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Exploring Interactions between the 49 kDa and ND1 Subunits in Mitochondrial NADH-Ubiquinone Oxidoreductase (Complex I) by Photoaffinity Labeling

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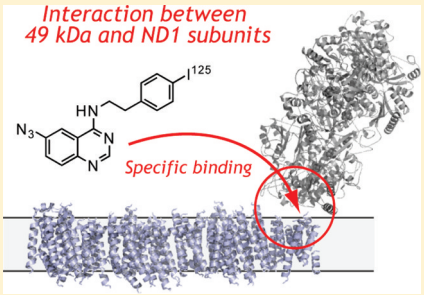
Supporting Information

ABSTRACT: Quinazolines are strong inhibitors of NADH-ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. Using a photoreactive quinazoline, [^{125}I]AzQ, and bovine heart submitochondrial particles (SMPs), we demonstrated previously that [^{125}I]AzQ binds at the interface of the 49 kDa and ND1 subunits in complex I; it labeled a site in the N-terminal (Asp41−Arg63) region of the 49 kDa subunit, suggesting that this region contacts the ND1 subunit [Murai, M., et al. (2009) *Biochemistry 48*, 688−698]. The labeled region of ND1 could not be identified because it is highly hydrophobic, and the SMPs did not yield sufficient amounts of labeled protein. Here, we describe how photoaffinity labeling of isolated complex I by [^{125}I]AzQ yielded sufficient material for identification of the labeled region of the ND1 subunit. The inhibition of the isolated enzyme by AzQ is comparable to that of SMPs. Our results reveal that the labeled site in ND1 is between Asp199 and Lys262, mostly likely in the third matrix loop that connects the fifth and sixth transmembrane helices. Thus, our results reveal new information about the interface between the hydrophilic and hydrophobic domains of complex I, a region that is thought to be important for ubiquinone reduction and energy transduction.



Proton-translocating NADH-quinone oxidoreductase (complex I) catalyzes the reduction of quinone by NADH, coupled to the generation of an electrochemical proton motive force across a membrane, to drive energy-consuming processes such as ATP synthesis.[1,2] Complex I is the largest of the multisubunit respiratory-chain enzymes. For instance, the enzyme from bovine heart mitochondria is composed of 45 different subunits with a combined mass of ∼1 MDa.[3] The prokaryotic enzyme is simpler, generally consisting of 14 core subunits that are conserved from bacteria to humans. The structure of the hydrophilic arm (peripheral arm) of complex I from *Thermus thermophilus* has been described at 3.3 Å resolution, revealing the core subunit arrangement and pathway for the transfer of electrons between the two substrate-binding sites.[4] Recently, electron density maps were presented for the intact complex I forms from *T. thermophilus*[5] and *Yarrowia lipolytica*,[6] at 4.5 and 6.3 Å resolution, respectively, and the arrangement of the transmembrane helices (TMHs) in the prokaryotic enzyme was described. However, information about the interface between the two arms, thought to constitute a large quinone/inhibitor binding pocket and to be critical for the function of the enzyme,[2,5,6] remains very limited because the structure in this region has not yet been resolved.

Detailed studies of the action mechanisms of specific inhibitors of complex I have provided important structural and functional insights about ubiquinone reduction and proton translocation.[1,2,7] In particular, photoaffinity labeling studies using photoreactive derivatives of complex I inhibitors have provided valuable insights into their binding sites.[8−13] The earlier studies indicated that the inhibitors bind to the interfacial region between the hydrophilic and membrane arms that is composed of the 49 kDa, PSST, and ND1 subunits. Extensive mutagenesis studies using *Y. lipolytica* complex I indicated that the reduction of ubiquinone by electrons from the terminal Fe−S cluster occurs in a large cleft formed by the 49 kDa and PSST subunits, though the specific binding sites for ubiquinone and different inhibitors may not be identical.[7,14] Thus, the interfacial region appears to be a "hot spot" for the binding of inhibitors and ubiquinone. We note that several photoaffinity labeling studies have suggested the presence of an additional binding site for quinone and inhibitors in the membrane arm besides the site in the interfacial region.[15−17] However, these suggestions are not supported by recent structural data,[5] and it is likely that the labeling methods employed were not fully specific.[7]

Quinazolines are strong inhibitors of complex I from bovine heart mitochondria.[18,19] Previously, we synthesized a photoreactive quinazoline, [^{125}I]AzQ [6-azido-4-(4-iodophenetylamino)quinazoline], and conducted photoaffinity labeling studies using bovine heart SMPs.[12] Our studies showed that [^{125}I]AzQ labeled both the 49 kDa (hydrophilic) and ND1 (hydrophobic) subunits, with a frequency of ∼4:1, and that the

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labeling of both subunits is completely blocked by other inhibitors such as rotenone and acetogenins. Thus, we proposed that [^{125}I]AzQ binds at the interface of the two subunits with high specificity. Furthermore, we localized the labeled site in the 49 kDa subunit to the 23 N-terminal amino acids between Asp41 and Arg63[12] and so proposed that this region is close to the ND1 subunit. Our proposal is consistent with structural data on complex I from *T. thermophilus*,[5] which suggested that the N-terminal β-sheet of Nqo4 (49 kDa, in the hydrophilic arm) is in the vicinity of the loops of Nqo8 (ND1, in the membrane arm). However, the loops of ND1 (Nqo8 in *T. thermophilus*) as well as most of the N-terminal section of the 49 kDa subunit (Nqo4), including the labeled region, have not yet been resolved structurally. It is conceivable that a loop from the ND1 subunit extends toward the hydrophilic arm and interacts with the 49 kDa subunit in a region of critical importance for the enzyme's function.[20,21]

Previously, identification of the cross-linked site in the ND1 subunit was unsuccessful because the protein is highly hydrophobic and yields only low sequence coverage (<15%) in peptide mass fingerprinting analyses,[12,22−25] and because an insufficient amount of labeled material could be recovered from the SMPs.[12] The use of isolated complex I is a promising alternative for overcoming the latter problem, but there are possible complications from the enzyme being detergent-solubilized, rather than membrane-bound (for example, decreased inhibitor sensitivity and/or changes in the labeling pattern). Here, we report photoaffinity labeling experiments on a well-characterized preparation of isolated bovine heart mitochondrial complex I.[26] Our results reveal that [^{125}I]AzQ binds to both the Asp41−Arg63 region of the 49 kDa subunit and the Asp199−Lys262 region of the ND1 subunit, indicating that they are both close to each other and that they both comprise the inhibitor-binding site. Thus, our study reveals important new information about the interface between the hydrophilic and membrane arms in complex I, a region in which dynamic interactions between the 49 kDa and ND1 subunits may be important for the energy transduction mechanism.

EXPERIMENTAL PROCEDURES

Materials. [^{125}I]AzQ was synthesized by the procedure reported previously.[12] Ubiquinone-1 (Q_1) was a kind gift from Eisai (Tokyo, Japan). Decyl-benzoquinone (DB), asolectin, L-α-phosphatidylcholine (PC, type XI-E from egg yolk), L-α-phosphatidylethanolamine (PE, type III from egg yolk), and cardiolipin (CL, from bovine heart) were from Sigma-Aldrich (St. Louis, MO). Protein standards (Precision Plus Protein standards and Kaleidoscope Polypeptide Standards) for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were from Bio-Rad (Hercules, CA). Other reagents were all of analytical grade.

Enzyme Assays. Complex I was purified from bovine heart mitochondria[26] and stored in 20 mM Tris-HCl (pH 7.5), 10% glycerol, 150 mM NaCl, and 0.03% *n*-dodecyl β-D-maltoside (DDM) at −80 °C. NADH-DB oxidoreductase activity was measured spectrometrically with a Shimadzu UV-3000 instrument (340 nm; ε = 6.2 mM^{-1} cm^{-1}). The reaction medium (2.5 mL) contained 20 mM Tris-HCl (pH 7.5), 0.4 mg/mL asolectin [from a 10 mg/mL stock solution in 2% (w/v) CHAPS], and 50 μM DB at 30 °C; the complex I concentration was 7.5 μg/mL, and the reaction was initiated with 50 μM NADH following a 4 min equilibration. NADH-Q_1 oxidoreductase activity was measured similarly.

Photoaffinity Labeling of Purified Complex I. Purified complex I (0.15 mg/mL, 50 μL) was incubated in a 1.5 mL Eppendorf tube with 6 nM [^{125}I]AzQ in a buffer containing 20 mM Tris-HCl (pH 7.5) and 0.4 mg/mL asolectin at room temperature for 10 min; 30 μM NADH and/or 30 μM DB was added for a further 10 min. Then, the mixture was irradiated with a long wavelength UV lamp (Black-lay model B-100A, UVP, Upland, CA) for 10 min on ice, positioned 10 cm from the light source. The reaction was quenched by addition of 17 μL of 4× Laemmli's sample buffer.

Separation of the Labeled Subunits by Electrophoresis. SDS−PAGE was performed using 12.5% Laemmli-type gels,[27] following incubation at 35 °C for 1 h in 4 volumes of Laemmli's sample buffer. Then, the gels were stained with Coomassie brilliant blue R-250 (CBB), dried, exposed to an imaging plate (BAS-MS2040, Fuji Film, Tokyo, Japan), and visualized with a Bio-Imaging Analyzer FLA-5100 instrument (Fuji Film). The radioactivity of each band was quantified from the digitalized data using Multi Gauge (Fuji Film) or directly from the dried gel slices using a γ-counting system (COBLA II, Packard).

Isolation and Proteolysis of the 49 kDa and ND1 Subunits. Partial digestion of the [^{125}I]AzQ-labeled 49 kDa subunit was conducted according to the method of Cleveland et al.[28] with some modifications from Omori et al.[29] The CBB-stained 49 kDa subunit separated as described above was excised from the gel. The excised slice was inserted into the loading well of a 15% Tris-EDTA SDS−PAGE mapping gel (90 mm × 80 mm × 1 mm), which had been preloaded with V8 protease (1 μg was loaded into each well and buried in the stacking gel by pre-electrophoresis). The V8 digestion was conducted at the boundary between the stacking gel and the separating gel for 30 min at room temperature; the peptides were visualized by CBB staining or by autoradiography.

For exhaustive digestion of the 49 kDa and ND1 subunits, the [^{125}I]AzQ-labeled subunits were isolated from excised gel slices by electroelution using D-Tube Dialyzers with an electroelution accessory (Merk4Biosciences, Darmstadt, Germany) in an elution buffer containing 10 mM Tris-HCl (pH 8.0) and 0.025% SDS (w/v). Typically, more than 90% of the radioactivity was recovered. The elution buffer was exchanged for the appropriate digestion buffer using an Amicon Ultra-0.5 apparatus (10 kDa cutoff, Millipore, Billerica, MA), and the isolated subunits were stored at −80 °C. Exhaustive protease digestion was performed overnight at 37 °C, as described previously,[12,13] using V8 protease, lysylendopeptidase (Lys-C), endoprotease (Asp-N), or trypsin in 50 mM ammonium bicarbonate buffer (containing 0.01% SDS), 50 mM Tris-HCl buffer (pH 8.5, containing 0.1% SDS), 50 mM sodium phosphate buffer (pH 8.5, containing 0.01% SDS), or 50 mM ammonium bicarbonate buffer (containing 0.01% SDS), respectively. The digests were analyzed by Tricine SDS−PAGE [16.5% total (acrylamide and bisacrylamide), containing 6% bisacrylamide[30]]. For comparison, theoretical digests of the 49 kDa (SwissProt entry P17694) and ND1 (SwissProt entry P03887) sequences were created using Peptide Mass (http://expasy.org/tools/peptide-mass.html).

N-Terminal Amino Acid Sequence Analysis. Protease digests of the 49 kDa and ND1 subunits were identified by

Edman degradation. They were transferred from the Tris-EDTA or Tricine gels described above to PVDF membranes (Immobilon-P^{SQ}, Millipore) in buffer containing 10 mM $NaHCO_3$, 3 mM Na_2CO_3, and 0.025% (w/v) SDS overnight at 35 V (100 mA) in a cold room. The blotted membranes were stained with 0.025% CBB in 40% methanol and destained with 50% methanol. Bands were excised, and their N-terminal amino acid residues were identified with a Procise 494 HT Protein Sequencing System (Applied Biosystems, Foster City, CA) at the APRO Life Science Institute, Inc. (Tokushima, Japan).

Peptide Mass Fingerprinting Analyses. Following SDS−PAGE and CBB staining as described above, the [^{125}I]AzQ-labeled bands were excised from the gel, destained with 50% acetonitrile and 25 mM NH_4HCO_3, and treated with dithiothreitol and iodoacetamide. The proteins were digested "in gel" with trypsin (Promega, Madison, WI) in a buffer containing 25 mM NH_4HCO_3 at 37 °C overnight. They were twice extracted from the gel using a solution containing 50% acetonitrile and 5% aqueous trifluoroacetic acid, and then the combined extracts were concentrated and desalted using a $ZipTip_{C18}$ (Millipore). Peptide mass fingerprinting was conducted using a Bruker Autoflex III Smartbeam instrument (MALDI-TOF MS system, Bruker Daltonics, Billerica, MA). The mass spectra were scanned in positive-ion mode from m/z 800 to 4000 [calibrated using Peptide Calibration Standard II (Bruker Daltonics)] with α-cyano-4-hydroxycinnamic acid as the matrix.[12] Obtained monoisotopic masses were compared against SwissProt (http://www.expasy.org/sprot/) using MASCOT (http://www.matrixscience.com), with a peptide mass tolerance of 100 ppm and a maximum missed cleavage of 1. The carbamidomethylation of cysteine residues and oxidation of methionine residues were set as variable modifications.

RESULTS

Characterization of the AzQ Sensitivity of Catalysis by Isolated Complex I. Sharpley et al.[26] described and characterized a preparation of complex I from bovine heart mitochondria that retains high inhibitor-sensitive NADH-DB oxidoreductase activity, provided that phospholipids are maintained during the preparation and present in the assay buffer. Taking into account these findings, we determined the effect of AzQ on the NADH-DB oxidoreductase activity of the isolated enzyme (using 7.5 μg of protein/mL = 7.6 nM complex I in 0.4 mg of asolectin/mL). The IC_{50} value (the concentration needed to halve the activity) of 14 ± 3 nM is higher than that determined using bovine SMPs (30 μg of protein/mL ≈ 2.5 nM complex I[31]) of 4.5 ± 0.6 nM, but direct comparison of the values determined using different experimental systems is confounded by differences in the volume of the hydrophobic phase and by tight binding effects: we conclude that the isolated enzyme maintains its high sensitivity to AzQ. Furthermore, it is well-known that a part of the NADH-exogenous quinone oxidoreductase activity of complex I is insensitive to specific inhibitors. The inhibitor-insensitive reaction occurs at the flavin site[32] and involves the production of reactive oxygen species; its extent depends on the alkyl chain of the quinone.[32,33] The AzQ-insensitive activity measured here for the isolated enzyme in excess AzQ (1 μM) was comparable to that observed in SMPs (~5%).

Photoaffinity Labeling of Isolated Complex I. Figure 1 shows that asolectin was crucial for the specific labeling of the isolated enzyme by [^{125}I]AzQ (6 nM, 0.15 mg of protein/mL). If asolectin was not included, then significant nonspecific labeling was observed; the asolectin concentration of 0.4 mg/mL was deemed most appropriate for further experiments. In 0.4 mg/mL asolectin, radioactivity was distributed predominantly into two bands, at ~45 and ~25 kDa, with relative intensities of 4 to 1. They were confirmed as the 49 kDa and ND1 subunits, respectively, by N-terminal sequence analysis, as described below. Radioactivity in the weaker bands observed between the 49 kDa and ND1 bands contributed less than 20% of the ND1 signal. Thus, although there are small variations in the specificity of labeling, we conclude that the labeling pattern, as well as the inhibitory effect of [^{125}I]AzQ, is similar in the isolated complex I and in SMPs.[12] Increasing concentrations of asolectin steadily suppressed the labeling of the 49 kDa and ND1 subunits. This is probably because an increase in the volume of the lipidic phase effectively decreases the concentration of the [^{125}I]AzQ reagent.

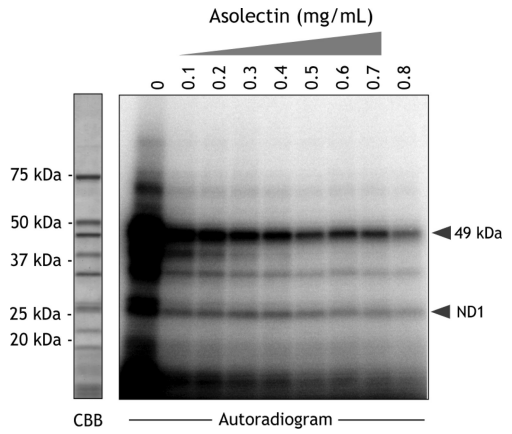

Figure 1. Effect of phospholipids on the labeling of complex I by [^{125}I]AzQ. Purified bovine complex I [0.15 mg of protein/mL in 10 mM Tris-HCl buffer (pH 7.6)] was labeled with [^{125}I]AzQ (6 nM) in the presence of different concentrations of asolectin. The labeling was quenched by the addition of 4× Laemmli's sample buffer, and SDS−PAGE (12.5% Laemmli-type gel, 2.8 μg/well) analysis was conducted. In 0.4 mg/mL asolectin, the amounts of radioactivity incorporated in the 49 kDa and ND1 bands were approximately 3500 and 1000 cpm, respectively. Data shown are representative of three independent experiments.

The efficiency of photochemical and chemical labeling of complex I is often enhanced by NADH.[15,34,35] Therefore, we examined the effects of NADH (30 μM) and/or ubiquinone (DB, 30 μM) on the labeling. The substrate(s) was added to a mixture of the enzyme and [^{125}I]AzQ and incubated for 10 min before UV irradiation (NADH and DB were completely consumed during the incubation when they were added together). There were no significant changes in the labeling pattern (Figure 2), although the specificity of the labeling appeared to be slightly improved in the presence of NADH and DB (Figure 2, left panel). However, the labeling suppression efficiency of another quinazoline-type inhibitor (6-aminoquinazoline) was considerably improved in the presence of NADH and DB (Figure 2, right). 6-Aminoquinazoline (present at ~300 times the [^{125}I]AzQ concentration) decreased the extent of labeling of the two subunits by more than 90% in the presence of NADH and DB, but in the absence of NADH and DB, 20−30% of the 49 kDa signal remained. The difference

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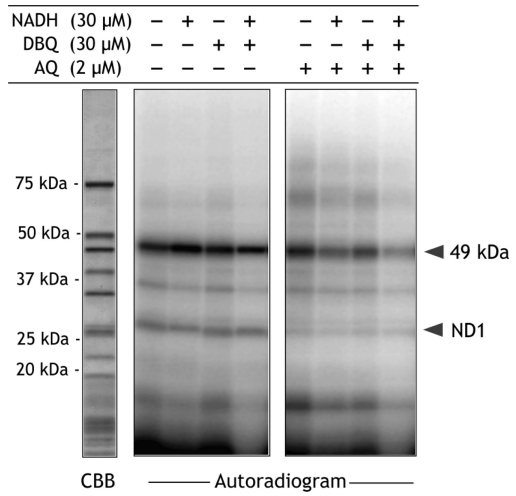

Figure 2. Effect of substrates on the specificity and suppression of the labeling of the 49 kDa and ND1 subunits. Purified bovine complex I (0.15 mg of protein/mL) was labeled with [^{125}I]AzQ (6 nM) in 0.4 mg/mL asolectin, in the presence of 30 μM NADH and/or 30 μM DB and/or an excess of 6-aminoquinazoline (2 μM), and then analyzed by SDS−PAGE (12.5% Laemmli-type gels, 2.8 μg/well). Data shown are representative of three independent experiments.

suggests that the enzyme adopts an active conformation upon addition of NADH and DB together. Finally, no significant difference in the labeling pattern was observed when phosphatidylcholine, phosphatidylethanolamine, or cardiolipin (major components of the mitochondrial inner membrane) were used instead of asolectin (data not shown).

Confirmation of the [^{125}I]AzQ Binding Site in the 49 kDa Subunit. Following the labeling of complex I with [^{125}I]AzQ, the 49 kDa subunit was isolated by SDS−PAGE, subjected to proteolysis by V8 protease, Lys-C, or Asp-N, and analyzed by electrophoresis (see Experimental Procedures). Partial digestion by V8 protease reproducibly produced ∼26 and ∼13 kDa radioactive fragments (Figure 3A). The N-terminal sequence for both bands was H_2N-T^{25}AHWK-, indicating that the labeled site resides in the region of Thr25−Glu143 [13.7 kDa (Figure 3C)]. When the subunit was exhaustively digested by V8 protease, a single radioactive band with an apparent molecular mass of ∼5 kDa was observed (Figure 3B). Analysis of the theoretical cleavage pattern of the 49 kDa subunit by V8 protease in the region of Thr25−Glu143 identified the Thr25−Glu67 peptide, with a calculated molecular mass of 4.8 kDa (Figure 3D).

Next, the 49 kDa protein was isolated by SDS−PAGE, excised and electroeluted from the gel, exhaustively digested with Lys-C or trypsin, and analyzed by electrophoresis. Dominant radioactive bands were observed at ∼4 and ∼3 kDa (Figure 3B). Careful examination of the predicted cleavage patterns for these proteases indicated that the Lys-C and tryptic fragments are the peptides Asp41−Lys75 (3.8 kDa) and Asp41−Arg63 (2.5 kDa), respectively. Therefore, as concluded in the previous work on SMPs,[12] the [^{125}I]AzQ-labeled site in the 49 kDa subunit is within the region of Asp41−Arg63 (summarized in Figure 3D).

Localization of the [^{125}I]AzQ Binding Site in the ND1 Subunit. The ND1 subunit, labeled with [^{125}I]AzQ, was isolated by SDS−PAGE, excision, and electroelution, subjected to proteolysis using Lys-C or Asp-N, and analyzed by electrophoresis. Digestion with Lys-C gave a single radioactive band, with an apparent molecular mass of ∼15 kDa, in Tricine gel analysis (Figure 4A, left). This fragment was detected by CBB staining on a PVDF membrane (Figure S1 of the Supporting Information), and we succeeded in determining a short stretch of the N-terminal sequence as H_2N-Y^{127}ALIG-. The apparent molecular mass and the expected Lys-C cleavage sites suggest that the fragment is Tyr127−Lys262 (15.2 kDa), which covers the fourth to seventh TMHs of ND1. Digestion by Asp-N also provided a single radioactive band, with an apparent mass of ∼10 kDa (Figure 4A, right). Unfortunately, the Asp-N digests were poorly stained with CBB, and analysis of the N-terminal sequence was unsuccessful. However, on the basis of the predicted cleavage sites, the Asp-N fragment is predicted to be Asp199−Tyr282 (9.7 kDa), covering the sixth to seventh transmembrane helices. Taken together, the data strongly suggest that the [^{125}I]AzQ-labeled site in the ND1 subunit is in the region of Asp199−Lys262 (summarized in Figure 4B). This region includes the sixth TMH, part of the seventh TMH, and the third matrix-side loop that connects the fifth and sixth TMHs (Figure 4C).

To attempt to pinpoint the amino acid residue(s) labeled with [^{125}I]AzQ, we conducted peptide mass fingerprinting analyses on the labeled subunits. However, neither of the labeled peptides was detected; generally, sequence coverage of the ND1 subunit is low in these analyses (∼15%),[12,22−25] and although total sequence coverage of the 49 kDa subunit is moderate (40−60%),[12,22,25] the peptide discussed here is not readily detected. We also conducted manual Edman radiosequencing of both labeled fragments (49 kDa Asp41−Arg63 and ND1 Asp199−Lys262), according to the method of Perodin et al.[36] No labeled residue was identified within the first 10 amino acids; more than ∼10 cycles were not possible because of the significant (90−95%) loss of radioactivity during the cycling. Finally, the nitrene and the azacycloheptatetraene intermediates produced by UV irradiation of the arylazido are strong electrophiles, which attack double bonds, heteroatoms (S, O, and N), and even aliphatic C−H bonds,[37,38] precluding prediction of the [^{125}I]AzQ-labeled residues on the basis of chemical functionality.

DISCUSSION

The positions of the 63 TMHs in complex I from *T. thermophilus* were recently modeled to data at 4.5 Å resolution,[6] showing clearly that Nqo8 (ND1) in the membrane arm is located right beneath the Nqo4 and Nqo6 (49 kDa and PSST) subunits in the hydrophilic arm. Previously, we showed that a quinazoline-type inhibitor [^{125}I]AzQ binds at the interface of the 49 kDa and ND1 subunits, and that the N-terminal region of Asp41−Arg63 in the 49 kDa subunit probably contacts the ND1 subunit at, or close to, the inhibitor-binding site.[12] Our present results confirmed these findings and revealed that the labeled site in the ND1 subunit is located within the region of Asp199−Lys262. Membrane topology models for ND1[20,21] indicate that this region contains the third matrix-side loop connecting the fifth and sixth TMHs, the sixth TMH, and part of the seventh TMH (Figure 4). The matrix loops of the ND1 subunit are highly enriched in conserved charged residues (particularly the first and third loops) and have been suggested to be involved in binding the hydrophilic core subunits;[21] our

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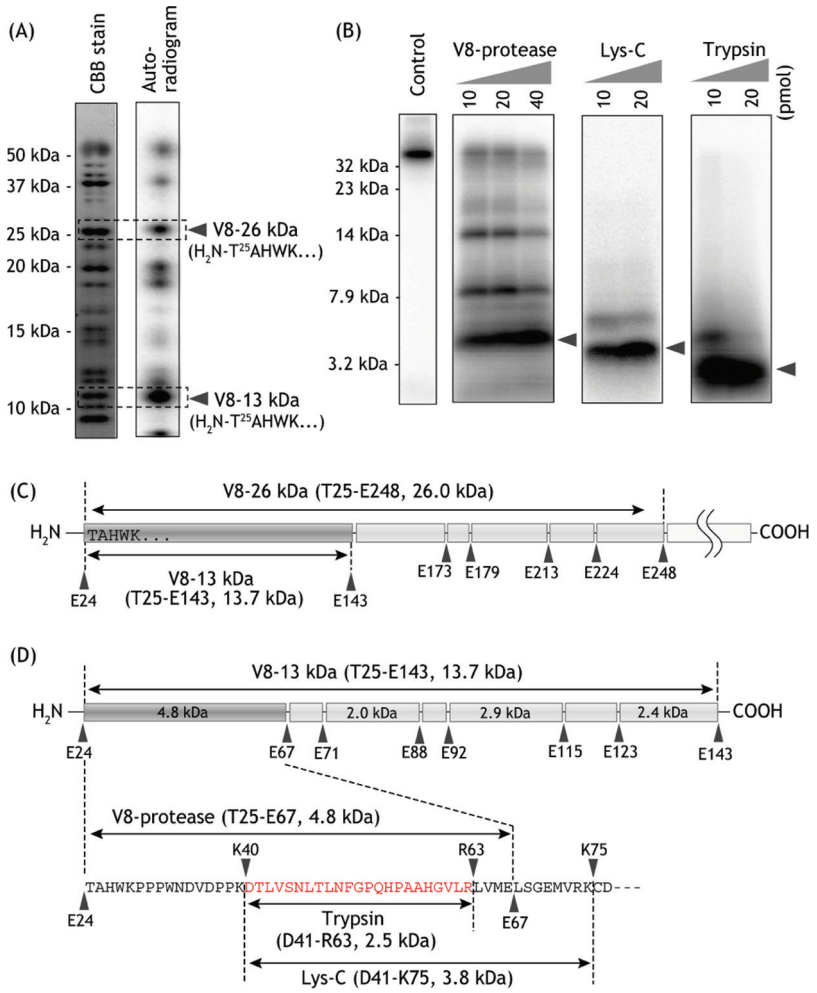

Figure 3. Analysis of the [^{125}I]AzQ-labeled site of the 49 kDa subunit by proteolysis. (A) Purified complex I (0.15 mg of protein/mL) was labeled with [^{125}I]AzQ (10 nM) in the presence of NADH (30 μM), DB (30 μM), and asolectin (0.4 mg/mL). The subunits were separated by SDS−PAGE (12.5% Laemmli-type gel, 18 μg/well), and the CBB-stained band containing the 49 kDa subunit was excised. The gel fragment was placed in the loading well of a 15% Tris-EDTA gel and subjected to partial digestion by V8 protease as described in Experimental Procedures. Panel A shows the Tris-EDTA gel, visualized by CBB staining and by autoradiography. The two bands marked V8-26 kDa and V8-13 kDa were analyzed by N-terminal sequencing, as described in Experimental Procedures, and a common N-terminal sequence (H_2N-T^{25}AHWK-) was identified. (B) The [^{125}I]AzQ-labeled 49 kDa subunit was electroeluted from the excised SDS−PAGE band and digested exhaustively with V8 protease, Lys-C, or trypsin (see Experimental Procedures). Panel B shows the analysis of the digests, along with an untreated control sample, by Tricine SDS−PAGE. The amounts of proteases in 30 μL of reaction buffer are indicated, and data shown are representative of three independent experiments. (C) Schematic representation of the partial digestion of the 49 kDa subunit by V8 protease. The predicted V8 cleavage sites between E143 and E248 are marked and denoted by their residue numbers in the mature sequence of the subunit (SwissProt entry P17694). (D) Schematic representation of the exhaustive digestion of the 49 kDa subunit by V8 protease, Lys-C, or trypsin. The top portion shows the V8-13 kDa peptide with the predicted V8 cleavage sites between E67 and E143 marked. The bottom portion shows the predicted trypsin and Lys-C cleavage sites within the T25−E67 V8 peptide with the shortest peptide that contains the labeled site (2.5 kDa, 23 amino acids) colored red.

results now reveal that the third matrix-side loop interacts with the N-terminal region of the 49 kDa subunit.

The interface between the hydrophilic and hydrophobic domains of complex I is already known to be important. It accommodates the bis-THF ring moiety of natural-type acetogenin and Δlac-acetogenin inhibitors[13] and has been probed in several mutagenesis studies. Notably, mutations in the ND1 homologues of two prokaryotic complex I forms have indicated the functional significance of several conserved charged residues in the third matrix-side loop, both for enzyme activity and for the stability of the hydrophilic−hydrophobic interface [for example, Glu220 and Glu228 in *Escherichia coli* (Glu206 and Glu214, respectively, in bovine)[21] and Glu212 and Glu247 in *Paracoccus denitrificans* (Glu192 and Glu227, respectively, in bovine)[39]]. On the "hydrophilic side", Grgic et al.[40] showed that mutations of the strictly conserved His91 and His95 residues in the 49 kDa subunit from *Y. lipolytica* (His55 and His59, respectively, in bovine), within the N-terminal region identified here, abolish the catalytic activity without affecting the EPR spectroscopic characteristics of the terminal Fe−S cluster. These data suggest that the connection between the two domains is critical for both enzyme stability and function. It is possible that the structure in this region forms the binding or access path for ubiquinone to the "ubiquinone cavity",[13] which is formed by the 49 kDa and PSST subunits.[7,14] Unfortunately, the N-terminal region of the 49 kDa subunit has

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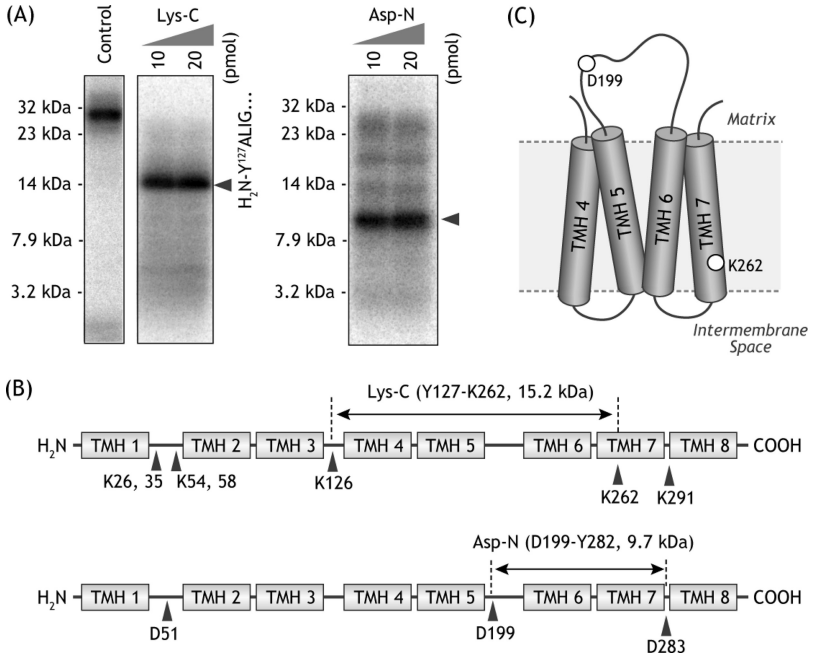

Figure 4. Analysis of the [^{125}I]AzQ-labeled site of the ND1 subunit by proteolysis. (A) Purified complex I (0.15 mg of protein/mL) was labeled with [^{125}I]AzQ (10 nM) in the presence of NADH (30 μM), DB (30 μM), and asolectin (0.4 mg/mL). The subunits were separated by SDS−PAGE (12.5% Laemmli-type gel, 18 μg/well), and the CBB-stained band containing the ND1 subunit was excised and electroeluted. Then, the isolated protein was exhaustively digested with Lys-C or Asp-N and analyzed by Tricine SDS−PAGE. The N-terminal sequence data determined from the Lys-C digest are shown. The amounts of the proteases in 30 μL of reaction buffer are indicated, and data shown are representative of three independent experiments. (B) Schematic representation of the exhaustive digestion of the ND1 subunit with Lys-C or Asp-N. The TMHs were assigned as described previously.[13,20] The residue numbers of the predicted cleavage sites refer to the sequence of the bovine ND1 subunit (SwissProt entry P03887). (C) The labeled region is shown in a topology model of the ND1 subunit.

not yet been completely resolved by structural analysis (see Figure S2 of the Supporting Information). Figure 5 shows how the current structural model for complex I is incomplete in the interfacial region, where the N-terminus of the 49 kDa subunit and the matrix loops of ND1 are located. Although this is consistent with significant structural mobility in this region, it precludes a more precise picture of the interaction between the 49 kDa and ND1 subunits being constructed at present. Additionally, we note that different organisms use different types of quinones (either ubiquinones or menaquinones) as electron carriers, but little is known about how the different quinones differ in their binding (or reaction) with complex I. Our interpretations are (by necessity) largely based on the crystal structure of *T. thermophilus* complex I,[5] which uses menaquinone, whereas the bovine enzyme that we study uses ubiquinone.

Schuler et al.[9] showed that a photoreactive pyridaben derivative [^{3}H]TDP binds to the PSST and ND1 subunits at low and high concentrations, respectively; the former is directly responsible for the inhibition of the enzyme activity, but the latter is unrelated to the inhibition. Subsequently, Schuler and Casida[35] demonstrated that the labeling pattern varies significantly, in a complicated fashion, according to the experimental conditions, such as the presence of NADH or different inhibitors. They concluded that [^{3}H]TDP has two different binding sites, high- and low-affinity sites in the PSST and ND1 subunits, respectively, and that the labeling pattern depends on a functionally coupled structural change that is induced by the addition of substrate or different inhibitors.[35] Thus, like [^{125}I]AzQ, [^{3}H]TDP also binds to two different subunits at the interface between the hydrophilic and membrane arms, but we interpret the patterns of inhibition differently.

In the case of [^{125}I]AzQ, there are two azido conformers with similar stability[41] [the bond angle for an azido group (−N═N$^+$═N$^-$) bound to a phenyl ring is 165°, not 180°], and we propose that the concomitant labeling of the 49 kDa and ND1 subunits reflects the binding of the two conformers in the same site between them: a major conformer in the bound state reacts with the 49 kDa subunit and a minor conformer with the ND1 subunit. This notion is supported by the following. (i) The pattern of photoaffinity labeling was essentially conserved from 1 to 60 nM [^{125}I]AzQ[12] and not significantly affected by NADH and/or DB (Figure 2). (ii) Both labeling events lead directly to enzyme inhibition.[12] (iii) Various complex I inhibitors blocked both labeling events with the same degree of efficiency.[12] (iv) Structural data from *T. thermophilus* complex I,[5] which was not available when refs 9 and 35 were published, are consistent with both labeled sections being in close contact. Accordingly, the most straightforward explanation for the dual labeling is that the phenylazido group is located between the two labeled regions. Nevertheless, at this stage, we cannot rule out completely that complex I has two (or more) conformers, each of which exposes one of the labeled sections.

In conclusion, deepening our knowledge of the roles of the interface between the hydrophilic and membrane arms, in quinone binding and energy transduction, is an important step in elucidating the function of complex I. Here, we present data to propose contacts between the N-terminal Asp41−Arg63 region of the 49 kDa subunit and the third matrix-side loop of the ND1 subunit, at, or close to, the

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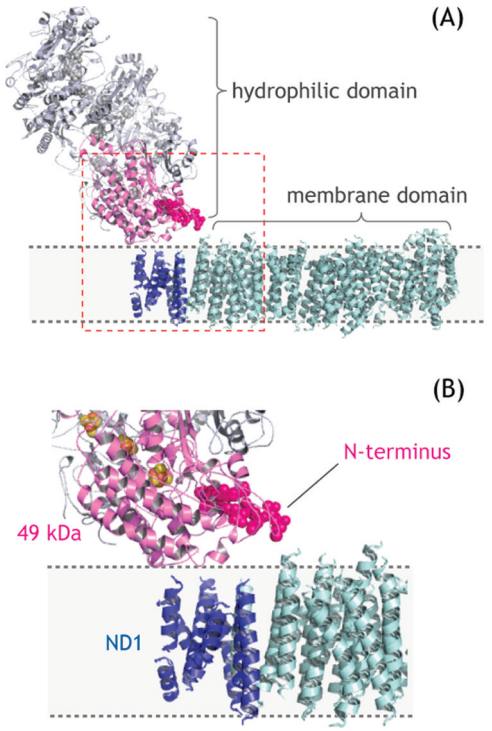

Figure 5. Knowledge about the structure of the interface between the hydrophilic and hydrophobic domains in complex I from the structural model of complex I from *T. thermophilus* (Protein Data Bank entry 3M9S[5]). (A) Nqo4 (the bacterial homologue of the 49 kDa subunit) and Nqo8 (ND1) are colored magenta and blue, respectively. (B) Close-up of the Nqo4 and Nqo8 subunits. Using a sequence alignment for the bovine 49 kDa and *T. thermophilus* Nqo4 subunits (Figure S2 of the Supporting Information), the labeled section of the 49 kDa subunit, which is resolved in the crystal structure (Met26−Gly31 and Gly39−Arg42), is indicated with spheres.

quinazoline inhibitor-binding site. Thus, we describe an important interaction, of potential structural and functional relevance, between two subunits at the interface between the two domains of complex I.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

AzQ, 6-azido-4-(4-iodophenetylamino)quinazoline; [^{125}I]AzQ, ^{125}I-labeled AzQ; CBB, Coomassie brilliant blue R250; complex I, proton-translocating NADH-quinone oxidoreductase; DB, decyl-benzoquinone (2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone); MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; PVDF, polyvinylidene fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SMP, submitochondrial particle; TMH, transmembrane helix.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on July 8, 2011, with an incorrect reference citation in Figure 4 caption. The corrected version was reposted on July 13, 2011.