 1  The structure of the dimeric \textit{bc}_1 complex depicted as ribbon diagram. Ligands are shown as ball and stick models. HHDBT (yellow) is bound at the Q_o site between the [2Fe-2S] cluster and the heme b_L. Tightly bound phospholipid molecules (grey) are mainly present in the matrix oriented leaflet of the phospholipid bilayer. The newly identified PC molecule (dark grey) at the intermembrane side marks the position of the enzyme with respect to the bilayer.

Fig. 2  Ionization of hydroxy-dioxobenzothiazole bound to yeast \textit{bc}_1 complex. A. Spectra of 5 µM THDBT at pH 6.0 and pH 8.7. Ionization of the 6-hydroxy group shifts the absorbance maximum from 284 nm to 272 nm. B. Difference spectra recorded after adding 1.6, 2.3 and 3.6 µM THDBT to yeast \textit{bc}_1 complex. The enzyme was diluted to a concentration of 2.84 µM in a buffer at pH 6.0 and divided between two cuvettes. Difference spectra were recorded as the inhibitor was added to one cuvette. Lowering THDBT concentration to sub-stoichiometric amounts shifts the absorbance maximum to 272 nm, indicating ionization of the bound inhibitor.

Fig. 3  HHDBT binding at the Q_o site of yeast \textit{bc}_1 complex. The difference density map was calculated (Fo-Fc) before inclusion of the ligand and is contoured at 3σ. It allows unambiguous orientation of HHDBT which is shown in the final refined model. Residues, which stabilize ligand binding and are visible in this orientation are labeled. His^{181} a coordinating ligand of the [2Fe-2S] cluster is within hydrogen bond distance to the hydroxyl oxygen. The carbonyl oxygen atom forms a hydrogen bond to water molecule Wat^{518}. Atoms are shown in standard colors. Atom numbering of the hydroxyquinone anion is depicted in the insert.
Fig. 4 Lineweaver-Burk plot for determination of the apparent $K_m$ of yeast $bc_1$ complex for ubiquinol in the absence and presence of UHDBT. The concentration of DBH$_2$ was varied from 5 to 54 µM, and the activity measured in the absence (solid diamonds) or presence of 24 (solid squares), 54 (solid triangles) or 108 (solid circles) nM UHDBT. After adding substrate and UHDBT to the reaction mixture, the reaction was started by addition of the complex to a concentration of 2.5 nM. The double-reciprocal line in the absence of the inhibitor was constructed with a $K_m$ for DBH$_2$ of 11 µM and a $V_{max}$ of 272 s$^{-1}$. The double reciprocal lines in the presence of UHDBT were constructed considering UHDBT as a competitive inhibitor with $V_{max}$ unaltered. The resulting $K_{m,app}$ values were 37.7, 48.5, and 169 µM, for 24 nM, 54 nM and 108 nM UHDBT, respectively.

Fig. 5 Comparison of the position of cytochrome $b$ residues from stigmatellin and HHDBT inhibited $bc_1$ complex. The root mean square deviation (rmsd, Å) of the $C_\alpha$ trace and of all atoms is depicted in red and blue, respectively. The average rmsd of $C_\alpha$ atoms and of all atoms was 0.234 Å and 0.365 Å, respectively. Glu$^{272}$ is labeled with an asterisk. The major displacements are observed in the ef loop between trans-membrane helices E and F, and subtle differences are observed for the two short helices cd1 and cd2 between C and D, notably these residues are part of the Q$_o$ site.

Fig. 6 Apparent hydrogen bond network at the Q$_o$ site with the hydroxyquinone anion inhibitor bound. Glu$^{272}$ is hydrogen bonded to the heme $b_L$ propionate A from which a proton exit pathway is formed by a chain of hydrogen bonded water molecules, as depicted with the dotted lines. The arrow from Wat$^{274}$ shows the proton exit pathway to bulk solvent. Hydrogen bonds stabilising the ligand are shown as stippled lines. The position of Glu$^{272}$ in the stigmatellin inhibited $bc_1$ complex is indicated in yellow.
Fig. 7 Mechanism of ubiquinol oxidation as deduced from structural analysis of hydroxyquinone anion and stigmatellin binding at the Q\(_{0}\) site. The oxidised [2Fe-2S] is indicated with black circles, the reduced in grey. Hydrogen bonds stabilising the enzyme-substrate complex as well as the b-position of the Rieske catalytic domain are indicated with dotted lines. Panel 1. Empty Q\(_{0}\) site with Glu\(^{272}\) directed out of the binding pocket. Panel 2. Initial stabilization of ubiquinol by cytochrome b residues. Panel 3. The electron donor complex ("enzyme-substrate complex") with the Rieske protein docked in the b-position. 4. Coupled electron-proton transfer to the Rieske protein as deduced from the binding mode of stigmatellin. Panel 5. Stabilisation of the anti-ferromagnetically coupled ubisemiquinone anion and rotational displacement of protonated Glu\(^{272}\) as seen for HHDBT binding. Panel 6. Release of the reduced and protonated Rieske protein and of the oxidised ubiquinone accompanied by displacement of Tyr\(^{279}\). The steps 4 and 5 have to be interpreted either as intermediates of a sequential reaction or as transition states intermediates as proposed by the concerted mechanism hypothesis.
### TABLE I

**Data collection and refinement statistics**

| Description                                                                 | Value                        |
|-----------------------------------------------------------------------------|------------------------------|
| Space group                                                                 | C2                           |
| a, b, c [Å]                                                                 | 215.0, 165.1, 147.5          |
| β [°]                                                                       | 117.3                        |
| **No. non-hydrogen atoms in the model**                                     |                              |
| atoms                                                                       | 18069                        |
| amino acid residues                                                         | 2169                         |
| Non-protein molecules                                                       | 13                           |
| solvent molecules                                                           | 326                          |
| **Data collection**                                                         |                              |
| Resolution range (outer shell) [Å]                                          | 25.0-2.50 (2.56-2.50)        |
| Measured reflections                                                        | 372220 (19194)               |
| Unique reflections                                                          | 149103 (9128)                |
| Completeness [%]                                                            | 92.4 (84.6)                  |
| R<sub>merge</sub> [%]<sup>1</sup>                                           | 6.6 (37.3)                   |
| I/σ(I) < 1                                                                 | 13.4 (1.2)                   |
| **Refinement**                                                              |                              |
| Resolution range (outer shell) [Å]                                          | 25.0-2.50 (2.52-2.50)        |
| R factor [%]<sup>*</sup>                                                    | 22.8 (41.0)                  |
| R<sub>free</sub> [%]<sup>2</sup>                                            | 25.2 (41.1)                  |
| B<sub>wilson</sub> [Å<sup>2</sup>]                                          | 50.3                         |
| Average B-factor                                                            | 72.0                         |
| **Root mean square deviations from ideal values**                           |                              |
| Bond lengths [Å]                                                            | 0.007                        |
| Bond angles [°]                                                             | 1.298                        |
| **Ramachandran plot (non-Gly, non-Pro)**                                    |                              |
| Most favored regions [%]                                                    | 86.8                         |
| Additional allowed [%]                                                      | 12.5                         |
| Generously allowed [%]                                                      | 0.5                          |
| Disallowed [%]                                                              | 0.2                          |

Diffraction data collected at ESRF, ID14-EH3 at 4°C, λ 0.9299 Å. <sup>1</sup>R<sub>merge</sub> = Σ<sub>h</sub>Σ<sub>i</sub> | I<sub>i(h)</sub> - <I<sub>mean</sub>(h) | Σ<sub>h</sub> I<sub>h</sub>(h), I<sub>i(h)</sub>: intensity of i-th measurement, <I<sub>mean</sub>(h): average intensity of a reflection, R factor = Σ<sub>h</sub>|F<sub>h</sub>_<sub>obs</sub>| - | F<sub>h</sub>_<sub>calc</sub>| Σ<sub>h</sub> | F<sub>h</sub>|, <sup>2</sup>R<sub>free</sub> calculated for 2.5% of reflections. <sup>*</sup>B<sub>wilson</sub> was calculated using TRUNCATE, CCP4 package (53). PDB entry: 1P84.
Table 2 Contacts between cytochrome $bc_1$ complex residues and inhibitor (inh) atoms. Distances less than 3.9 Å are shown.

| $C_{obp}$ | Å inh. | $C_{obp}$ | Å inh. |
|-----------|--------|-----------|--------|
| M139 (O)  | 3.6    | P271 (C$\beta$) | 3.4    |
| W142 (C)  | 3.9    | C2        | 3.7    |
|           | 3.9    | S1        | 3.7    |
| W142 (C$\beta$) | 3.8   | P271 (C$\gamma$) | 3.6    |
| W142 (O)  | 3.9    | S1        | 3.8    |
| G143 (N)  | 3.4    | C2        | 3.9    |
|           | 3.8    | N3        | 3.7    |
| W142 (C$\beta$) | 3.8  | S1        | 3.7    |
| G143 (C$\alpha$) | 3.5  | N3        | 3.7    |
| V146 (C$\beta$) | 3.8  | C2        | 3.9    |
|           | 3.9    | C7        | 3.7    |
| V146 (C$\gamma$) | 3.6  | O7        | 3.7    |
|           | 3.7    | C7        | 3.7    |
| V146 (C$\gamma$) | 3.6  | O6        | 3.7    |
|           | 3.7    | C6        | 3.7    |
| V146 (C$\gamma$) | 3.2  | O7        | 3.7    |
|           | 3.7    | C7        | 3.9    |
| I147 (C$\delta$) | 3.4  | C9        | 2.8    |
| I269 (C$\delta$) | 3.7  | S1        | 3.5    |
|           | 3.6    | C7A       | 3.8    |
|           | 3.8    | C7        | 2.9    |
|           | 3.9    | O7        | 2.9    |

$\text{Rip1p}$  Å inh.

| C180 (C$\beta$) | 3.8    |
| H181 (C$\epsilon$) | 3.4    |
| H181 (C$\delta$) | 3.9    |
| H181 (N$\epsilon$) | 2.8    |
| S1             | 3.5    |
| C7A            | 3.8    |
| Water          | 2.9    |
| W 518          | 2.9    |
Fig. 1

Fig. 2
Fig. 3

Fig. 4
Fig. 7
Structure of the yeast cytochrome bc1 complex with a hydroxyquinone anion Qo site inhibitor bound
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J. Biol. Chem. published online June 2, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302195200

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