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# The Carboxin-Binding Site on *Paracoccus denitrificans* Succinate:Quinone Reductase Identified by Mutations

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Succinate:quinone reductase catalyzes electron transfer from succinate to quinone in aerobic respiration. Carboxin is a specific inhibitor of this enzyme from several different organisms. We have isolated mutant strains of the bacterium *Paracoccus denitrificans* that are resistant to carboxin due to mutations in the succinate:quinone reductase. The mutations identify two amino acid residues, His228 in SdhB and Asp89 in SdhD, that most likely constitute part of a carboxin-binding site. This site is in the same region of the enzyme as the proposed active site for ubiquinone reduction. From the combined mutant data and structural information derived from *Escherichia coli* and *Wolinella succinogenes* quinol:fumarate reductase, we suggest that carboxin acts by blocking binding of ubiquinone to the active site. The block would be either by direct exclusion of ubiquinone from the active site or by occlusion of a pore that leads to the active site.

**KEY WORDS:** Succinate:quinone reductase; succinate dehydrogenase; carboxin; TTFA; ubiquinone; *Paracoccus denitrificans*.

## INTRODUCTION

Succinate:quinone reductase (SQR; EC 1.3.5.1) is a membrane-bound enzyme that catalyzes electron transfer from succinate to quinone in aerobic respiration (for a review see Hägerhäll, 1997). SQR is composed of a membrane-peripheral domain and a membrane-anchor domain. Succinate is oxidized on the peripheral domain. This domain is attached to the electronegative side of the respiratory membrane and consists of two polypeptides (SdhA and SdhB), one covalently bound FAD group, and three iron–sulfur clusters, one each of [2Fe–2S], [4Fe–4S], and [3Fe–4S] type. The SdhB polypeptide contains three cysteinyl-rich segments, which serve as ligands for the three iron–sulfur clusters. The membrane anchor is required for quinone reduction and is composed of one or two polypeptides and one or two *b*-type hemes. The exact composition of the anchor is correlated to the type of quinone that the organism uses in aerobic respiration. For example, the anchor of succinate:ubiquinone reductases generally consists of two polypeptides (SdhC and SdhD) and one *b*-type heme. Electron transfer from succinate to quinone occurs in a linear pathway, including FAD, [2Fe–2S], [4Fe–4S], and [3Fe–4S] as electron carriers in the given order (Iverson *et al*., 1999; Lancaster *et al*., 1999). Heme seems not absolutely required for reduction of ubiquinone as indicated by mutant data with *Escherichia coli* SQR (Vibat *et al*., 1998). Despite considerable recent progress in the understanding of the structure of SQR (Ackrell, 2000; Lancaster and Kröger, 2000; Ohnishi *et al*., 2000), little is still known about how ubiquinone is bound to the enzyme and how it is reduced.

5,6-Dihydro-2-methyl-1,4-oxathin-3-carboxanilide (carboxin) specifically inhibits SQR from several different organisms, including mammals, plants, fungi, yeast, and the bacterium *Paracoccus denitrificans* (Ackrell *et al*., 1992 and references therein). Carboxin sensitivity is only found among those SQR that use ubiquinone as electron acceptor. The inhibitor interferes with electron transfer from the [3Fe–4S] cluster to quinone (Ackrell *et al*., 1977). Photolabeling experiments with intact SQR complex have shown that carboxin binds to the membrane-anchor domain (Ramsay *et al*., 1981), but the

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binding site has not been identified. The inhibitor does not bind to isolated membrane anchor, suggesting that some part of the membrane-peripheral domain of SQR contributes to the carboxin-binding site.

There is no X-ray crystal structure available for any SQR. However, the crystal structures of quinol:fumarate reductase (QFR) from *Wolinella succinogenes* (Lancaster *et al*., 1999) and *E. coli* (Iverson *et al*., 1999) were recently reported at 2.2 and 3.3 Å resolution, respectively. SQR and QFR are closely related enzymes, being very similar in structure and composition (Hägerhäll, 1997). *In vitro*, they can catalyze the same reactions but are optimized to *in vivo* catalyze the reverse reactions. As shown by the two QFR structures, the SdhA polypeptide is not in contact with the membrane anchor and is, therefore, not expected to take part in carboxin binding.

4,4,4-Trifluoro-1-(2-thienyl)-butane-1,3-dione (TTFA) is another inhibitor of SQR activity. Enzymes that are sensitive to carboxin are usually also sensitive to TTFA, but generally carboxin is the most potent inhibitor. TTFA is known to compete with carboxin for binding to SQR (Coles *et al*., 1978; Ramsay *et al*., 1981; Grivennikova and Vinogradov, 1985) indicating that these two inhibitors have overlapping binding sites.

With the objective to determine the mechanism of action of carboxin and increase our understanding of ubiquinone binding to SQR, we have isolated and analyzed *P. denitrificans* mutants resistant to carboxin. The respective mutations define a carboxin-binding site located close to the [3Fe–4S] cluster at the interface between the membrane-peripheral domain and the membrane-anchor domain of SQR.

## EXPERIMENTAL PROCEDURES

### Carboxin-Resistant Mutants

*Paracoccus denitrificans* strains Cbx2004, Cbx2005, and Cbx2006, isolated in this work, and KTA4, isolated previously (Matsson *et al*., 1998), are carboxin-resistant mutants. The Cbx strains are derived from PD1222 ($Rif^r$, $Spc^r$) (de Vries *et al*., 1989), whereas the KTA4 strain is derived from ATCC 13543.

### Dot Blot Hybridization

The *sdhCD* gene fragment (nucleotide position 1594-3011; GenBank accession no. U31902) from different *P. denitrificans* strains was amplified directly from dilute bacterial cell suspensions using a standard PCR with custom made primers. The PCR fragments were screened for mutations using dot blot hybridization with Hybond-N membranes (Amersham Pharmacia Biotech, Sweden) and two different $^{32}$P-end-labeled DNA probes. One probe (5′-GGAAATAG**C**CGTCGATC-3′) was KTA4 specific, i.e., it was complementary to the region of *sdhD* in KTA4 that contains a mutation conferring resistance to carboxin. The other probe (5′-GGAAATAG**T**CGTCGATC-3′) was complementary to the wild-type sequence of the same region of *sdhD*. High-stringency washing of the membranes, optimized by variation in time and temperature, was used to remove all probe molecules but those binding with a perfect match.

### Cloning of *sdh* Genes

The *sdhCD* genes of strains PD1222, Cbx2004, and Cbx2005, amplified by PCR, were cloned by cutting the DNA fragments with *Apa*I and *Pvu*II and then inserting the resulting 1.2-kbp fragments into *Apa*I and *Hinc*II cut pBluescript II SK(-) (Stratagene). The entire *sdhCDAB* operon from PD1222, Cbx2004, and Cbx2005, respectively, was cloned as a 10-kbp *Eco*RI fragment in pBluescript II KS(-) (Stratagene) as described earlier (Matsson *et al*., 1998). The resulting plasmids were used in DNA sequence analyses of *sdh* genes. Plasmids were propagated in *E. coli* SURE (Stratagene).

### Expression of Cloned *sdh* Genes in *P. denitrificans*

The *sdhCDAB* operon from different strains was expressed in *P. denitrificans* strain PD1222 by cloning in the broad host-range vector pEG400 ($Sm^r$, $Spc^r$) (Gerhus *et al*., 1990). The *sdhCDAB* gene clusters first cloned in pBluescript were transferred as *Eco*RI fragments to pEG400 and the resulting plasmids were introduced into PD1222 by conjugation with *E. coli* strain SM10 (Simon *et al*., 1983) as follows. Overnight cultures of PD1222 in LB and SM10 (containing a pEG400 derivative) in LB supplemented with 25 $\mu$g/ml streptomycin were diluted ten-fold in antibiotic-free medium and grown for 2 h at 30°C, 200 rpm. The PD1222 culture (400 $\mu$l) was mixed with the SM10 culture (100 $\mu$l) and cells were then pelleted by centrifugation and resuspended in LB (100 $\mu$l). The cell mixture was spotted onto a LB agar plate, which was incubated at 30°C for 6 h to allow conjugation. The cells on the plate were then suspended in LB and transformants were selected on LB agar plates, containing 100 $\mu$g/ml rifampicin and 40 $\mu$g/ml streptomycin.

### Enzyme Activity Measurements

Membranes were isolated from *P. denitrificans* cells grown in LB supplemented with 50 mM succinate

(Matsson *et al*., 1998). Protein was determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard. Succinate:phenazine methosulfate (PMS) reductase activity and succinate:quinone reductase activity were measured essentially as described before (Matsson *et al*., 1998). Decylubiquinone (Sigma Chemical Co., St. Louis, MO, USA) was used in the succinate:quinone reductase assay.

## RESULTS AND DISCUSSION

### Isolation of Carboxin-Resistant Mutants

SQR is important for aerobic growth of *P. denitrificans* and 3′-methyl carboxin specifically inhibits this enzyme *in vivo* (Matsson *et al*., 1998). This provides the possibility to select for mutant clones with carboxin-resistant SQR. In a previous study, such mutants were isolated after chemical mutagenesis using *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (Matsson *et al*., 1998). The mutation responsible for carboxin resistance was found to be identical in the four independent isolates that were analyzed. It is an A-to-G mutation in *sdhD* resulting in the replacement of Asp89 for Gly in the SdhD anchor polypeptide. Strain KTA4 contains this mutation.

In this work, we aimed to find novel types of mutations that confer carboxin resistance. Therefore, a different approach was used to isolate carboxin-resistant mutants. PD1222 cells, not treated with any mutagen, were spread on LB agar plates containing 200 $\mu$M 3′-methyl carboxin. Colonies appeared after several days of incubation at 30°C. Eight carboxin-resistant clones were isolated from single colonies. Isolated membranes from all clones showed carboxin-resistant succinate:quinone reductase activity indicating that the resistance is caused by mutations in the *sdhCDAB* operon.

### Mapping of Mutations

Carboxin resistance is presumably caused by impaired binding of the inhibitor to SQR and amino acid replacements that confer resistance are expected to be located at the binding site for the inhibitor. As mentioned in the introduction, this site does not involve the SdhA polypeptide. Mutations conferring resistance to carboxin were, therefore, sought in the *sdhB*, *sdhC*, and *sdhD* genes.

The carboxin-resistant clones were first screened to determine if they carried the same mutation as KTA4. The *sdhCD* genes from PD1222, KTA4, and the eight new carboxin-resistant clones were amplified by PCR and the DNA fragments were analyzed in dot blot hybridizations using an oligonucleotide specific for the KTA4 mutation as probe. The *sdhCD* fragment from one of the carboxin-resistant clones, Cbx2006, showed the same high binding affinity for the probe as the fragment from KTA4. As a control experiment, the hybridization analysis was also done using a wild-type specific probe complementary to the same region of *sdhD* as the KTA4 specific probe. The *sdhCD* fragments from all the mutants except KTA4 and Cbx2006 hybridized well with the wild-type specific probe. This demonstrated that seven of the isolated carboxin-resistant clones contain novel mutations.

Two clones, Cbx2004 and Cbx2005, were selected for further characterization. The *sdhCD* region from PD1222, Cbx2004, and Cbx2005, respectively, was cloned in pBluescript in *E. coli* SURE and the DNA sequences of the entire cloned fragments were determined. The *sdhC* and *sdhD* genes of both Cbx2004 and Cbx2005 were found to be identical to those in PD1222, i.e., as wild-type. From these results, we expected strains Cbx2004 and Cbx2005 to be mutated in *sdhB*.

### Identification of SdhB Mutations

The *sdhCDAB* operon from PD1222, Cbx2004, and Cbx2005, respectively, was cloned in pBluescript in *E. coli* SURE and the DNA sequence of the entire *sdhB* gene was determined. In *sdhB* of Cbx2004 a transversion was found in the codon for His228 (CAC) changing it to a codon for Asn (AAC). The *sdhB* gene of Cbx2005 contained a transition in the His228 codon changing it to a codon for Tyr (TAC).

To confirm that the identified mutations in SdhB confer resistance to carboxin, the *sdhCDAB* operon from PD1222, Cbx2004, and Cbx2005, respectively, was inserted into the broad host-range vector pEG400. The resulting plasmids, pPSD1200, pPSD1204, and pPSD1205, respectively, were conjugated into PD1222. Cells containing plasmids with the *sdhCDAB* operon from Cbx2004 or Cbx2005 were resistant to carboxin, i.e., they grew on LB agar plates containing 200 $\mu$M 3′-methyl carboxin, whereas those carrying plasmids with the wild-type operon from PD1222 were sensitive.

### Properties of Mutant *P. denitrificans* SQR

Two different assays were used to study the effect of 3′-methyl carboxin on succinate oxidation by membrane-bound SQR. In the succinate:PMS reductase assay, electrons are transferred from succinate by way of SQR to the artificial electron acceptor PMS. It is not known exactly from where on the enzyme electrons are donated to PMS,

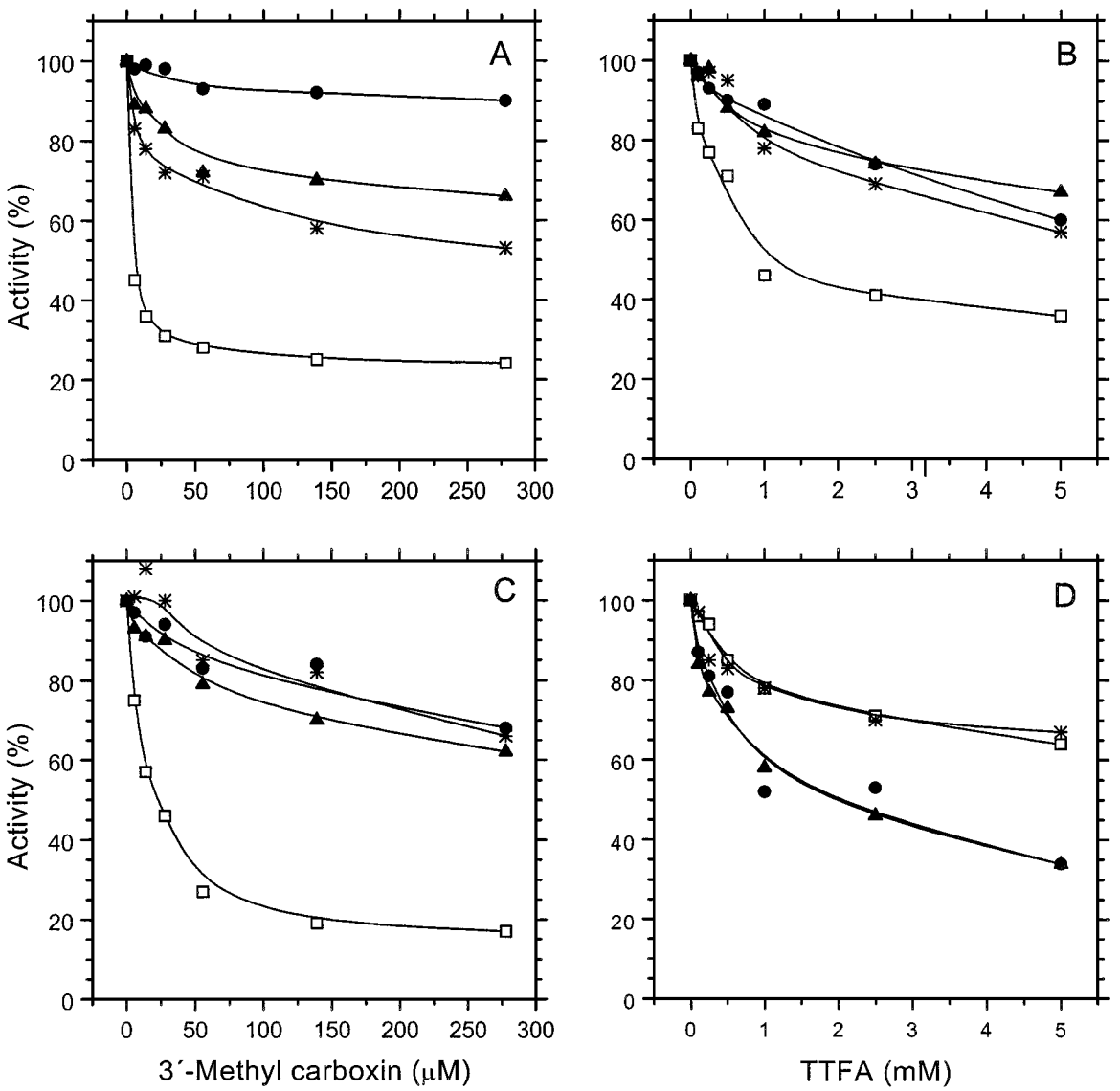


**Fig. 1.** Inhibition of enzyme activity by carboxin and TTFA. Effects of 3′-methyl carboxin (panels A and C) and TTFA (panels B and D) on enzyme activities in isolated membranes from *P. denitrificans* strains PD1222 (□, wild type), KTA4 (●, SdhD–D89G), Cbx2004 (∗, SdhB–H228N), and Cbx2005 (▲, SdhB–H228Y). Succinate:PMS reductase (panels A and B) and succinate:quinone reductase (panels C and D) activities were measured using 1 mM PMS and 25 $\mu$M decylubiquinone, respectively, as primary electron acceptors. The 100% activity ($\mu$mol succinate oxidized per minute and mg protein) in membranes from PD1222, KTA4, Cbx2004, and Cbx2005 was 1.1, 0.7, 0.7, and 0.6, respectively, in the succinate:PMS reductase assay and 0.6, 0.2, 0.4, and 0.2, respectively, in the succinate:quinone reductase assay.

but it probably occurs at several different sites. In the succinate:quinone reductase assay electrons are transferred from succinate to decylubiquinone, which is a ubiquinone analog. Decylubiquinone is expected to accept electrons predominantly from the quinone reduction site(s) on SQR.

All three variants of membrane-bound mutant *P. denitrificans* SQR (SdhD-D89G, SdhB-H228N, and SdhB-H228Y) were, *in vitro*, less sensitive to 3′-methyl carboxin than the wild-type enzyme. This was found using both the succinate:PMS (Fig. 1, panel A) and the succinate:quinone (Fig. 1, panel C) reductase assays. The fact that the mutant enzymes were inhibited by high concentrations of carboxin shows that they can bind the inhibitor, but the binding affinity is decreased compared to wild type.

Since carboxin and TTFA are thought to bind to the same or overlapping sites on SQR, the effect of the

mutations on TTFA sensitivity was also analyzed. The succinate:quinone reductase activity of the mutant enzymes showed the same (SdhB-H228N) or increased (SdhB-H228Y and SdhD-D89G) sensitivity compared to wild type (Fig. 1, panel D). In contrast, the succinate:PMS reductase activity of all three variants of mutant enzymes showed resistance to TTFA (Fig. 1, panel B). We have no explanation for this difference in the observed effect of TTFA, depending on the assay, but the phenomenon is important to consider when results from inhibitor studies are compared. The fact that the carboxin-resistant mutants are not cross resistant to TTFA in the succinate:quinone reductase assay shows that the binding sites for carboxin and TTFA are not identical.

### Summary of Mutations Known to Confer Resistance to Carboxin

The most comprehensive set of identified mutations conferring resistance to carboxin is now available for *P. denitrificans*. Mutations have been found in both the SdhB and SdhD polypeptide of SQR. SdhB mutations conferring resistance to carboxin have been found also in the basidiomycete *Ustilago maydis* (Keon *et al*., 1991; Broomfield and Hargreaves, 1992) and the ascomycete *Mycosphaerella graminicola* (Skinner *et al*., 1998). The SdhB mutations are compiled in Fig. 2. All are substitutions of a His residue that is invariant in succinate:ubiquinone reductases and is positioned next to one of the cysteinyl ligands to the [3Fe–4S] cluster (Hägerhäll, 1997). The only known SdhD mutation conferring resistance to carboxin is the Asp89 to Gly substitution in *P. denitrificans*. Asp89 is invariant in succinate:ubiquinone reductases and is predicted to be located in a cytoplasmic loop connecting transmembrane segments number V and VI (Hägerhäll and Hederstedt, 1996).

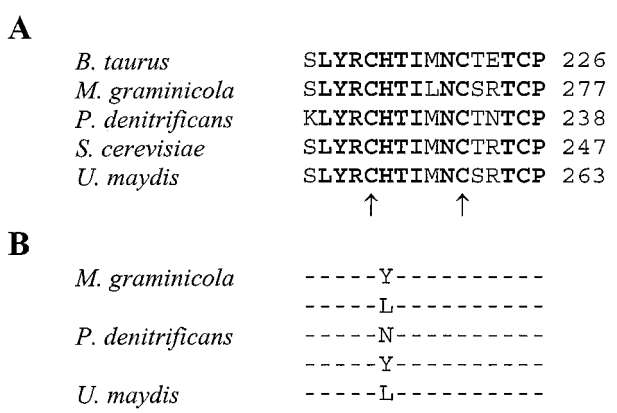


**Fig. 2.** SdhB mutations conferring resistance to carboxin. (A) Alignment of the third cysteinyl-rich segment in SdhB from *Bos taurus*, *Mycosphaerella graminicola*, *Paracoccus denitrificans*, *Saccharomyces cerevisiae*, and *Ustilago maydis*, which are known to have a carboxin-sensitive SQR. The first two conserved cysteine residues (indicated by arrows) are ligands to the [3Fe–4S] cluster whereas the third conserved cysteine residue is a ligand to the [4Fe–4S] cluster. (B) Summary of SdhB mutations known to confer resistance to carboxin in *M. graminicola* (Skinner *et al*., 1998), *P. denitrificans* (this work), and *U. maydis* (Keon *et al*., 1991; Broomfield and Hargreaves, 1992).

The indicated invariant His residue in SdhB and the Asp residue in SdhD are both most likely involved, directly or indirectly, in binding of carboxin. Additional residues probably contribute to carboxin binding but they might not be identified using a mutant approach with *in vivo* selection for carboxin resistant SQR. This is because the selection procedure relies on a functional SQR and it will not detect carboxin-binding residues if they are also required for assembly of the enzyme or enzyme activity.

### Which Is the Mechanism of Enzyme Inhibition by Carboxin?

3′-Methyl carboxin inhibits *P. denitrificans* SQR in a partly competitive manner with respect to quinone (Matsson *et al*., 1998). With bovine heart SQR, carboxin is known to quench an enzyme-associated semiquinone radical signal detectable by EPR (Grigolava *et al*., 1982). These findings combined suggest that ubiquinone and carboxin bind to the same site, or separate but somehow overlapping sites, on the enzyme. Carboxin and ubiquinone show some structural similarity, but are not obvious structural analogs. It is noteworthy that carboxin specifically inhibits succinate:ubiquinone reductases, whereas other enzymes that reduce ubiquinone, such as the NADH:quinone reductases, are resistant. Probably SQR contains ubiquinone-binding sites of a rather general structure, i.e., has binding sites similar to those found in other enzymes that use ubiquinone as a substrate. If so, the high specificity of carboxin binding to SQR indicates that different structural elements are responsible for tight binding of carboxin and ubiquinone, respectively. This view is supported by the fact that carboxin-resistant mutants with apparent close to normal affinity for quinone can be isolated (Matsson *et al*., 1998).

The crystal structure of *E. coli* QFR seemingly contains two menaquinone molecules bound to the membrane anchor; one proximal quinone ($Q_P$) located close to the [3Fe–4S] cluster and one distal quinone ($Q_D$) located toward the periplasmic side of the membrane (Iverson *et al*., 1999) (Fig. 3A). The distance between $Q_P$ and $Q_D$ is ~27 Å. The *W. succinogenes* QFR crystal structure contains no bound quinone molecules (Lancaster *et al*., 1999). Results from photoaffinity labeling and mutant analysis

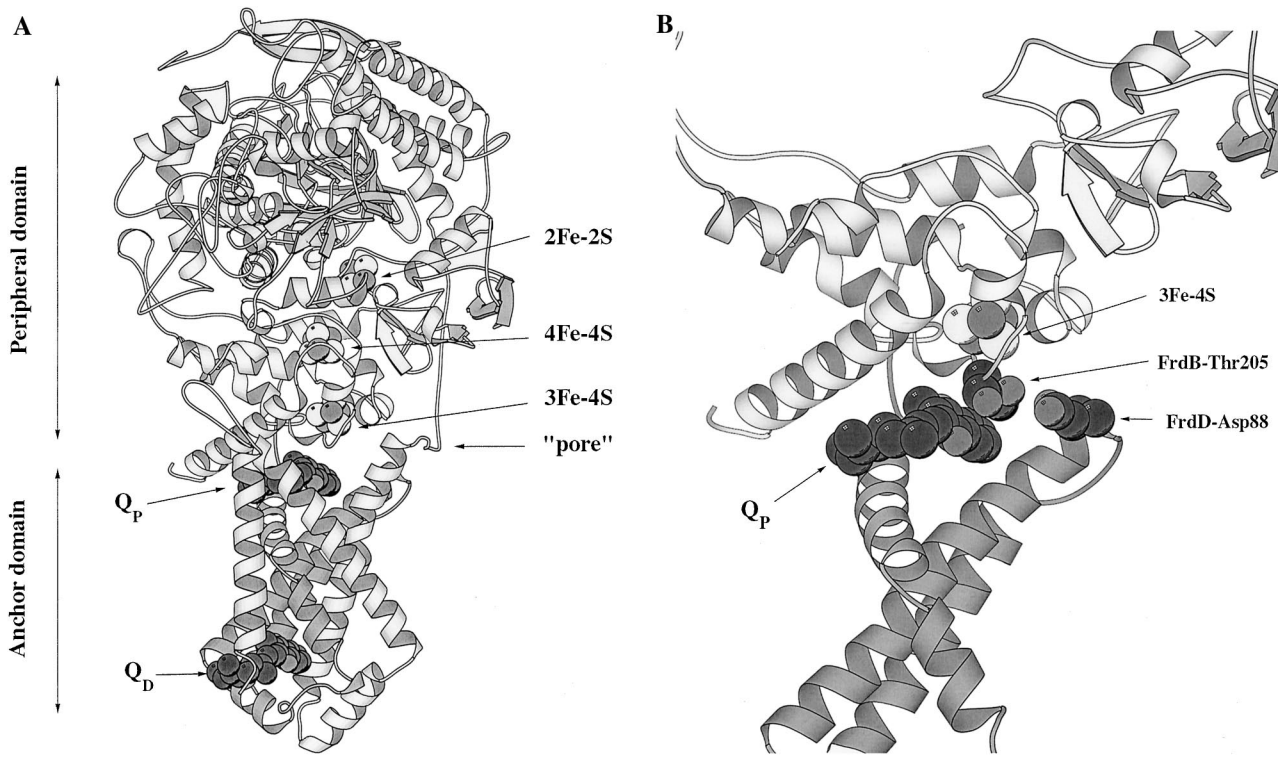


**Fig. 3.** Structure of *Escherichia coli* quinol:fumarate reductase. (A) Crystal structure of *E. coli* QFR in ribbon presentation (PDB entry 1FUM). The three iron–sulfur clusters of the FrdB subunit of the membrane-peripheral domain and two bound menaquinone molecules ($Q_P$ and $Q_D$) of the membrane-anchor domain are shown as spacefill structures. (B) Close up view of the region containing the [3Fe–4S] cluster and the $Q_P$ site, with one bound menaquinone molecule. The side chains of residues FrdB–Thr205 and FrdD–Asp88, which are predicted to correspond to SdhB–His228 and SdhD–Asp89 in *P. denitrificans* SQR, are highlighted. Only the FrdB (light grey) and the FrdD (dark grey) polypeptides are shown to clearly visualize the details that are discussed in this work.

using, e.g., bovine heart SQR (Shenoy *et al*., 1999 and references therein) indicate a $Q_P$ and a $Q_D$ site also in succinate:ubiquinone reductases. A major difference between *E. coli* QFR and succinate:ubiquinone reductases is that the former enzyme lacks heme, whereas the latter enzymes contain one heme molecule in the anchor domain. This heme is most likely positioned close to the [3Fe–4S] cluster, as judged from the *W. succinogenes* QFR structure, which contains heme in the membrane anchor. The [3Fe–4S] cluster is the immediate electron donor to quinone and the $Q_P$ site is probably the functional quinone reduction site in succinate:ubiquinone reductases (Schirawski and Unden, 1998). The $Q_P$ site is also thought to be the active site (quinol reduction site) of *E. coli* QFR, whereas the active site of *W. succinogenes* QFR is probably the $Q_D$ site (Lancaster and Kröger, 2000; Ohnishi *et al*., 2000).

We have used the *E. coli* QFR crystal structure as a framework to interpret data obtained with mutant *P. denitrificans* SQR. We assume that *E. coli* QFR and *P. denitrificans* SQR are similar in structure, despite the fact that the former enzyme lacks heme and binds menaquinone instead of ubiquinone. The structure of the iron–sulfur protein subunit is probably very similar in all SQRs and QFRs because the amino acid sequence of the SdhB/FrdB polypeptide is well conserved and the ligation of three iron–sulfur clusters within the relatively small polypeptide allows little structural variation. Residue SdhB–His228 of *P. denitrificans* SQR clearly corresponds to FrdB–Thr205 of *E. coli* QFR and these residues most likely have a nearly identical position in the two enzymes, because the adjacent cysteine residue is a ligand to the [3Fe–4S] cluster (Fig. 2). The membrane anchor polypeptides of SQR and QFR are poorly conserved, but common sequence patterns allow reasonable alignments (Hägerhäll and Hederstedt, 1996). SdhD–Asp89 of *P. denitrificans* SQR corresponds to FrdD–Asp88 of *E. coli* QFR, which is located in a turn connecting transmembrane helices V and VI. The side chains of FrdB–Thr205 and FrdD–Asp88 in *E. coli* QFR are close in space and

point into a "pore" that leads from the $Q_P$ site to the surface of the enzyme (Fig. 3A, B). Quinone probably enters the enzyme via this "pore." Our results with the carboxin-resistant mutants (Matsson *et al.*, 1998 and this work) suggest that the side chains of SdhB–His228 and SdhD–Asp89 in *P. denitrificans* SQR are important for tight binding of carboxin, but of little role for binding of ubiquinone. Based on the structure of *E. coli* QFR, the two residues are probably positioned close to each other and might directly bind carboxin. Notably, also in *W. succinogenes* QFR, which contains heme, the two residues (FrdB–Met209 and FrdC–Lys193) corresponding to SdhB–His228 and SdhD–Asp89 in *P. denitrificans* SQR are close in space (PDB entry 1QLA).

From the combined information available on carboxin-resistant mutants and the QFR structural data, we propose two alternative, although similar, models to explain how carboxin acts as a specific inhibitor of SQR. Both models are compatible with the assumption that residues SdhB–His228 and SdhD–Asp89 are involved in binding carboxin, but are not important for tight binding of ubiquinone. In one model, bound carboxin excludes the binding of ubiquinone to the active site (the $Q_P$ site) where quinone is being reduced by electrons from the [3Fe–4S] cluster. In this model, the carboxin-binding site overlaps the active site. In the alternative model, carboxin competes with ubiquinone for binding to low-affinity ubiquinone binding sites located in a "pore" leading up to the active site. These low-affinity sites would be transient prebinding sites that guide ubiquinone into the active site. In this model the effect of carboxin would not be at the active site, but by blocking a "pore" leading up to this site.

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