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# Exploring the inhibitor binding pocket of respiratory complex I

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ABSTRACT

Numerous hydrophobic and amphipathic compounds including several detergents are known to inhibit the ubiquinone reductase reaction of respiratory chain complex I (proton pumping NADH:ubiquinone oxidoreductase). Guided by the X-ray structure of the peripheral arm of complex I from *Thermus thermophilus* we have generated a large collection of site-directed mutants in the yeast *Yarrowia lipolytica* targeting the proposed ubiquinone and inhibitor binding pocket of this huge multiprotein complex at the interface of the 49-kDa and PSST subunits. We could identify a number of residues where mutations changed $I_{50}$ values for representatives from all three groups of hydrophobic inhibitors. Many mutations around the domain of the 49-kDa subunit that is homologous to the [NiFe] centre binding region of hydrogenase conferred resistance to DQA (class I/type A) and rotenone (class II/type B) indicating a wider overlap of the binding sites for these two types of inhibitors. In contrast, a region near iron–sulfur cluster N2, where the binding of the *n*-alkyl-polyoxyethylene-ether detergent $C_{12}E_8$ (type C) was exclusively affected, appeared comparably well separated. Taken together, our data provide structure-based support for the presence of distinct but overlapping binding sites for hydrophobic inhibitors possibly extending into the ubiquinone reduction site of mitochondrial complex I.



## 1. Introduction

Respiratory chain complex I is the largest enzyme complex of the inner mitochondrial or bacterial plasma membrane. It couples the transfer of two electrons from NADH to ubiquinone to the translocation of four protons across the bioenergetic membrane [1]. In the strictly aerobic yeast *Yarrowia lipolytica* complex I is composed of at least 40 different subunits with a total molecular mass of 947 kDa [22]. Electron microscopic single particle analysis of complex I from *Y. lipolytica* [2] revealed that like complex I from other organisms, the enzyme is L-shaped with a hydrophobic membrane embedded arm and a hydrophilic peripheral arm extending into the mitochondrial matrix.

A large number of hydrophobic and amphipathic compounds, including natural products, synthetic compounds and even some commonly used detergents like Triton X-100 and *n*-alkyl-polyoxyethylene-ethers are known to inhibit the ubiquinone reductase reaction of complex I [3–7]. Several classification schemes have been proposed for this plethora of inhibitors that were mostly based on their behaviour in steady-state inhibition kinetics. In a study using bovine heart SMP and ubiquinone-2 as a substrate Friedrich et al. proposed two inhibitor classes that differed in enzyme specificity and mode of action [8]: class I inhibitors, exemplified by the natural compound piericidin A inhibit complex I in a partially competitive manner and are also competitive inhibitors of glucose:ubiquinone oxidoreductase from *Gluconobacter oxidans*. Class II inhibitors, exemplified by the natural compound rotenone, act on complex I in a non-competitive manner and are ineffective on bacterial glucose:ubiquinone oxidoreductase.

Another somewhat more hypothetical classification by Degli Esposti that was based on an extensive literature survey postulates three functionally different types of complex I inhibitors with type A inhibitors acting as antagonists of the substrate ubiquinone, type B inhibitors acting to displace the intermediate ubisemiquinone and type C inhibitors acting as antagonists of the product ubiquinol [9]. Type A and type B inhibitors in most cases correspond to class I and class II inhibitors, respectively. Type C inhibitors, exemplified by the capsaicins and their synthetic analogues like CC44 [10], form a third distinct class. The *n*-alkyl-polyoxyethylene-ether detergent Thesit ($C_{12}E_9$) was shown to compete with the capsaicinoid CC44 and was thus classified as a type C inhibitor [7]. In contrast to capsaicin and its derivatives, polyoxyethylene-ether detergents also inhibit complex I from *Y. lipolytica*.

Initially, it had been suggested that the various types or classes of complex I inhibitors would bind to at least two discrete domains [8,9]. However it was demonstrated by direct competition binding assays that representatives from all inhibitor classes bind to partially

*Abbreviations:* CC44, *N*-methyl-*N*-(3,4-dimethoxybenzyl)-4-(*p*-tert-butylphenoxy) benzamide; DBQ, *n*-decylubiquinone; dNADH, deamino-NADH (reduced form); DQA, 2-decyl-4-quinazolinylamine; HAR, hexaammineruthenium(III)-chloride; Mops, 3-(*N*-morpholino) propanesulphonic acid; PMSF, phenylmethylsulfonyl fluoride; SMP, submitochondrial particles; Tris, Tris(hydroxymethyl)aminomethane

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**Table 1**
Effects of point mutations introduced into the 49-kDa subunit

| Strain | Complex I content[a] | Complex I activity[b] | Apparent $K_m$ for DBQ | $I_{50}$ | | |
|---|---|---|---|---|---|---|
| | | | | $C_{12}E_8$ | DQA | Rotenone |
| | % | % | μM | μM | nM | |
| Parental | 100±3[e] | 100±5[e] | 15[e] | 3.3 | 16[e] | 530[e] |
| R141A | 130[c] | 17[c] | 10[f] | n.d. | 21[c] | 570[c] |
| R141K | 140[d] | 45[d] | 13[d] | 3.4 | 55[d] | 1500[d] |
| D143A | 118±3 | 30±3 | 18 | 2.8 | 180 | 10,000 |
| D143C | 97±2 | 73±4 | 13 | 2.7 | 30[c] | 580[c] |
| D143E | 112±4 | 69±6 | 14 | 3.1 | 38[c] | 630[c] |
| D143N | 91±3 | 42±5 | 15 | 2.5 | 60 | 5000 |
| S146C | 100[g] | 100[g] | 12[g] | 3.4 | 80[g] | 1500[g] |
| S146V | 94±4 | 94±6 | 16 | 3.2 | 29 | 1100 |
| M188C | 98±3 | 104±6 | 18 | 3.0 | 28 | 2500 |
| M188L | 102±2 | 120±5 | 23 | 2.9 | 16 | 780 |
| F203E | 97±3 | 28±3 | 14 | 2.8 | 70 | 2300 |
| F203W | 103±3 | 98±6 | 15 | 0.8 | 14 | 450 |
| F207H | 117±4 | 92±8 | 15 | 5.0 | 45 | 3000 |
| F207W | 99±2[e] | 58±2[e] | 12[e] | 2.6 | 21[e] | 850[e] |
| R224D | 97±2[e] | 107±4[e] | 13[e] | 2.3 | 12[e] | 550[e] |
| R224I | 93±3[e] | 102±5[e] | 14[e] | 2.0 | 12[e] | 650[e] |
| R224K | 86±2[e] | 103±3[e] | 10[e] | 2.5 | 11[e] | 550[e] |
| R224N | 124±2[e] | 99±7[e] | 14[e] | 2.4 | 15[e] | 700[e] |
| L225A | 102±3[e] | 103±6[e] | 13[e] | 2.5 | 13[e] | 550[e] |
| L225F | 95±3[e] | 110±6[e] | 13[e] | 2.0 | 11[e] | 460[e] |
| L225H | 84±3[e] | 107±5[e] | 12[e] | 2.6 | 14[e] | 700[e] |
| L225V | 95±2[e] | 91±3[e] | 12[e] | 2.3 | 12[e] | 700[e] |
| K407H | 80±2[e] | 78±2[e] | 13 | 3.4 | 13[e] | 500[e] |
| K407R | 93±3[e] | 105±4[e] | 12[e] | 3.1 | 75[e] | 550[e] |
| D458A | 91±2 | 48±3 | 12[f] | 3.3 | 520[c] | 5200[c] |
| D458E | 83±1 | 89±3 | 13 | 3.4 | 30 | 2200 |
| V460L | 83±4[e] | 21±2[e] | n.d. | n.d. | 130 | 9000 |
| V460M | 100±2[e] | 16±1[e] | 9[f] | n.d. | 53[c] | 760[c] |
| E463Q | 78±1 | 52±1 | 5[f] | 2.3 | 34[c] | 780[c] |
| E463T | 78±2 | 35±2 | 11 | 2.8 | 8 | 800 |

n.d., not determined.
[a] 100% of complex I content corresponds to 1.25 μmol $min^{-1}$ $mg^{-1}$ NADH:HAR oxidoreductase activity.
[b] Normalized for complex I content. Normalized NADH:DBQ oxidoreductase activity of the parental strain was 0.58 μmol $min^{-1}$ $mg^{-1}$.
[c] Data from [13].
[d] Data from [17].
[e] Data from [21].
[f] Data from Kashani-Poor (unpublished data).
[g] Data from Grgic (unpublished data).

overlapping sites in a single, large pocket [5]. This strongly suggested the presence of a single ubiquinone reduction site within complex I, thereby ruling out several hypothetical reaction schemes involving a reversal of the ubiquinone cycle of the cytochrome $bc_1$ complex [11,12].

Several lines of evidence have indicated that a significant part of the ubiquinone reducing catalytic core of complex I is made up of the 49-kDa and PSST subunits (the bovine heart complex I subunit nomenclature will be used throughout), which are evolutionary related to the large and small subunits of water-soluble [NiFe] hydrogenases, respectively [13]. A photoreactive derivative of the complex I inhibitor pyridaben led to specific labelling of the PSST subunit in membranes from *Paracoccus denitrificans* and *Thermus thermophilus* [14]. A genetic screen in *Rhodobacter capsulatus* for mutants showing resistance to the inhibitor piericidin A yielded a missense mutation leading to substitution V407M (equivalent to V460M in *Y. lipolytica*) in the 49-kDa subunit [15]. These observations have been corroborated in the following by the generation and characterization of a number of site-directed mutations, mainly in *R. capsulatus* [16] and in *Y. lipolytica* [13,17,18]. Significantly, exchanges of those residues that correspond to the hydrogenase [NiFe] cluster ligands (D143, S146, V460, E463, *Y. lipolytica* numbering) or of several nearby residues yielded resistance to rotenone, to DQA, or to both inhibitors. On the basis of these findings we proposed that the interface between the 49-kDa and PSST subunits has evolved from water-soluble [NiFe] hydrogenases to form a significant part of the ubiquinone binding pocket of complex I [19]. This includes a cavity in the 49-kDa subunit that corresponds to the [NiFe] site in the large subunit of the hydrogenase.

Recently, the structure of the peripheral arm of complex I from *T. thermophilus* has been solved by X-ray crystallography [20]. It confirmed that this part of the complex contains all known redox prosthetic groups, namely one FMN, non-covalently bound to the 51-kDa subunit, and a chain of bi- and tetranuclear iron–sulfur clusters that terminates in the ubiquinone reducing [$Fe_4S_4$] cluster N2 located in the PSST subunit at the interface with the 49-kDa subunit. Taking advantage of the X-ray structure of the peripheral arm of complex I from *T. thermophilus* [20] we have mapped the ubiquinone binding pocket in an extensive site-directed mutagenesis study and identified a likely entry path for the substrate [21]. Using the same approach, we have now focussed on the localization of the inhibitor binding sites. To this end, we further expanded our collection of mutations around iron–sulfur cluster N2 and analyzed the pattern of changes in affinity for the class I/type A inhibitor DQA, the class II/type B inhibitor rotenone and the type C inhibitor *n*-alkyl-polyoxyethylene-ether $C_{12}E_8$. Our results indicate that representatives from all three inhibitor classes bind to defined, but partially overlapping sites in a single, large pocket. This strongly supports our earlier view derived from inhibitor binding studies [5].

## 2. Materials and methods

Site-directed mutagenesis, small scale preparation of mitochondrial membranes, measurement of NADH:HAR and dNADH:DBQ catalytic activities and preparation of structural images were performed essentially as described in [21]. In brief, deletion strains of *Y. lipolytica* lacking the *NUCM* gene which encodes the 49-kDa subunit or lacking the *NUKM* gene which encodes the PSST subunit were complemented with plasmid-borne site-directed mutant copies of the respective genes under the control of their natural promoters. Strains complemented with unchanged gene copies are referred to as parental strains. The *n*-alkyl-polyoxyethylene-ether detergent $C_{12}E_8$ was purchased from Sigma-Aldrich GmbH, Munich and diluted as required for $I_{50}$ determination in 20 mM $Na^+$/Mops, pH 7.4, 50 mM NaCl, 2 mM KCN. Reactions were started by the addition of 70 μM DBQ to a mixture that had been pre-incubated for at least 30 s at 30 °C and contained 50 μg/ml mitochondrial membrane protein, 0.1 mM dNADH in 20 mM $Na^+$/Mops, pH 7.4, 50 mM NaCl, 2 mM KCN and variable concentrations of $C_{12}E_8$.

## 3. Results

For a more detailed structure/function analysis of the large ubiquinone reduction pocket around iron–sulfur cluster N2 of complex I, it was necessary to define the inhibitor binding domains by site-directed mutagenesis of the *NUCM* and *NUKM* genes, encoding the 49-kDa and PSST subunits of the peripheral arm of complex I. We here analyzed the $I_{50}$ values for the inhibitors DQA (class I/type A), rotenone (class II/type B) and $C_{12}E_8$ (type C) to cover all three major

**Table 2**
Effects of point mutations introduced into the PSST subunit

| Strain | Complex I content[a] | Complex I activity[b] | Apparent $K_m$ of DBQ | $I_{50}$ | | |
|---|---|---|---|---|---|---|
| | | | | $C_{12}E_8$ | DQA | Rotenone |
| | % | % | μM | μM | nM | |
| Parental | 100±2[c] | 100±3[c] | 14[c] | 3.0 | 13[c] | 450[c] |
| V88L | 94±4[c] | 92±5[c] | 15[c] | 1.8 | 25[c] | 500[c] |
| V88M | 95±2[c] | 56±3[c] | 13[c] | 1.5 | 32[c] | 330[c] |
| M91C | 114±2 | 31±4 | 11 | 1.3 | 500 | 15000 |
| M91K | 86±3 | 10±2 | n.d. | n.d. | n.d. | n.d. |

n.d., not determined.
[a] 100% of complex I content corresponds to 1.25 μmol $min^{-1}$ $mg^{-1}$ NADH:HAR oxidoreductase activity.
[b] Normalized for complex I content. Normalized NADH:DBQ oxidoreductase of the parental strain was 0.6 μmol $min^{-1}$ $mg^{-1}$.
[c] Data from [21].