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Direct Demonstration of Half-of-the-sites Reactivity in the Dimeric Cytochrome bc_1 Complex

*ENZYME WITH ONE INACTIVE MONOMER IS FULLY ACTIVE BUT UNABLE TO ACTIVATE THE SECOND UBIQUINOL OXIDATION SITE IN RESPONSE TO LIGAND BINDING AT THE UBIQUINONE REDUCTION SITE**

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We previously proposed that the dimeric cytochrome bc_1 complex exhibits half-of-the-sites reactivity for ubiquinol oxidation and rapid electron transfer between bc_1 monomers (Covian, R., Kleinschroth, T., Ludwig, B., and Trumpower, B. L. (2007) *J. Biol. Chem.* 282, 22289–22297). Here, we demonstrate the previously proposed half-of-the-sites reactivity and intermonomeric electron transfer by characterizing the kinetics of ubiquinol oxidation in the dimeric bc_1 complex from *Paracoccus denitrificans* that contains an inactivating Y147S mutation in one or both cytochrome *b* subunits. The enzyme with a Y147S mutation in one cytochrome *b* subunit was catalytically fully active, whereas the activity of the enzyme with a Y147S mutation in both cytochrome *b* subunits was only 10–16% of that of the enzyme with fully wild-type or heterodimeric cytochrome *b* subunits. Enzyme with one inactive cytochrome *b* subunit was also indistinguishable from the dimer with two wild-type cytochrome *b* subunits in rate and extent of reduction of cytochromes *b* and c_1 by ubiquinol under pre-steady-state conditions in the presence of antimycin. However, the enzyme with only one mutated cytochrome *b* subunit did not show the stimulation in the steady-state rate that was observed in the wild-type dimeric enzyme at low concentrations of antimycin, confirming that the half-of-the-sites reactivity for ubiquinol oxidation can be regulated in the wild-type dimer by binding of inhibitor to one ubiquinone reduction site.

The cytochrome bc_1 complex is a multisubunit enzyme that generates a transmembrane protonmotive force by transferring electrons from ubiquinol to cytochrome *c*. Energy conservation in this enzyme complex is ensured by the oxidation of ubiquinol and the reduction of ubiquinone at active sites located on opposite sides of the membrane, as described by the protonmotive Q-cycle (1). High resolution structures have shown that the bc_1 complex is a dimer composed of two copies of cytochrome *b*, the Rieske iron-sulfur protein, and cytochrome c_1, which are the only polypeptides present in some bacterial enzymes (2), in addition to six to eight additional subunits present exclusively in each monomer of the eukaryotic complex (3–5).

The functional relevance of this dimeric arrangement has been supported by an extensive body of kinetic evidence (for review, see Ref. 6), which includes the key observations that only one ubiquinol oxidation site, or center P, is active when the two ubiquinone reduction, or center N, sites are occupied by inhibitors (7), that electrons rapidly equilibrate between the cytochrome *b* subunits (8, 9), and that there is conformational communication between center P and center N sites (10). We have proposed that this half-of-the sites activity at center P also exists under normal steady-state conditions in the absence of inhibitors and that this mechanism minimizes the leakage of electrons to oxygen under conditions that would favor the accumulation of electrons in the cytochrome *b* hemes (8, 11). Evidence consistent with this proposed dimeric mechanism has been obtained independently by other research groups (12–14). However, other interpretations of these data have been proposed to support a strictly monomeric mechanism of action in the bc_1 complex (15).

We have recently used the relatively simple three-subunit bc_1 complex from *Paracoccus denitrificans* to show that the inhibitor stigmatellin, which is considered to be a mimetic of ubiquinol bound at center P, binds with different rates and spectral effects to one of the center P sites in the dimer, thereby supporting the functional relevance of the dimeric structure of this enzyme (16). In the present work, we have generated *P. denitrificans* bc_1 heterodimers in which one center P site is largely inactivated by a mutation in cytochrome *b*, and we have determined its kinetics in comparison with wild-type and mutant homodimers. Our results directly demonstrate the half-of-the sites activity of the bc_1 complex dimer both in the presence and absence of center N inhibitors and confirm other postulates of the dimeric mechanism that we have proposed (6), such as the existence of intermonomeric electron transfer and the activa-

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tion of the second center P site upon binding of inhibitor to one center N site in the dimer.

EXPERIMENTAL PROCEDURES

Materials—Dodecylmaltoside was obtained from Anatrace. Stigmatellin, antimycin, decylubiquinone, and horse heart cytochrome *c* were purchased from Sigma. Decylubiquinol was prepared from decylubiquinone as described before (17). All inhibitors as well as decylubiquinol were quantified by UV spectroscopy (18) using reported extinction coefficients (19, 20).

Cloning Procedures—For the construction of the *fbc* operon with the deletion of the N-terminal acidic domain in cytochrome c_1, a 1.5-kb SmaI-PstI cassette was subcloned into pTZ18R (Pharmacia). The resulting plasmid was then cut with XhoI and NotI in a double digestion, followed by the removal of the sticky ends using mung bean nuclease. This double digestion removed the sequence encoding the acidic domain (amino acids 39–203 in cytochrome c_1) in-frame. After re-ligation of the vector and transformation, the newly obtained SmaI-PstI cassette was then subcloned into the cloning vectors described below.

To clone the His tag into the *fbc* operon in which the acidic domain of the cytochrome c_1 is deleted, a splicing by overlap extension PCR (SOEing PCR)[5] was used. First, a cassette containing the *fbc*B and *fbc*C genes was cloned via HindIII and Pfl23II restriction sites in an empty pSL1180 vector. The resulting plasmid was the template for the PCR. The primers used were as follows: SOEing4, 5′-AGCGGATAACAATTTCACACAGG-3′; SOEing1, 5′-CACGACGTTGTAAAACGACGGCCAG-3′; 5′-3′PCytbC-termHis-t_ext_ord, 5′-ACCCATCCTGCCGAGCATCACCATCACCATCACCATCACCATCACTAAGGAGAGGCAAC-3′; and 3′-5′PCytbC-termHis-t_ext_ord, 5′-GTTGCCTCTCCTTAGTGATGGTGATGGTGATGGTGATGGTGATGCTCGGCAGGATGGGT-3′.

The PCR products were SOE1 and SOE2 (580 and 1287 bp, respectively), obtained using the primer 5′-3′PCytbC-termHis-t_ext_ord and SOEing1 for the first fragment and 3′-5′PCytbC-termHis-t_ext_ord and SOEing4 for the second fragment. The two products, annealing via 30 bp at the 3′-end of SOE1 and the 5′ terminus of SOE2, were then mixed together as templates for the third PCR, with the primers SOEing1 and SOEing4. This created a product of 1867 bp. The fragment was blunt-end-ligated in an empty pSL1180 vector and then cloned by Pfl23II and HindIII restriction into the expression vector pRI2 (21) containing the *fbc* operon. This plasmid (pMC1) was used to transform competent DH5α cells, and the resulting strain was used in a triparental mating procedure to transfer the expression vector into the MK6 *P. denitrificans* strain (22).

The Strep tag II was introduced in the *fbc* operon using the same procedure as the His tag. In addition to the same primers SOEing1 and SOEing4, the other two primers were 5′-3′-CytbC-termstrep-tagII (5′-AACTGGTCGCATCCGCAGTTCGAGAAGTAAGGAGAGGCAACAATGAC-3′) and 3′-5′-CytbC-termStrep-tagII (5′-CTTCTCGAACTGCGGATGCGACCAGTTCTCGGCAGGATGGGTTTCAG-3′). The final *fbc* operon with Strep tag II was cloned into the expression vector pBBR1-MCS5 (23), resulting in pTK56 (Fig. 1).

The Y147S mutation was obtained by one-primer site-directed mutagenesis of the wild-type *fbc* operon with the primer bY147SEco24I (5′-CCGCCTTCATGGGCT**CG**GT**C**CTGCCCTGG-3′). Sequencing confirmed that mutagenesis was successful. The mutation was then subcloned via SacI-Pfl23II into a pUC18 vector containing a copy of the *fbc* operon with the deletion of the acidic domain in Cyt c_1 and the His tag. The clones obtained were screened by Eco24I digestion because the introduction of the Y147S mutation eliminates a restriction site for this enzyme in the operon. The clones showing the desired restriction pattern were then sequenced, and the newly obtained vector was digested with HindIII and SacI to subclone the *fbc* operon with mutation Y147S into the expression vector pEG400 (24). Clones were screened by digestion with the previously mentioned enzymes, and the resulting plasmid was named pMC12 (Fig. 1). The two plasmids, pMC12 and pTK56, were used to transform competent DH5α cells, and the resulting strains were used in a four-strain mating procedure to transfer the expression vectors simultaneously into the MK6 *P. denitrificans* strain (22) selected on rifampicin, kanamycin, gentamycin, and streptomycin, resulting in a strain stably containing both vectors.

Purification of Cytochrome bc_1 Complex—Cells from an overnight growth were harvested at an OD value of 3–5 and resuspended in a buffer containing 100 mM sodium phosphate, pH 8, and 1 mM EDTA, and frozen. Membranes were obtained as described previously (22). To solubilize the bc_1 complex, membranes were diluted to a protein concentration of 35 mg/ml. The suspension was then diluted 1:1 with solubilization buffer (50 mM sodium phosphate, pH 8, 300 mM sucrose, 1.2 M NaCl, with addition of trace amounts of avidine to titrate endogenous biotin compounds in the case of the Strep-tagged protein), and dodecylmaltoside was added to a final ratio 1.15:1 with respect to total protein. Solubilization of the complex was done on ice with stirring for 1 h. After centrifugation at 100,000 $\times$ *g* for 1 h, the supernatant was filtered and loaded onto the appropriate column.

Mutant homodimer purification was performed using a Ni^{2+}-NTA column, previously equilibrated with 6 column volumes of buffer A (50 mM sodium phosphate, pH 8, 300 mM NaCl, 0.02% dodecylmaltoside). The column was then washed with 1 column volume, and a 0–200 mM histidine gradient in the same buffer (5 column volumes) was used to elute the complex. Wild-type homodimers were bound to a StrepTactin column (IBA), equilibrated with 5 column volumes of buffer A, and washed with 2 column volumes. A one-step elution was used with 1 column volume of buffer A containing 2.5 mM desthiobiotin. The heterodimer was purified using the 2 columns sequentially in tandem purification (first Ni^{2+}-NTA, then StrepTactin), following the protocols described above. Fractions from the purifications were collected and analyzed by redox difference spectra, SDS-PAGE, and Western blotting, to define purity and protein composition. Protein concentrations were calculated using the extinction coefficients of 23.2 mM^{-1} cm^{-1} at 553–539 nm (25) and 25.6 mM^{-1} cm^{-1} for the average

[5] The abbreviations used are: SOEing PCR, splicing by overlap extension PCR; NTA, nitrilotriacetic acid; Strep, a synthetic peptide of 8 amino acids, Trp-Ser-His-Pro-Gln-Phe-Glu-Lys.

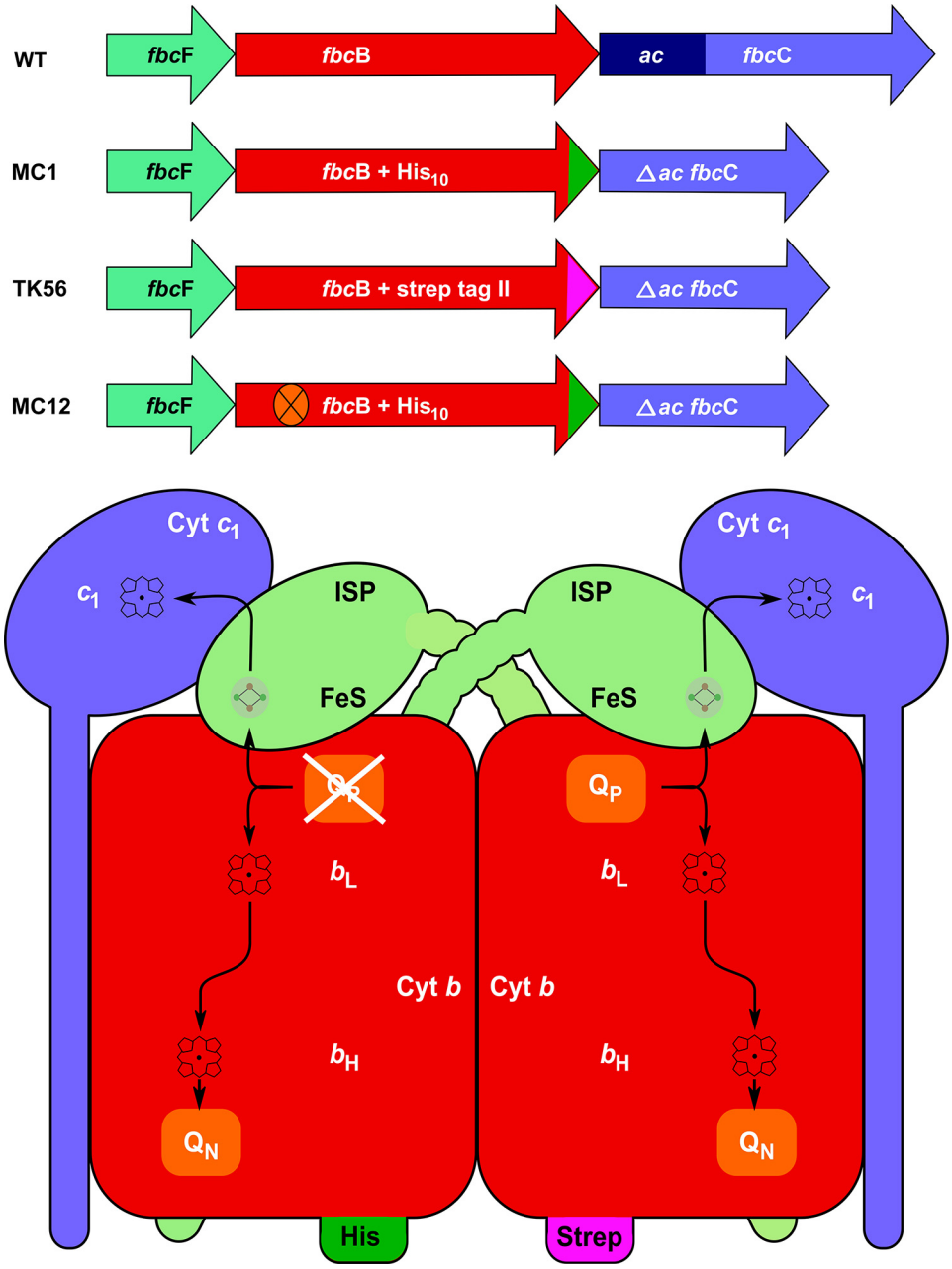

FIGURE 1. ***fbc* operon and mutagenesis of the operon to construct a heterodimeric cytochrome bc_1 complex with one active and one inactive monomer.** *Upper panel*, *P. denitrificans fbc* operon and its three constituent genes, *fbc*F, encoding the Rieske iron-sulfur protein; *fbc*B, encoding cytochrome *b*; and *fbc*C, encoding cytochrome c_1. *WT* is the operon with no mutations and includes the acidic N terminus that is normally present on cytochrome c_1. *MC1* is the construct in which the acidic N terminus has been deleted. *TK56* is the construct in which the acidic N terminus has been deleted and the Strep tag has been encoded into the cytochrome *b* gene and with no mutations in *fbc*B. *MC12* is the N-terminal deletion construct in which a His tag has been encoded into the *fbc*B gene and a Y147S mutation has been introduced into the cytochrome *b* gene. *Lower panel*, three redox subunits of the heterodimeric cytochrome bc_1 complex in which one cytochrome *b* subunit carries a His tag and contains a Y147S mutation that extensively inactivates ubiquinol oxidation at the center P (Q_p) site. The other cytochrome *b* subunit carries a Strep tag II and no cytochrome *b* mutation.

of the two hemes in cytochrome *b* (26). All enzyme samples yielded ratios very close to 2 *b* hemes:cytochrome c_1.

Pre-steady-state Reduction of bc_1 Complexes—Pre-steady-state reduction of cytochrome c_1 and cytochrome *b* was followed at room temperature by stopped flow rapid scanning spectroscopy using the OLIS Rapid Scanning Monochromator as described before (7, 27). Reactions were started by rapid mixing of 2 μM enzyme with or without 2.5 μM antimycin in assay buffer containing 100 mM Tris-HCl, pH 8.0, 2 mM sodium azide, 0.2 mM EDTA, and 0.05% dodecylmaltoside against an equal volume of the same buffer containing the indicated concentration of decylubiquinol. For each experiment, 8 data sets were averaged after subtracting the oxidized spectrum. The time course of absorbance change at the appropriate wavelengths was extracted using software from OLIS and exported to the Origin 5.0 program (OriginLab Corp.). The contribution of cytochrome *b* to c_1 absorbance was calculated and corrected for as previously described (7). Absorbance changes as a function of time were fitted to monophasic or biphasic exponential functions in Origin.

Steady-state Activities of bc_1 Complexes—Horse heart cytochrome *c* reduction was followed at room temperature by stopped flow rapid scanning spectroscopy using the OLIS Rapid Scanning Monochromator. Reactions were started by rapid mixing of 100 nM enzyme and 20 μM cytochrome *c* in assay buffer against an equal volume of the same buffer containing the indicated concentration of decylubiquinol. For each experiment, 6 data sets were averaged after subtracting the oxidized spectrum. The time course of absorbance change between 550 and 539 nm was extracted using software from OLIS and exported to the Origin 5.0 program, and the initial increase in absorbance was fitted to a linear regression function and expressed as turnover number (moles of cytochrome *c* reduced/mol of bc_1 monomer/s) using an extinction coefficient of 21.5 $mM^{-1}cm^{-1}$ (28). Initial rates at different decylubiquinol concentrations were fitted to the Michaelis-Menten equation to obtain V_m and K_m values.

For the antimycin titration experiments, the indicated concentration of inhibitor was added to a 10 μM solution of enzyme on ice and incubated for at least 2 min before dilution in assay buffer at room temperature to a concentration of 100 nM enzyme in the presence of 20 μM cytochrome *c*, and rapid mixing against an equal volume of buffer containing 80 μM decylubiquinol. The very low K_d for antimycin (~30 pM) (19) allows for the stoichiometric binding of the inhibitor to be preserved

even after diluting the enzyme to 100 nM. The time course of absorbance change at 550 *versus* 539 nm from 6 individual data sets/antimycin concentration was extracted using software from OLIS and exported to the Origin 5.0 program, where rates were calculated as described above. Initial rates at each antimycin concentration were fitted to kinetic models published previously (7) that assume that dimers with one antimycin bound contribute equally or twice as much as free dimers to the overall steady-state rate.

RESULTS

Construction of a Fully Wild-type Dimeric Cytochrome bc_1 Complex, a Mutant/Wild-type Heterodimeric bc_1 Complex, and a Homodimeric Mutant bc_1 Complex by Mutagenesis of the P. denitrificans fbc Operon—The *fbc* operon of *P. denitrificans* is composed of three structural genes, encoding for the three subunits in the complex: the Rieske iron-sulfur protein, cytochrome *b*, and cytochrome c_1 (Fig. 1). Three different domains can be identified in the last protein: an N-terminal, acidic domain, a "core domain" coordinating the *c* type heme, and a C-terminal transmembrane domain. In this work, a cytochrome c_1 mutant has been used in which the acidic domain was deleted (24) to convert the enzyme from a dimer of dimers (29) to a dimer, as judged by the molecular mass obtained by gel filtration and mass spectrometry when this domain is deleted, without affecting the catalytic rate at center P, as will be described in detail in a future publication.[6]

The construction of a heterodimeric bc_1 complex (Fig. 1) in *P. denitrificans* was achieved by transforming a bc_1 complex-deficient strain (MK6) with two plasmids: pMC12 and pTK56. The two plasmids were conjugated at the same time, using a four-strain mating procedure. Plasmid DNA obtained from the resulting colonies was screened with the restriction endonucleases BglII and Bpu1102I, cutting inside the antibiotic resistance genes of pTK56 (Gm) and pMC12 (Sm), respectively. Four positive clones were identified, followed by large scale expression and membrane preparation.

Two different affinity columns were used to achieve purification of the heterodimer, a Ni^{2+}-NTA and a StrepTactin column (Fig. 2*A*). From the clones transformed with both plasmids, three differently tagged dimers can be expressed (two His-tagged monomers, two Strep-tagged monomers, and the heterodimeric complex with one His and one Strep tag). The first column (Ni^{2+}-NTA), retains the His-tagged dimer and the heterodimeric bc_1 complex. These could subsequently be separated using the StrepTactin column. In Fig. 2*B*, the Western blot analysis of the different fractions from the purification shows the presence of the expected tags in the fractions obtained from each column, with the heterodimer containing both tags.

Crystallographic structures (2–5) show that the bc_1 complex dimer is stabilized by the transmembrane helices of the Rieske iron-sulfur protein, which traverse from one monomer to the other, and by the N terminus helical region of each cytochrome *b* subunit. The tilted arrangement of the iron-sulfur proteins is in turn stabilized by multiple interactions with cytochrome *b* and cytochrome c_1. Therefore, re-equilibration of cytochrome *b* subunits between different dimers is not possible because it would require complete dissociation of the subunits of the two monomers of the complex and reassembly by *in vivo* factors that are not present in the purified enzyme solution.

Pre-steady-state Oxidation of Ubiquinol at Center P of the Fully Wild-type Dimeric, the Mutant/Wild-type Heterodimeric, and the Homodimeric Mutant bc_1 Complexes—In the absence of inhibitors, decylubiquinol reduced cytochrome c_1 under pre-steady-state conditions through center P with the rates shown in Fig. 3. Both the fully wild-type dimer and the mutant homodimers with the Y147S/Y147S cytochrome *b* mutations exhibited monophasic kinetics on the time scale shown but with a rate 6-fold lower in the case of the mutant dimer. The heterodimer, in contrast, exhibited biphasic kinetics with a fast and a slow rate identical to that obtained in the wild-type and mutant homodimers, respectively. All three dimer samples showed an initial lag in the reduction of cytochrome c_1, which lasted longer in the case of the mutant homodimer. This lag phase could reflect a relatively slow binding or partitioning of the substrate toward center P immediately after rapid mixing.

The extent of reduction of each phase in the heterodimer was approximately half of the total absorbance change, indicating that the fast rate corresponds to the catalysis of the wild-type monomer and the slow phase to that of the monomer with the Y147S cytochrome *b* mutation. This implies that, with center N free to reoxidize cytochrome *b*, the slower catalysis of the mutant center P site was revealed after the more active wild-type site became unable to continue catalysis due to the accumulation of electrons in the Rieske iron-sulfur protein and in cytochrome c_1. The total absorbance change was identical in all three enzymes and corresponded to the total concentration of cytochrome c_1 present in the assay, indicating that no significant fraction of enzyme in any of the three dimer preparations was inactivated during the purification procedure.

In the presence of antimycin, which blocks reoxidation of the b_H hemes through center N, both cytochrome *b* and cytochrome c_1 undergo reduction exclusively through center P, although the observed rate of cytochrome c_1 reduction is slightly slower than that of cytochrome *b* because of the higher redox potential of the Rieske protein, which causes a delay in the appearance of the electron in the c_1 heme (as fully discussed in Ref. 30). Nevertheless, both c_1 and *b* reduction report the rate of quinol oxidation at center P with antimycin present. As shown in Fig. 4, the wild-type homodimer and the heterodimer showed very similar rates and extents of reduction for both cytochrome *b* and c_1, whereas the mutant homodimer showed reduction rates ~7-fold lower, yielding a significantly decreased and incomplete extent of reduction on the time scale shown. The kinetic traces obtained showed that only one c_1 heme/dimer underwent reduction in the fully wild-type and heterodimeric enzymes, indicating that only one center P/dimer (the wild-type in the case of the heterodimer) was able to oxidize ubiquinol when antimycin was bound to both center N sites. However, the spectra showed reduction of close to 70% of the total cytochrome *b* present, without showing the shoulder at 566 nm that is indicative of b_L reduction. These observations indicate that both b_H hemes in the dimer were reduced

[6] M. Castellani, T. Kleinschroth, and B. Ludwig, unpublished data.

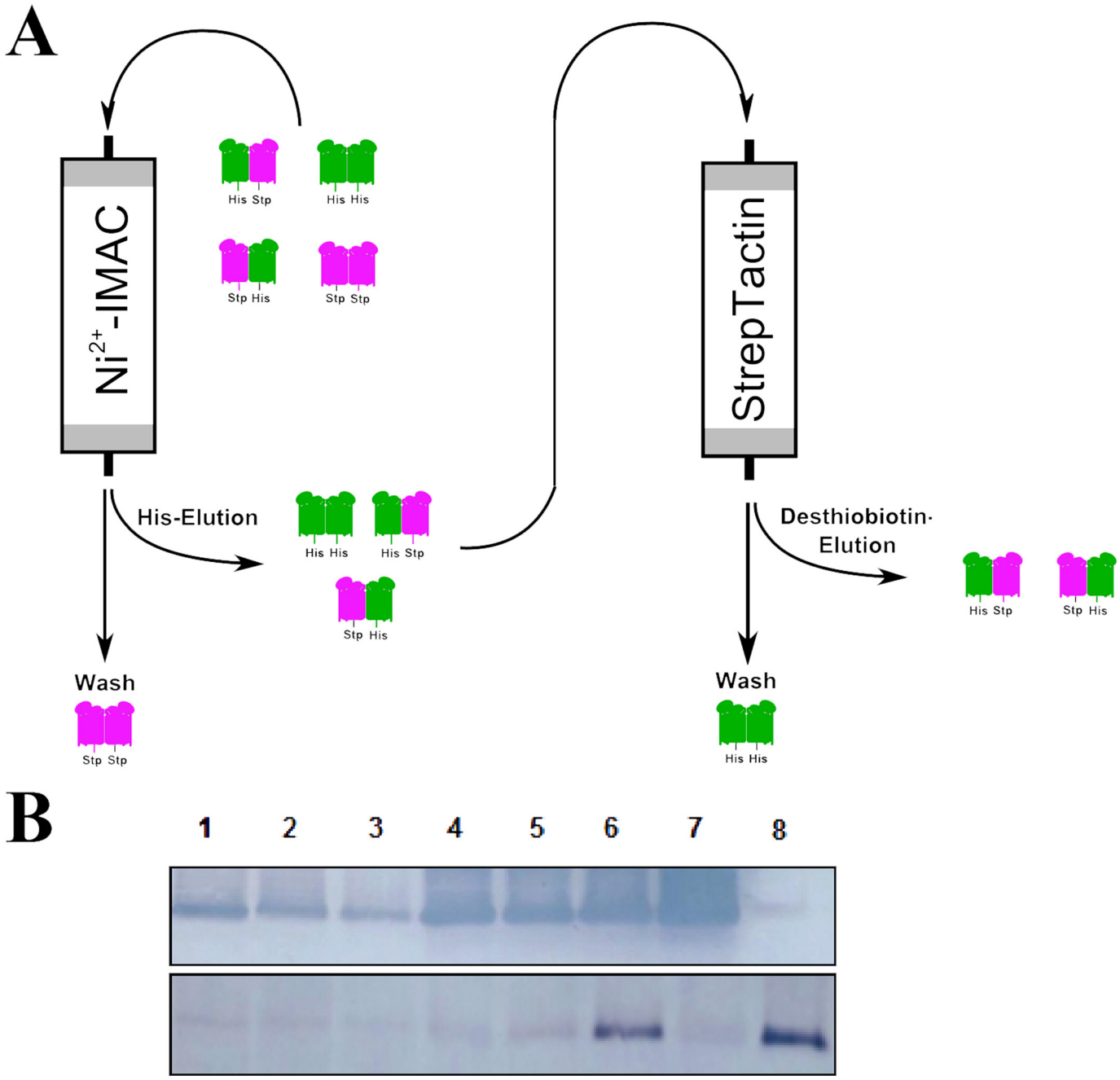

FIGURE 2. **Chromatography purification protocol and Western blots of the chromatography fractions.** *A*, schematic depiction of the chromatography procedure in which two affinity columns were used in sequence to purify the various populations of bc_1 complexes. The His-tagged monomer with the Y147S cytochrome *b* mutation is colored *green*, and the Strep-tagged monomer with wild-type cytochrome *b* is colored *magenta*. *B*, Western blot against the His tag (*upper*) and Strep tag II (*lower*), both at the C terminus of the cytochrome *b* subunit. *Lane 1*, solubilized extract; *lane 2*, flow-through from the Ni^{2+}-NTA column; *lane 3*, eluate from the Ni^{2+}-NTA wash step; *lane 4*, flow-through from the StrepTactin column; *lane 5*, eluate after the StrepTactin wash step; *lane 6*, bc_1 heterodimer; *lane 7*, MC1; *lane 8*, Strep-tagged wild-type bc_1 complex. *Lane 1* shows a signal, as expected, for both tags. *Lanes 2* and *3* also show signals for both tags, corresponding, respectively, to the Strep-tagged complex not binding to the column and the His-tagged complex not binding due to slight overloading of the column. The flow-through of the StrepTactin column (*lane 4*) and the washing step (*lane 5*) show, as expected, a strong signal for the His tag because it contains mostly His-tagged complex, being washed away. The heterodimeric complex (*lane 6*) after tandem purification delivers a strong signal in both panels. The Strep-tagged, but otherwise wild-type complex (*lane 8*) was used as negative (*upper panel*) and positive (*lower panel*) control.

through a single center P site by intermonomeric electron transfer between cytochrome *b* subunits.

Steady-state Activity of the Fully Wild-type Dimeric, the Mutant/Wild-type Heterodimeric, and the Homodimeric Mutant bc_1 Complexes—In the presence of an excess of cytochrome *c* and without inhibitors, a steady state that allows multiple turnovers of the enzyme occurs as it operates catalytically. As shown in Fig. 5, the rate of this steady-state activity in the heterodimer, as well as its affinity for substrate, was essentially identical to that of the wild-type homodimer. In contrast, the mutant homodimer showed a ~10-fold lower V_m together with an increased apparent affinity for ubiquinol, possibly caused by the smaller relative contribution in the mutant of the catalytic rate constant (k_{cat}) to the K_m, which does not necessarily rep-

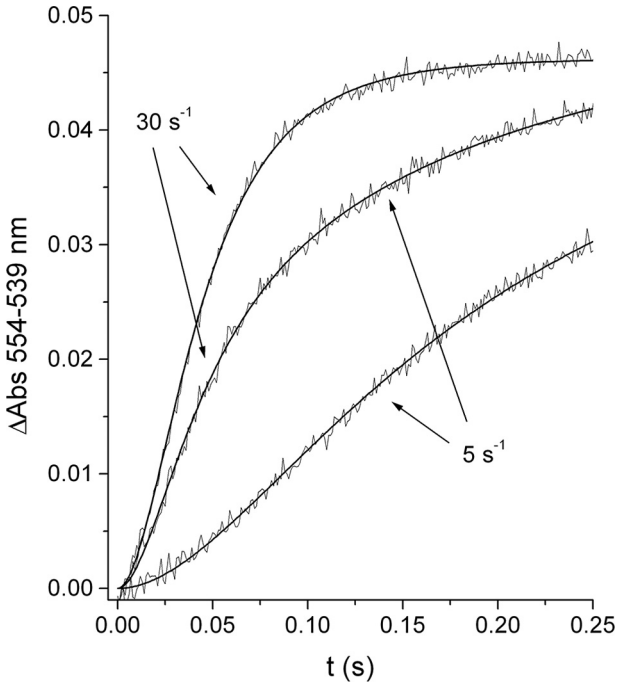

FIGURE 3. **Pre-steady-state reduction of cytochrome c_1 in uninhibited *P. denitrificans* bc_1 complex dimers.** Fully wild-type (*top trace*), heterodimeric mutant/wild-type (*middle trace*), or homodimeric mutant (*bottom trace*) bc_1 complex (2 μM) was reduced with 40 μM decylubiquinol, and the resulting traces were fitted (*solid curves*) to a one- or two-phase exponential equation, resulting in the indicated rates of cytochrome c_1 reduction through center P.

resents a true dissociation constant for substrate binding. The virtually identical activity between the fully wild-type and heterodimers demonstrates that only one of the two center P sites is active at a time in the homodimer, and in the heterodimer the one wild-type monomer oxidizes ubiquinol continuously and is able to catalyze the same steady-state rate as in the wild-type dimer. This identical activity also disproves the remote possibility that slow cytochrome *b* re-equilibration between dimers occurred in the heterodimeric preparation to form wild-type and mutant homodimers because the generation of the latter would have decreased the overall turnover rate.

Antimycin Titration of the Steady-state Activity of the Wild-type and Heterodimeric bc_1 Complex Dimers—As shown in Fig. 6*A*, the tightly bound center N inhibitor antimycin (K_d ~30 pM) (Ref. 19) exhibits a paradoxical effect on the steady-state activity of the wild-type dimer, consisting of a stimulation of the observed rate of cytochrome *c* reduction that peaks at an inhibitor: monomer ratio of 25% followed by a decrease in the rate. This effect, which we have already observed in the yeast bc_1 complex with antimycin or ilicicolin H (8, 9), could be fitted by assuming that, whereas free dimers have only one center P site active at a time, binding of antimycin to one center N activates the second center P site, allowing ubiquinol oxidation to proceed in both monomers simultaneously (Fig. 6*A*, *inset*). Consequently, a dimer with an inhibitor bound at one center N has twice as much activity as a free dimer due to activation of the otherwise silent center P site, being able to use a single center N site to catalyze quinone reduction due to rapid electron equilibration between cytochrome *b* subunits at the level of the b_L hemes.

This interpretation was supported by the results shown in Fig. 6*B* using the heterodimeric enzyme in which one center P site is inactivated. The stimulation of the activity by antimycin was virtually absent in the heterodimer, consistent with the assumption that in the free dimer activity is sustained only by the wild-type center P site and that binding of antimycin to one center N results in the activation of the mutant monomer that, because of its low intrinsic activity (~10% of the wild-type, as shown in Fig. 5) conveyed by the Y147S cytochrome *b* mutation, contributes by a correspondingly small fraction to the total rate. Therefore, the activity of dimers with only one antimycin bound is equal to that of free dimers (Fig. 6*B*, *inset*), yielding a titration curve that lacks the stimulation observed with the wild-type homodimer. Because electrons coming from the wild-type center P site in heterodimers with one monomer occupied by antimycin are still able to reach the unbound center N, the titration curve exhibits a nonlinear hysteresis, given that a dimer would become fully inactive only when antimycin was bound to both center N sites. If electrons could not be transferred between cytochrome *b* subunits, wild-type center P sites with antimycin bound at the center N site in the same monomer would be inactive under steady-state conditions, and the titration curve would be linear (*dotted line* in Fig. 6*B*), which was clearly not the case.

In both the wild-type and heterodimeric enzymes, antimycin at concentrations at or above 100% per monomer did not inhibit the rate of cytochrome *c* reduction beyond ~70%. Pre-steady-state experiments with higher enzyme concentrations in the presence of cytochrome *c* and saturating antimycin showed that the ~30% residual activity corresponded to bypass reactions in which electrons reach cytochrome *c* without cytochrome *b* undergoing reduction. These bypass reactions were associated with superoxide production, being ~50% sensitive to superoxide dismutase (results not shown). This was expected considering that (as discussed in Ref. 31), after electrons accumulate in cytochrome *b* due to the antimycin blockage, center P transfers one electron from quinol to cytochrome *c* via the iron-sulfur protein and cytochrome c_1, whereas the remaining electron in the substrate is directly transferred to oxygen to form superoxide, which by itself is a good reductant of cytochrome *c*. Thus, superoxide dismutase only inhibits reduction of cytochrome *c* by superoxide but is not able to block reduction by the electron coming from the iron-sulfur protein and cytochrome c_1, yielding a maximal 50% inhibition in the total antimycin-insensitive reduction of cytochrome *c*.

DISCUSSION

The relative simplicity of the three-subunit bc_1 complex in *P. denitrificans* compared with its eukaryotic counterparts allowed us in the present work to tag differentially the C terminus of the cytochrome *b* subunits containing a largely inactivating center P mutation to purify heterodimers where only one monomer had the mutation. This would have been extremely difficult to achieve in a eukaryotic enzyme because the C terminus of the mitochondrially encoded cytochrome *b* is surrounded by a polypeptide of 14 kDa (4, 5, 12), which would have masked or even sterically clashed with the tag extension. Furthermore, we have already demonstrated that the half-

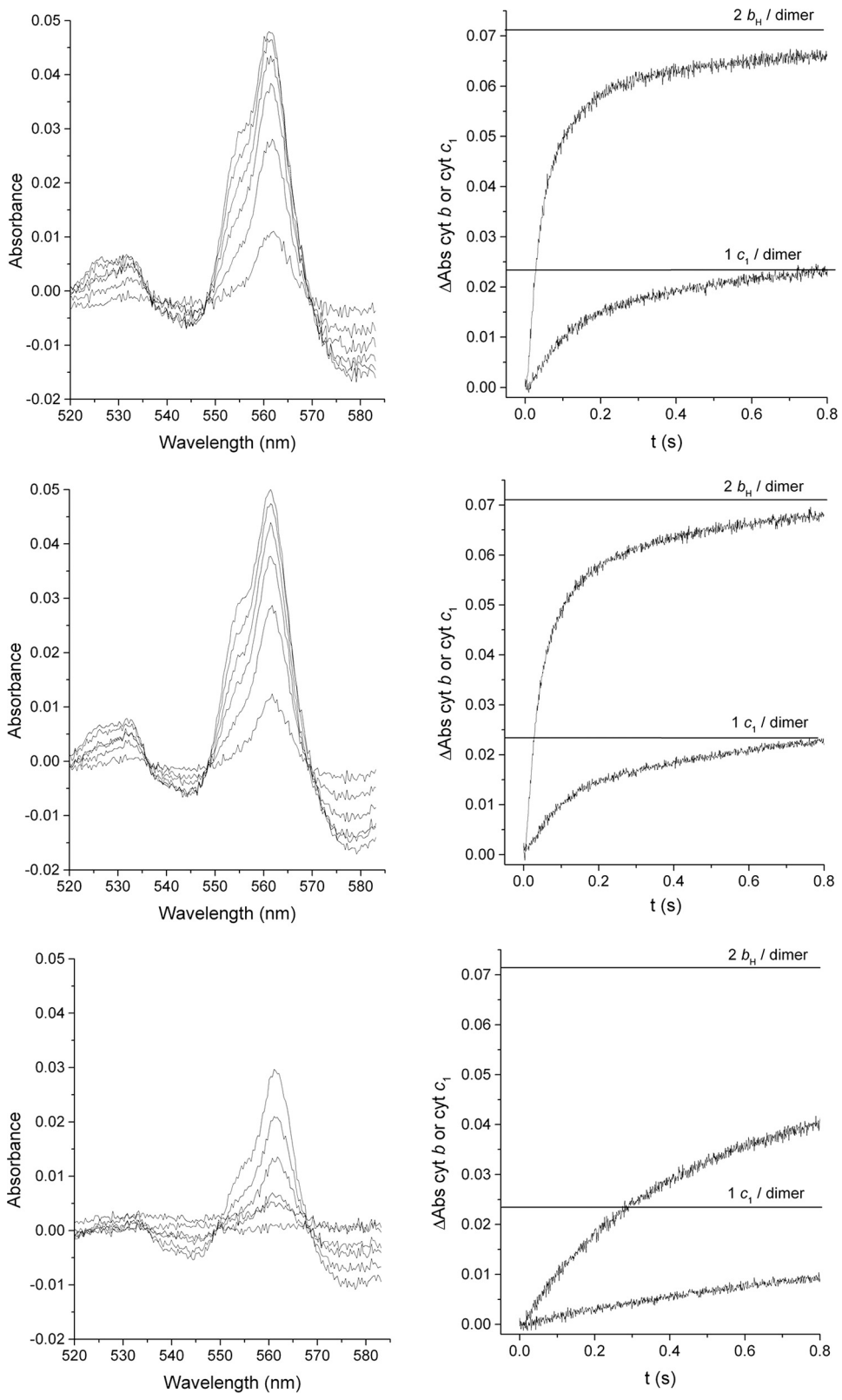

FIGURE 4. **Pre-steady-state oxidation of ubiquinol at center P of *P. denitrificans* bc_1 complex dimers in the presence of antimycin.** Fully wild-type (*top panels*), heterodimeric mutant/wild-type (*middle panels*), or homodimeric mutant (*bottom panels*) bc_1 complex (2 μM) preincubated with 2.5 μM antimycin was reduced with 40 μM decylubiquinol. *Left panels*, spectra obtained at 20, 50, 100, 200, 400, and 800 ms after mixing with the substrate. *Right panels*, reduction kinetics of cytochrome *b* (*upper traces*) and cytochrome c_1 (*lower traces*) compared with the indicated extent of reduction of b_H and c_1 hemes/dimer. The initial rates of cytochrome *b* and c_1 reduction, respectively, were 20 s^{-1} and 7.4 s^{-1} for the wild-type dimer, 20.9 s^{-1} and 8.2 s^{-1} for heterodimer, and 3.1 s^{-1} and 1.3 s^{-1} for the homodimeric mutant enzyme.

of-the sites activity at center P observed in the yeast enzyme is also evident in the complex isolated from *P. denitrificans* (16).

It was necessary to delete an acidic N-terminal extension in the cytochrome c_1 subunit peculiar to this bacterium that results in the stable aggregation of pairs of dimers, as will be published elsewhere, to purify heterodimers with the Y147S mutation in one cytochrome *b* subunit. Nevertheless, pre-steady-state kinetic experiments indicated no difference in the rates of cytochrome *b* or c_1 reduction through center P between bc_1 complexes containing the wild-type cytochrome *b* with or without the c_1 acidic extension, nor were differences observed when comparing the effect of tagging the C terminus of wild-type or Y147S cytochrome *b* with His or Strep extensions (results not shown).

Our present results demonstrate directly that only one center P site is active in both the wild-type and heterodimeric enzymes when antimycin is bound at both center N sites (see Fig. 4) and under steady-state conditions (see Fig. 5) in which, as we have previously proposed (8), both center N sites are likely occupied with semiquinone most of the time. The identical turnover rates obtained in the heterodimer, in which one ubiquinol oxidation site was largely inactivated by mutation, relative to the wild-type enzyme (see Fig. 5), is clear evidence that one active center P site is sufficient to sustain full catalysis in the bc_1 complex dimer. The Y147S cytochrome *b* substitution does not preclude the binding of substrate, as evidenced by EPR studies that show normal occupancy at center P (32). This agrees with the even higher apparent affinity of the mutant homodimer for decylubiquinol (see Fig. 5) relative to the wild-type homodimer, and with its fast and tight binding of stigmatellin (results not shown), an inhibitor that occupies the same site as the substrate. Therefore, the observation that the wild-type monomer is the only one

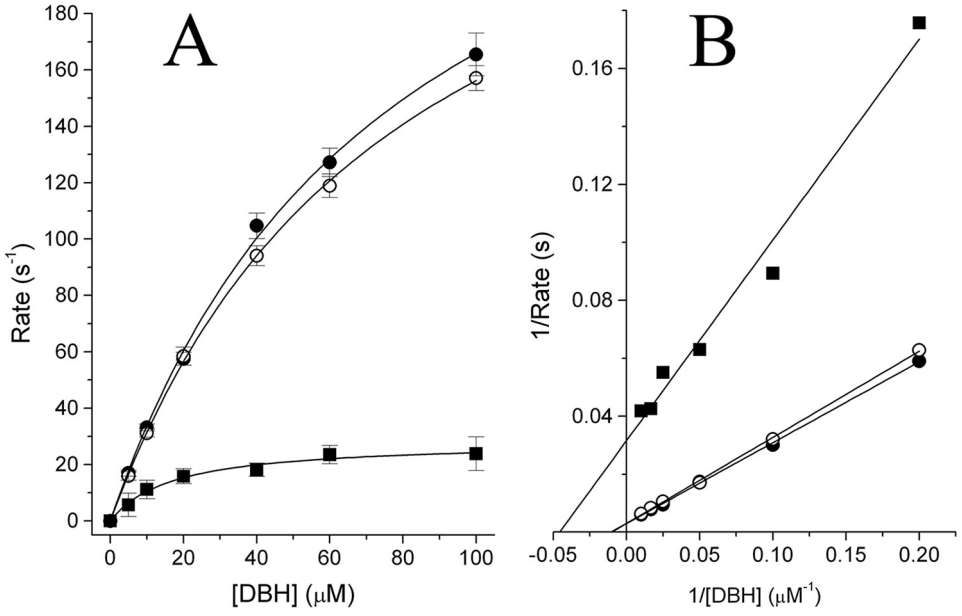

FIGURE 5. **Kinetic parameters of the steady-state activity of *P. denitrificans* bc_1 complex dimers.** The catalytic rates of reduction of 10 μM horse heart cytochrome *c* by 50 nM wild-type (*filled circles*), heterodimeric (*open circles*), or homodimeric mutant (*filled* squares) bc_1 complexes obtained by mixing with the indicated final concentrations of decylubiquinol (*DBH*) were fitted to the Michaelis-Menten equation (*solid curves* in *A*). The fitted V_m and K_m values, respectively, were 294.6 ± 14 s^{-1} and 77.5 ± 6.6 μM for the wild-type dimer, 279.3 ± 7.4 s^{-1} and 78.8 ± 3.7 μM for the heterodimeric enzymes, and 28.2 ± 1.6 s^{-1} and 31.7 ± 2.9 μM for the homodimeric mutant enzyme. The reciprocal plots of the rates obtained at each substrate concentration (*B*) illustrate more clearly the large difference in V_m and K_m values obtained with the homodimeric mutant dimer relative to the wild-type and heterodimeric bc_1 complexes, which are virtually identical to each other.

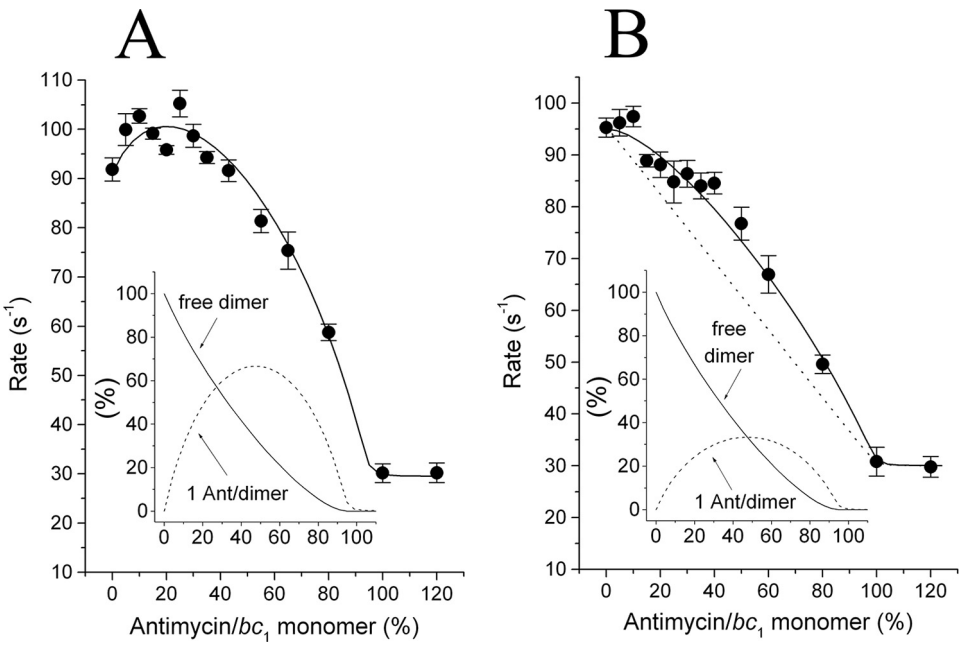

FIGURE 6. **Antimycin titration of the steady-state activity of *P. denitrificans* bc_1 complex dimers.** The catalytic rates of reduction of 10 μM horse heart cytochrome *c* by 50 nM wild-type (*A*) or heterodimeric (*B*) bc_1 complex were obtained by mixing with a final concentration of 40 μM decylubiquinol after incubating in the presence of the indicated ratio of antimycin/monomer. *Solid curves* correspond to the simulated activity expected assuming that activation of the previously silent center P site in dimers with only one antimycin molecule bound contribute to the activity twice as much as free dimers in the case of the wild-type enzyme (*A*, *inset*) or to the same extent as free dimers in the heterodimeric enzyme (*B*, *inset*), where the activated second center P site is too slow to contribute significantly to the rate. In both cases, intermonomeric electron transfer is assumed to allow either center N site in the dimer to recycle electrons from center P in the opposite monomer. Without this assumption, a linear titration curve (*dotted line* in *B*) would be expected. Antimycin at concentrations higher than 100%/monomer did not inhibit the activity beyond a rate of ~30 s^{-1}, indicating stoichiometric binding as expected from the very low K_d (~30 pM) of this inhibitor.

to manifest activity in the case of the WT/Y147S heterodimer suggests that ubiquinol oxidation at the faster center P site, and not merely its binding, contributes to the complete inactivation of the second center P site. This agrees with the proposal that electron transfer from b_L to b_H affects the mobility of the Rieske iron-sulfur protein, as evidenced by the much slower cytochrome c_1 reduction in enzyme that lacks the b_H hemes (13).

The half-of-the-sites dimeric mechanism we have proposed for the functioning of the bc_1 complex (6) also postulates that an asymmetry in binding of ligands to the center N sites allows simultaneous activity at the center P sites, as evidenced by the stimulation exerted by low concentration of antimycin on the steady-state activity of the dimer. Our present data showing the absence of this stimulation in the heterodimer (see Fig. 6) are a clear indication that activation of the second center P site is indeed responsible for this paradoxical effect of center N inhibitors. Our pre-steady-state results in the absence of inhibitors and cytochrome *c* (see Fig. 3) are also consistent with this aspect of the dimeric mechanism. Given that center N was allowed to oscillate freely between states with different semiquinone occupancies, the second center P site in the dimer was allowed to manifest its intrinsic activity once the first site was prevented from further ubiquinol oxidation events by the accumulation of electrons in cytochrome c_1 and the Rieske protein. This effect was evidenced by the biphasic c_1 reduction kinetics obtained with the heterodimeric enzyme. However, under normal turnover conditions in which cytochrome *c* continuously oxidizes c_1, only one monomer would be active at a time, as observed under steady-state conditions. This also supports our previous proposal that dimeric states in which semiquinone is present only at one center N site are probably transient under normal turnover conditions (6), having the effect of

randomly switching which monomer will be active in the next cycle of ubiquinol oxidation.

Our present kinetic results also convincingly demonstrate the existence of non-rate-limiting electron equilibration between cytochrome *b* subunits, which allows both b_H hemes to undergo reduction through only one active center P site (see Fig. 4) and permits the recycling of electrons coming from either or both center P sites through only one center N (see Fig. 6). We have already provided direct evidence for rapid electron transfer (8, 9) at the level of the b_L hemes, given the close distance between them as shown in crystallographic structures, which is optimal to sustain fast electron tunneling rates (8, 33). Therefore, our present work clearly demonstrates that the bc_1 complex does not function as independent monomeric units, as has been argued recently (15), and supports our previous conclusion (10) that a strong selective pressure has preserved the dimeric structure and function of this energy-transducing enzyme to regulate its activity in response to the redox poise of the substrate pool and minimize formation of reactive oxygen species.

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