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Characterization of the Ubiquinone Binding Site in the Alternative NADH-Quinone Oxidoreductase of *Saccharomyces cerevisiae* by Photoaffinity Labeling[†]

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ABSTRACT: The Ndi1 enzyme found in the mitochondrial membrane of *Saccharomyces cerevisiae* is an NDH-2-type alternative NADH-quinone oxidoreductase. As Ndi1 is expected to be a possible remedy for complex I defects of mammalian mitochondria, a detailed biochemical characterization of the enzyme is needed. To identify the ubiquinone (UQ) binding site in Ndi1, we conducted photoaffinity labeling using a photoreactive biotinylated UQ mimic (compound **2**) synthesized following a concept of the least possible modification of the substituents on the quinone ring. Cleavage with CNBr of Ndi1 cross-linked by **2** revealed the UQ ring of **2** to be specifically cross-linked to the Phe281−Met410 region (130 amino acids). Digestion of the CNBr fragment with V8 protease and lysylendopeptidase (Lys-C) gave ∼8 and ∼4 kDa peptides, respectively. The ∼8 kDa V8 digest was identified as the Thr329−Glu399 region (71 amino acids) by an N-terminal sequence analysis. Although the ∼4 kDa Lys-C digest could not be identified by N-terminal sequence analysis, the band was thought to cover the Gly374−Lys405 region (32 amino acids). Taken together, the binding site of the Q ring of **2** must be located in a common region of the V8 protease, and Lys-C digests Gly374−Glu399 (26 amino acids). Superimposition of the Ndi1 sequence onto a three-dimensional structural model of NDH-2 from *Escherichia coli* suggested that the C-terminal portion of this region is close to the isoalloxazine ring of FAD.

The alternative NADH-quinone oxidoreductase (NDH-2)[1] catalyzes transfer of an electron from NADH via FAD to quinone without proton pumping. The NDH-2 enzymes are found in bacteria and in mitochondria of plants and fungi, but not in mammalian mitochondria. Various species accomplishing respiration with NADH as an electron source use either NDH-1 (complex I, proton-pumping NADH-Q oxidoreductase), NDH-2, or both (*1*, *2*). In general, NDH-2 is much more insensitive to the inhibition by rotenone than NDH-1 (*1*). Plant and fungal mitochondria possess two types of NDH-2 (*3*); one is directed to the matrix and catalyzes NADH oxidation in the matrix (designated the internal NADH dehydrogenase or Ndi), and the other faces the intermembrane space and oxidizes NADH in the cytoplasmic space (designated the external NADH dehydrogenase or Nde). The Ndi1, an NDH-2-type enzyme, found in the mitochondria of *Saccharomyces cerevisiae* is composed of a single polypeptide of 53 kDa (630 amino acids) and contains noncovalently bound FAD and no iron−sulfur cluster (*4*). This enzyme is similar to complex I in terms of the reoxidation of matrix NADH produced in the Krebs cycle (*1*, *3*).

A series of studies by Yagi and colleagues suggest that the *S. cerevisiae NDI1* gene encoding Ndi1 may function as a therapeutic agent for mitochondrial diseases caused by complex I deficiencies (*5*−*13*). Actually, the Ndi1 expressed in the substantia nigra of the mouse brains has protective effects against Parkinsonian symptoms caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment (*12*, *14*). The expressed Ndi1 enzyme may play a dual role in rescuing complex I-deficient cells (*15*), one role being to restore the NADH oxidase activity and the other being to decrease the level of oxidative damage caused by complex I inhibition. To further test the idea of using Ndi1 to treat complex I defects, it is important to clarify the mechanism of the protective effects. For this purpose, a detailed biochemical characterization of the Ndi1 enzyme is indispensable.

Previously (*16*), we successfully overexpressed Ndi1 in *Escherichia coli* membranes using an N-terminal His_{10} tag fusion system and purified the functional enzyme. The purified recombinant Ndi1 had properties similar to those of the authentic Ndi1 enzyme isolated from *S. cerevisiae* mitochondria (*4*). The Ndi1 enzyme extracted with Triton X-100 contained no UQ_8, but the enzyme extracted with dodecyl β-D-maltoside (DM) contained a substoichiometric amount of UQ_8 (∼0.2 mol of UQ_8/mol of Ndi1). Exogenous UQ, such as UQ_6, could be incorporated into the

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[1]Abbreviations: CBB, Coomassie brilliant blue; CNBr, cyanogen bromide; complex I, mitochondrial proton-pumping NADH-ubiquinone oxidoreductase; DM, dodecyl β-D-maltoside; Lys-C, lysylendopeptidase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; NDH-1, bacterial proton-pumping NADH-quinone oxidoreductase; NDH-2, alternative NADH-quinone oxidoreductase; Ndi1, internal rotenone insensitive NADH-quinone oxidoreductase (NDH-2) from *S. cerevisiae*; PVDF, polyvinylidene fluoride; SELDI-TOF, surface-enhanced laser desorption ionization time-of-flight; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Q, quinone; UQ, ubiquinone.

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Ndi1 extracted with Triton X-100 to a level where the ratio of bound UQ to Ndi1 was ~1. From comparative biochemical studies of the UQ-bound and UQ-free Ndi1 enzymes, we suggested that the bound UQ site is distinct from the UQ catalytic site (*16*), while a semiquinone radical bound to the enzyme has not yet been detected. The existence of a bound UQ site has been demonstrated in *E. coli* membrane-bound glucose dehydrogenase (*17*, *18*) and *bo*-type ubiquinol oxidase (*19*−*22*) with definitive spectroscopic evidence. Identification of the UQ binding site(s) in Ndi1, whose X-ray crystal structure is unavailable at present, is crucial to understanding the molecular mechanism of electron transfer in the enzyme. To this end, we synthesized several photoreactive azido-Qs possessing a biotin at the terminal end of the side chain and conducted photoaffinity labeling. This study revealed that the binding site of the Q ring is located in the Gly374−Glu399 region (26 amino acids). On the basis of the structural model of NDH-2 from *E. coli*, the C-terminal portion of this region may be close to the isoalloxazine ring of FAD.

EXPERIMENTAL PROCEDURES

Materials. Ubiquinone-1 (UQ_1) and ubiquinoe-2 (UQ_2) were generously provided by Eisai (Tokyo, Japan). Ubiquinone-6 (UQ_6) and streptavidin-agarose were purchased from Sigma (St. Louis, MO). Protein standards for SDS−PAGE (the Precision plus blue standard and the Kaleidoscope polypeptide standard) were from Bio-Rad (Hercules, CA). Other reagents were of analytical grade.

Synthesis of Biotinylated Azido-Qs. The structures of each biotinylated azido-Q are shown in Figure 1. The synthesis of and spectral data for compounds **1**−**3** are given as Supporting Information.

Preparation and Assay of S. cerevisiae Ndi1. The expression and purification of *S. cerevisiae* Ndi1 were conducted using Triton X-100 as described previously (*16*). The Ndi1 enzyme prepared with Triton X-100 did not contain UQ_6 (Q-free Ndi1). The UQ_6-bound Ndi1 possessing a stoichiometric UQ_6 (~1:1 UQ_6:Ndi1) was prepared using DM in place of Triton X-100 as reported previously (*16*). Electron transfer activities of UQ analogues were measured spectrometrically with a Shimadzu UV-3000 instrument (340 nm, ε = 6.2 mM^{-1} cm^{-1}). The reaction medium (2.5 mL) contained 50 mM NaP_i buffer (pH 6.0) and 1 mM EDTA, and the protein concentration was 0.066 μg/mL. The reaction was started via addition of 100 μM NADH after the equilibration of the enzyme with the UQ analogues.

Photoaffinity Labeling of Ndi1. The purified Ndi1 (0.1−0.3 mg of protein/mL) was incubated with azido-Q in a buffer containing 50 mM MOPS-KOH (pH 7.0), 0.1 mM EDTA, and 10% glycerol for 10 min at room temperature. The molar ratio of azido-Q to Ndi1 was varied in accordance with the purpose of the respective experiment and described in the figure legends. The reconstituted Ndi1 was UV-irradiated with a long wavelength UV lamp (Black-lay model B-100A, UVP, Upland, CA) for 10−20 min on ice 10 cm from the light source. When suppression of the cross-linking was tested, a competitor was added and incubated for 10 min prior to the treatment with azido-Q. The cross-linking was quenched by adding 4× Laemmli's sample buffer or acetone precipitation, and the samples were subjected to further analysis.

Electrophoresis and Biotin Detection. SDS−PAGE was performed according to the methods of Laemmli (*23*) and Schägger (*24*). For the analysis of undigested Ndi1 samples, denatured samples were separated on 10% Laemmli's gel and subjected to CBB staining or electroblotting. For the analysis of CNBr-cleaved or enzymatically digested Ndi1 samples, the samples were separated on Schägger's Tricine gel (16.5% T/6% T) and subjected to further analysis.

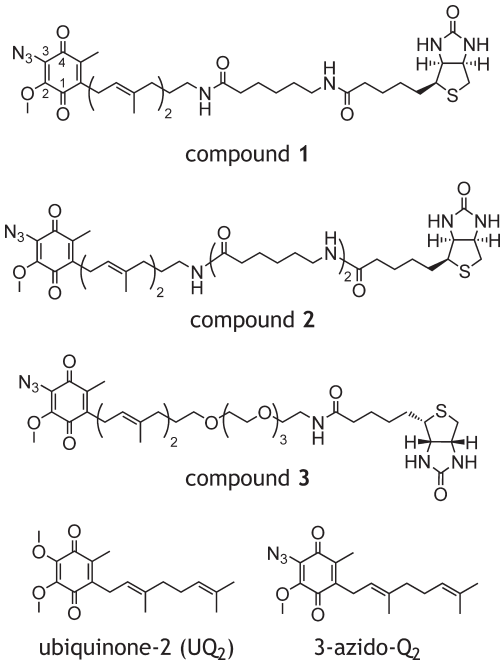

FIGURE 1: Structures of the quinones studied in this work. Compounds **1**−**3** were newly synthesized for this study.

Electrophoresed proteins were transferred onto a PVDF membrane (Immn-blot PVDF membrane, 0.2 μm, Bio-Rad) in a buffer containing 10 mM $NaHCO_3$, 3 mM $NaCO_3$, and 0.025% (w/v) SDS overnight at 35 V (100 mA) in a cold room. The blotted membrane was blocked with 1% gelatin in Tween TBS [10 mM Tris-HCl (pH 7.4), 0.9% NaCl, and 0.05% Tween 20] for 1 h at room temperature, followed by incubation for an additional 1 h with streptavidin-AP (Dako Cytomation, Glostrup, Denmark) at room temperature. The treated membrane was washed with Tween TBS (3 × 10 min) and developed with NBT/BCIP chromogenic substrates (AP color development kit, Bio-Rad).

Limited Proteolysis. Ndi1 (0.1−0.3 mg of protein/mL) was photo-cross-linked by a 2−4-fold molar excess of azido-Q in the buffer described above. The reaction was terminated by acetone precipitation, and the pellet was subjected to cleavage by CNBr or digestion with trypsin. For CNBr cleavage, the pellet was resuspended in 70% formic acid and digested with CNBr at a 300-fold excess of the Met residues contained. The digestion was continued overnight at 37 °C in darkness. Cleavage was quenched by addition of water and vacuum centrifugal evaporation, and the digests were dissolved in 1× Schägger's sample buffer and subjected to Tricine/SDS−PAGE. When the cleaved peptides were further digested by proteases, the CNBr digests were recovered from the tricine gel by electrophoretic elution (electroelution) using a Centrilutor equipped with a Centricon YM-10 (Millipore, Billerica, MA).

For secondary enzymatic digestion, the electroeluted peptides were digested with V8 protease (Glu-C, Roche Applied Science, Penzberg, Germany) and lysylendopeptidase (Lys-C, Wako, Osaka, Japan) in 50 mM ammonium bicarbonate buffer and 20 mM Tris-HCl buffer (pH 9.0), respectively. The protease to

substrate ratio was set to 1:30 (v/v), and digestion was continued overnight at 37 °C.

For in-gel digestion, the CBB-stained Ndi1 bands were digested overnight at 37 °C with trypsin (Promega) or chymotrypsin (Roche) in a buffer containing 50 mM NH_4HCO_3 and 5 mM $CaCl_2$. The digests were extracted from the gel once in 0.1% formic acid and twice in a 50% acetonitrile/0.1% formic acid mixture. The combined extracts were concentrated using a vacuum centrifugal evaporator and subjected to LC−ESI-MS analysis.

Electrospray Ionization Mass Spectroscopy (ESI-MS). The mass spectra of the enzyme digests were recorded on a triple quadrupole mass spectrometer (API-3000, Applied Biosystems, Foster City, CA) in the Q1 positive ion mode. The peptide samples were separated by an "on-line" HPLC system (Agilent Technologies, Santa Clara, CA) using a reverse-phase column (Supelco Discovery BIO Wide Pore C18, 2.1 mm × 150 mm, Sigma-Aldrich, Bellefonte, PA) and subjected to mass spectrometric analysis. The mobile phase was comprised of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile), delivered at a flow rate of 0.2 mL/min, and a linear gradient of solvent B was formed from 5 to 55% (60 min). The spectra were scanned from m/z 500 to 1500 with a step size of 0.1−0.2 and dwell time of 1.0 ms. BioMultiview (Sciex) was used for the reconstruction of multiply charged ions, and calculated molecular masses were compared with theoretical digests from the published protein sequences of *S. cerevisiae* Ndi1 (Swiss-Prot entry P32340) using Peptide Mass (http://www.expasy.org/tools/peptide-mass.html).

N-Terminal Amino Acid Sequence Analysis. The CNBr and enzymatic digests of the cross-linked Ndi1 were separated by Tricine/SDS−PAGE and blotted onto a PVDF membrane as described above. The membrane was stained with 0.025% CBB-R250 in 40% MeOH and destained with 50% MeOH. The identified band corresponding to the cross-linked peptide fragment was excised, and the N-terminal sequence was analyzed using the PROCISE 491HT system (Applied Biosystems).

Enrichment of Biotinylated Ndi1 and Mass Spectrometric Analysis. The cross-linked (i.e., biotinylated) Ndi1 was purified using immobilized streptavidin. The Q-free Ndi1 (0.34 mg of protein/mL, total of 68 μg) was photoirradiated with a 20-fold molar excess of azido-Q, and the excess probe was removed by acetone precipitation. The labeled Ndi1 was resolubilized via incubation in 2% SDS (60 μL) at 40 °C for 1 h. Then, the Ndi1 was diluted with 500 μL of 1% Triton X-100 in TBS buffer [0.9% NaCl, 10 mM Tris-HCl (pH 7.4), and 0.21% SDS] and incubated with a 50 μL slurry of streptavidin-agarose (Sigma) overnight in a cold room. The avidin resin was washed twice with 0.5% Triton X-100 in TBS buffer and twice with TBS buffer. The Ndi1 captured on the avidin resin was eluted in Laemmli's SDS−PAGE buffer at 90 °C. These capture−release procedures using avidin resin were monitored by SDS−PAGE and Western blotting.

Peptide mass fingerprinting of the labeled Ndi1 using the SELDI (surface-enhanced laser desorption ionization)-TOF MS system was performed at ProteNova Co., Ltd. (Takamatsu, Japan). CBB-stained gel bands were treated with 10 mM DTT and 55 mM iodoacetamide, followed by in-gel digestion by TPCK-trypsin (Promega, Madison, WI) in a buffer containing 50 mM NH_4HCO_3 and 0.02% octyl glucoside (pH 8.0) at 35 °C for 5 h. The digested peptides were extracted with 0.6% TFA in 10% acetonitrile and analyzed with the ProteinChip SELDI system (Bio-Rad) on an NP 20 chip.

Sequence Alignment and Prediction of the UQ Binding Site. Multiple-sequence alignment was performed with Clustal W (*25*), using the amino acid sequences of alternative NADH-Q oxidoreductases from various prokaryotic and eukaryotic organisms as follows: *E. coli* (CAA23586.1), *Azotobacter vinelandii* (AAK19737.1), *S. cerevisiae* (CAA43787.1), *Yarrowia lipolytica* (CAA07265.1), *Solanum tuberosum* (CAB52796.1), *Arabidopsis thaliana* (AAM61225.1), *Trypanosoma brucei* (AAM95239.1), *Synechocystis* sp. PCC 6803 (BAA17787.1), and *Thermosynechococcus elongatus* BP-1 (BAC08688.1). These sequences, including those of *S. serevisiae* Ndi1 and *E. coli* NDH-2, are classified as group A NDH-2 forms on the basis of their conserved binding motifs (*2*).

For the prediction of the UQ binding site in Ndi1, the in silico three-dimensional structure of the NDH-2 from *E. coli* (*26*) was obtained from the Protein Data Bank (PDB) as entry 1OZK. Visualization of the putative UQ binding site was conducted using Mac Pymol (version 0.99, DeLano Scientific LLC, Palo Alto, CA).

RESULTS

Design Concept for Photoreactive Biotinylated Q. We synthesized three photoreactive biotinylated azido-Qs [**1**−**3** (Figure 1)] following a concept of least possible modification of the substituents on the quinone ring. ^{3}H-labeled azido-Qs have been powerful chemical probes for identifying the UQ binding site in various respiratory enzymes (*27*−*31*). The substitution pattern on the Q ring used in earlier studies was limited to 2-methyl-3-azido-5-methoxy-(6-alkyl tail)-1,4-benzoquinone (*27*−*31*), while the substitution pattern of natural UQ is 2,3-dimethoxy-5-methyl-6-(alkyl tail)-1,4-benzoquinone. Any photoaffinity labeling probe should ideally be as similar in structure as possible to the original biologically active chemical. Therefore, we here synthesized 2-methoxy-3-azido-5-methyl-6-(alkyl tail)-1,4-benzoquinone according to our previous procedure (*32*). This Q ring structure was shown to react as an efficient photoreactive Q mimic with the cytochrome *bd* complex in *E. coli* (*33*).

With regard to the hydrophobic isoprene tail moiety, it is noteworthy that, although the tail moiety has been generally believed to merely enhance the hydrophobicity of the UQ molecule, the isoprene moiety in the vicinity of the Q ring (i.e., first and/or second isoprene) plays a specific role as a substrate for respiratory enzymes such as mitochondrial complex I and the cytochrome *bo* complex in *E. coli* (*34*). Therefore, to ensure inherent binding of the Q ring to the enzyme, two isoprenyl groups were directly attached to the Q ring in compounds **1**−**3**.

Moreover, the incorporation of a radioisotope into the probe molecule is useful as a tag for monitoring the cross-linked protein in photoaffinity labeling experiments; however, this is sometimes accompanied by inconvenience in experimental handling. We therefore attached a biotin tag at the terminal end of the tail through a linker, such as amide (**1** and **2**) and diethyl glycol (**3**), to enable several applications in the detection and isolation procedures. In addition, the linker's structure was varied since this moiety may affect the reactivity of the probe molecule with the enzyme and/or avidin.

Electron Accepting Ability of the Azido-Qs. To verify the electron accepting ability of the biotinylated azido-Qs, we determined kinetic parameters with UQ-free Ndi1 using UQ_1 and UQ_2 as references (Table 1). Judging from the intrinsic electron accepting efficiency in terms of the V_{max}/K_m value, we

Table 1: Summary of the Kinetic Data of the Quinones Studied in This Study

	K_m (μM)[a]	V_{max} [μmol min^{-1} (mg of protein)$^{-1}$][a]	V_{max}/K_m
compound **1**	22.0 ± 3.4	28.0 ± 1.8	1.3
compound **2**[b]	18.1 ± 4.8	224 ± 29	12.4
compound **3**	33.0 ± 6.5	1120 ± 107	33.9
UQ_1	15.2 ± 5.8	491 ± 73	32.3
UQ_2	7.9 ± 2.7	742 ± 83	93.9

[a]Values (means ± standard error) were calculated with Prism (version 4, GraphPad). [b]Tween 20 (0.3%) was added to improve the solubility of **2**.

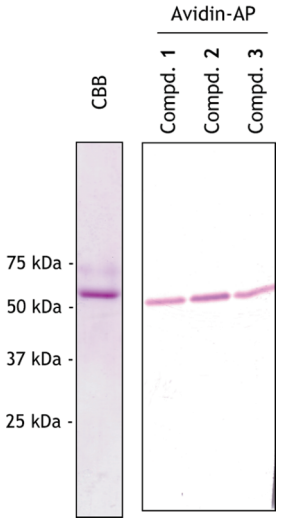

FIGURE 2: Photoaffinity labeling of Ndi1 by the biotinylated azido-Qs (**1**−**3**). The UQ-free Ndi1 was photoirradiated with each azido-Q at an equivalent molar ratio (6.6 μg of protein/mL) and analyzed on 10% Laemmli's gel with CBB and streptavidin-AP (0.5 μg of protein/lane for CBB and 0.1 μg of protein/lane for Western blotting). Data shown are representative of four independent experiments.

found that compounds **2** and **3** work well as an electron acceptor, indicating that these compounds are suitable for photoaffinity labeling as UQ mimics. We also confirmed that a specific inhibitor, aurachin analogue AC0-10 (*16*), completely blocks the reduction of the three azido-Qs.

Photo-Cross-Linking of Ndi1. To determine the best azido-Q for the cross-linking reaction with Ndi1, we compared the extents of cross-linking among the three compounds. The UQ-free Ndi1 was incubated with each azido-Q for 10 min before photolysis with an azido-Q:Ndi1 ratio of 1. The reconstituted azido-Q was UV-irradiated to generate the nitrene, which reacts to an amino acid residue in the proximity of the Q ring. The cross-linking was detected by Western blotting using AP-conjugated streptavidin (Figure 2). The signal quantity indicated that the extent of cross-linking by **2** is the best among the three. It is, however, difficult to establish whether this can be attributed to the affinity of **2** for the enzyme, the reactivity against AP-conjugated streptavidin, or both. Although **3** is the best candidate with regard to electron accepting ability, given the extent of cross-linking, we decided to use **2** as a photoaffinity probe in the subsequent experiments.

To specify the cross-linking by **2**, we examined whether other short chain UQ analogues (UQ_2 and azido-Q_2) suppress cross-linking. As shown in Figure 3, both UQ analogues suppressed cross-linking in a concentration-dependent manner, verifying the specific binding of **2**.

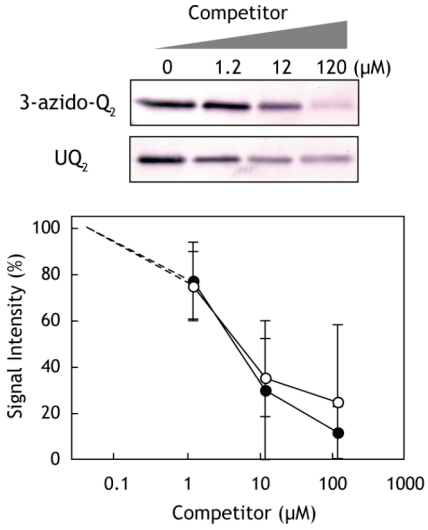

FIGURE 3: Effect of short chain Q on the specific labeling of Ndi1. The UQ-free Ndi1 (6.6 μg of protein/mL) was photoirradiated with **2** at an equivalent molar ratio (0.12 μM) in the presence of UQ_2 (○) or 3-azido-Q_2 (●) and subjected to SDS−PAGE and Western blotting (0.1 μg/lane). Signal intensity was quantified with Image J (http://rsbweb.nih.gov/ij/). Data are means of four independent measurements ± the standard deviation. Representative results of Western blotting are shown at the top.

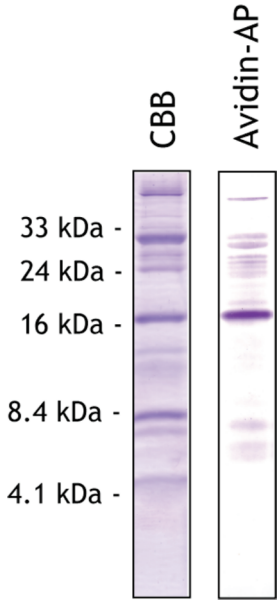

FIGURE 4: CNBr cleavage and gel electrophoresis of the cross-linked Ndi1. The UQ-free Ndi1 was photoirradiated with a 4-fold excess of **2**, cleaved by CNBr, and subjected to Tricine/SDS−PAGE (16.5% T/5% C) and Western blotting (4 μg of protein/lane for CBB and 2 μg of protein/lane for Western blotting). Data shown are representative of five independent experiments. The avidin-AP reactive ~18 kDa band was subjected to an N-terminal sequence analysis.

Analysis of the Cross-Linked Peptide. To identify the UQ binding site, the Ndi1 photoirradiated with **2** was cleaved by CNBr into several fragments (Figure 4). The cleavage yield was >90%, as judged by gel electrophoresis. The cross-linking was predominantly found in the ~18 kDa fragment regardless of the **2**:Ndi1 ratio (1−20). The specificity of the cross-linking was also confirmed by digestion with V8 and Lys-C as described below, indicating that random cross-linking during photolysis is negligible. On the basis of theoretical sites of cleavage by CNBr (summarized in Figure 6), this fragment was presumed to be

Phe281−Met410 (130 amino acids, calculated peptide mass of 14439.5 Da). To verify this, we analyzed the N-terminal sequence of the fragment on the PVDF membrane, which yielded H_2N-FEKKLSSYAQSHLENTSIKV, which corresponds to amino acid residues Phe281−V300.

The CNBr fragment was isolated by electroelution from the Tricine gel and further cleaved into smaller peptides by being treated with V8 protease or Lys-C. After the V8 cleavage, major cross-linking was found in the ~8 kDa fragment (Figure 5), and the N-terminal sequence analysis of the CBB-stained band on the membrane provided H_2N-T^{329}IPYG. Taking into consideration the theoretical cleavage site of V8 and the apparent molecular mass of the band, we identified this fragment as Thr329−Glu399 [71 amino acids, calculated peptide mass of 7648.1 Da (Figure 6)]. On the other hand, after the Lys-C cleavage, cross-linking was found solely in the ~4 kDa fragment. On the basis of suspected sites of cleavage by Lys-C, this fragment was thought to be Gly374−Lys405 [32 amino acids, calculated peptide mass of 3314.6 Da (Figure 6)]. We could not identify the CBB-stained band corresponding to the Western band on the PVDF membrane.

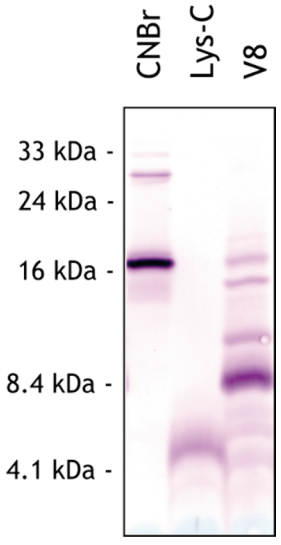

FIGURE 5: Protease digestion of the cross-linked CNBr fragment. The UQ-free Ndi1 was photoirradiated with a 4-fold excess of **2**, treated with CNBr, and subjected to Tricine/SDS−PAGE. The cross-linked CNBr fragment was recovered from the gel by electroelution. This fragment was further digested with lysylendopeptidase (Lys-C) and V8 protease (V8). Data shown are representative of five independent experiments.

To confirm the occurrence of the two fragments, we checked the digestion pattern of the nonlabeled ~18 kDa fragment by LC−ESI-MS (Table S1 of the Supporting Information). Actually, both fragments produced by V8 (Thr329−Glu399, 7652.6 Da, 0.06% error) and Lys-C (Gly374−Lys405, 3318.4 Da, 0.1% error) were determined. We concluded that the binding site of the Q ring of **2** must be located in a common region of the V8 and Lys-C digests (i.e., Gly374−Glu399, 26 amino acids).

Effect of Bound Q on Cross-Linking. A previous study suggested that Ndi1 bears two distinct UQ binding sites: one for bound UQ and the other for catalytic UQ (*16*). To test this hypothesis and examine the effect of bound UQ on photo-cross-linking, we compared the extent of cross-linking between the UQ_6-bound and UQ-free Ndi1 at a **2**:Ndi1 ratio of 1−4. The UQ_6-bound Ndi1 containing a stoichiometric amount of UQ_6 (~1 mol/mol) was prepared as described previously (*16*).

As shown in Figure S1 of the Supporting Information, there was no difference in the extent of cross-linking between the two enzyme preparations. We also compared the position of the CNBr-cleaved peptides, and the V8 and Lys-C digests between

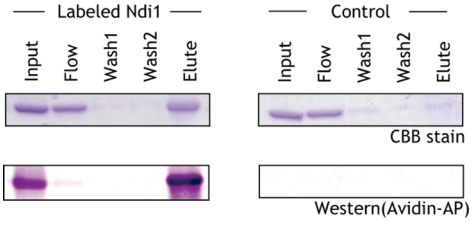

FIGURE 7: Purification of the Ndi1 cross-linked by **2**. The UQ-free Ndi1 (0.34 mg of protein/mL) was cross-linked by a 20-fold excess of **2** and subjected to acetone precipitation. The precipitate was solubilized in 4% SDS and subjected to the purification procedures described in Experimental Procedures by repeated rotation and centrifugation. The supernatant from each step was analyzed by SDS−PAGE and Western blotting: Input, supernatant before incubation with the avidin resin (1.2% of total volume, ~0.8 μg of protein/well); Flow, supernatant after the incubation with the avidin resin (1.2% of total volume); Wash1, supernatant after the wash with 0.5% Triton X-100 in TBS buffer; Wash2, supernatant after the wash with TBS buffer; Elute, supernatant after the treatment with Laemmli's buffer (50% of total volume). Data shown are representative of three independent experiments.

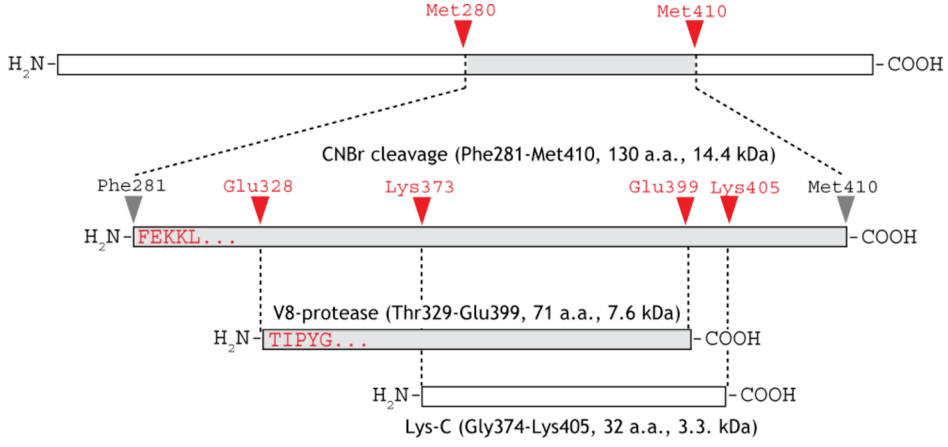

FIGURE 6: Schematic presentation of the CNBr/proteolic mapping of Ndi1. The results of SDS−PAGE and Western blotting of CNBr/proteolic digests are summarized. The fragments identified by the N-terminal sequence analysis are shown as gray rectangles with the initial sequences. Residue numbers of the theoretical cleaved site refer to the published sequence of the *S. cerevisiae* Ndi1 (P32340).

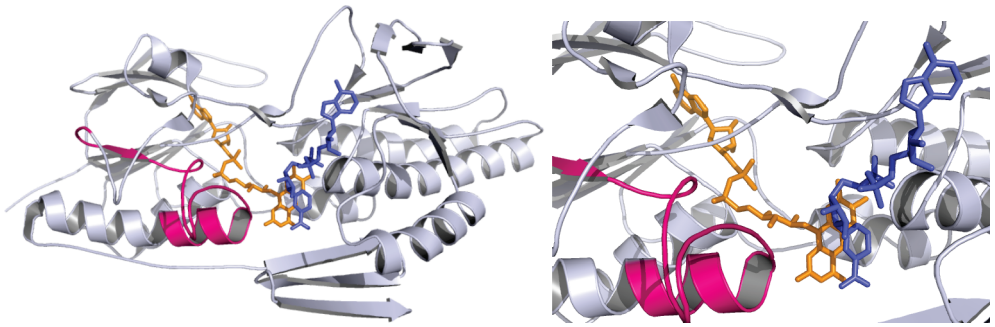

FIGURE 8: Ribbon representation of the modeled alternative NADH-Q oxidoreductase (NDH-2) from *E. coli* [PDB entry 1OZK (*26*)]. FAD and NADH are colored orange and blue, respectively. On the basis of an amino acid sequence alignment of Ndi1 from *S. cerevisiae* and NDH-2 from *E. coli*, the region cross-linked by **2** (Gly374−Glu399) is superimposed (colored red).

the UQ_6-bound and UQ-free Ndi1. Irrespective of the presence of bound UQ_6, photo-cross-linking occurred solely at the ~18 kDa band (Figure S1 of the Supporting Information), and the migration pattern of the other digests was also identical to that of UQ-free Ndi1 (Figure S2 of the Supporting Information). If **2** also binds to the site for bound UQ, which may have a higher affinity for UQ than the site for catalytic UQ, the extent of cross-linking should be suppressed in the presence of bound UQ. However, this was not the case. Altogether, this study did not provide evidence of the occurrence of the bound UQ site in Ndi1. We will further discuss this point.

Identification of a Cross-Linked Amino Acid. To pinpoint the amino acid cross-linked by **2**, we conducted a MALDI-TOF MS analysis of the triptic digests of the CNBr fragment prepared from Ndi1 photoirradiated with or without **2**. It should be noted that the Ndi1 photoirradiated with **2** is a mixture of cross-linked and non-cross-linked enzymes because the reaction yield of the cross-linking is far less than 100%. Although the peptide mass fingerprinting by MALDI-TOF MS covered 87% of the CNBr fragment, no significant difference was observed between the two Ndi1 preparations (data not shown).

We therefore tried to purify the cross-linked (i.e., biotinylated) enzyme using immobilized streptavidin, as described in Experimental Procedures. As confirmed by SDS−PAGE and Western analysis at each purification step (Figure 7), the Ndi1 cross-linked by **2** was specifically captured by the avidin resin and released by treatment with Laemmli's buffer containing 4% SDS. Judging from the protein bands on the CBB-stained gel, we figured that the recovery of the cross-linked Ndi1 from a mixture of cross-linked and non-cross-linked Ndi1 is ~5%.

With the purified cross-linked enzyme in hand, we compared peptide mass fingerprinting of the tryptic digests of the cross-linked and non-cross-linked Ndi1 with the SELDI-TOF MS system, which is superior in sensitivity, quantification performance, and reproducibility to the MALDI-TOF MS system (*35*). While the total sequence coverage was 82%, no significant difference was observed between the cross-linked and non-cross-linked enzymes (e.g., Figure S3 of the Supporting Information). In addition, we carried out LC−ESI-MS analysis of the tryptic, Lys-C, and chymotryptic digests of the cross-linked and non-cross-linked Ndi1. However, we could not detect the peaks corresponding to the peptides cross-linked by the quinone probe, as shown in Figure S4 of the Supporting Information, taking the triptic digests as an example.

DISCUSSION

We synthesized photoreactive Q probes bearing a biotin and conducted photoaffinity labeling to identify the UQ binding site in Ndi1. Our results strongly suggest that the Gly374−Glu399 region (26 amino acids) constructs the binding domain of the Q ring. In agreement with this, the region contains Asp383, a residue invariantly conserved among several NDH-2 enzymes (Figure S4 of the Supporting Information) and known to be essential for enzyme activity.[2] To further pinpoint the binding site, we purified the cross-linked Ndi1 and conducted peptide mass fingerprinting by MALDI-TOF MS and SELDI-TOF MS, and also LC−ESI-MS analysis. Unfortunately, we could not detect the peptide fragment cross-linked by **2** using these techniques. This is probably because compound **2** cross-linked to the enzyme may be highly unstable under the protease treatment conditions, and left the peptide. It should be noted that the lifetimes of nitrene and nitrenium ion (a protonated form of nitrene) are rather long, being on the order of microseconds (*36*). Therefore, we cannot exclude the possibility that the cross-linking may have occurred with multiple residues within the Gly374−Glu399 region.

Schmid and Gerloff (*26*) created a three-dimensional structural model for the alternative NADH-Q oxidoreductase (NDH-2) from *E. coli* through comparative modeling onto a template from SCOP family "FAD/NAD-linked reductases" (Figure 8). In the model, they suggested that the redox active parts of the cofactors (i.e., the nicotinamide ring of NADH and the isoalloxazine ring of FAD) are orientated in parallel, in the proximity of each other (< 3 Å), to facilitate electron transfer. In contrast to the FAD and NADH binding sites, details of the UQ binding site, such as residues involved in hydrogen bonding, cannot be derived directly from the structural mode. However, they suggested that the UQ binding site would be close to FAD for achieving efficient electron transfer. On the basis of an amino acid sequence alignment of Ndi1 from *S. cerevisiae* and NDH-2 from *E. coli* (see Figure S5 of the Supporting Information), we superimposed the region identified in this study (Gly374−Glu399) into the NDH-2 structure (colored red in Figure 8). The C-terminal portion of this region is close to the isoalloxazine ring of FAD, enabling facilitative electron transfer between the isoalloxazine ring and the UQ ring. It is likely that NDH2, in the orientation in which it is displayed in Figure 8, tops the membrane (*26*). Therefore, if the UQ ring is located in this region, the hydrophobic isoprene tail may favorably anchor in the membrane.

The Ndi1, overexpressed in *E. coli* membranes, extracted with Triton X-100 contains no UQ_8, but the enzyme extracted with

[2]From site-specific mutations of Ndi1, Yamashita et al. found that D383A, -N, and -Q mutants almost completely lose the NADH-UQ_1 oxidoreductase activity ($< 1\%$) (T. Yamashita, E. Nakamaru-Ogiso, A. Matsuno-Yagi, and T. Yagi, unpublished data).

DM contains a substoichiometric amount of UQ_8 (*16*). The Ndi1 extracted with Triton X-100 can accommodate exogenous UQ, such as UQ_6, at a molar ratio of ~1:1 (*16*). From these results along with other biochemical studies of the UQ-bound and UQ-free Ndi1 enzymes, we suggested that Ndi1 bears two distinct UQ binding sites [one for bound UQ and the other for catalytic UQ (*16*)], with analogy to *E. coli* membrane-bound glucose dehydrogenase (*17*, *18*) and *bo*-type ubiquinol oxidase (*19*−*22*). However, our photoaffinity labeling study did not support the occurrence of a bound UQ site in Ndi1; neither the extent nor the pattern of photo-cross-linking was affected irrespective of the presence of bound (i.e., reconstituted) UQ_6. One may speculate that the bound UQ site proposed previously is identical to the catalytic UQ site. In this case, the reconstituted UQ_6 may occupy the catalytic site of Ndi1. Given the observation that an excess amount of UQ_2 or 3-azido-Q_2 was needed to prevent cross-linking by **2** (Figure 3), the total amount of UQ_6 in the UQ_6-reconstitued Ndi1 may be too small to efficiently compete with **2**. We actually confirmed that the cross-linking by **2** is suppressed by UQ_6 in a concentration-dependent manner under experimental conditions identical to those used for the competition test by UQ_2 and azido-Q_2 (data not shown).

In conclusion, to identify the UQ binding site in Ndi1, we conducted photoaffinity labeling using a newly synthesized photoreactive UQ mimic, **2**. Our results reveal the binding site of the Q ring of **2** to be located in the Gly374−Glu399 region (26 amino acids), the C-terminal portion of which may be close to the isoalloxazine ring of FAD. It is likely that the bound UQ site proposed previously (*16*) is identical to the catalytic UQ site.

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SUPPORTING INFORMATION AVAILABLE

Synthesis of compounds **1**−**3**, Table S1, and Figures S1−S5. This material is available free of charge via the Internet at http://pubs.acs.org.

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