The Ubiquinone-binding Site in NADH:Ubiquinone Oxidoreductase from Escherichia coli*

An azido-ubiquinone derivative, 3-azido-2-methyl-5-methoxy[3H]-6-decyl-1,4-benzoquinone ([3H]azido-Q), was used to study the ubiquinone/protein interaction and to identify the ubiquinone-binding site in Escherichia coli NADH:ubiquinone oxidoreductase (complex I). The purified complex I showed no loss of activity after incubation with a 20-fold molar excess of [3H]azido-Q in the dark. Illumination of the incubated sample with long wavelength UV light for 10 min at 0 °C caused a 40% decrease of NADH:ubiquinone oxidoreductase activity. SDS-PAGE of the complex labeled with [3H]azido-Q followed by analysis of the radioactivity distribution among the subunits revealed that subunit NuoM was heavily labeled, suggesting that this protein houses the Q-binding site. When the [3H]azido-Q-labeled NuoM was purified from the labeled reductase by means of preparative SDS-PAGE, a 3-azido-2-methyl-5-methoxy-6-decyl-1,4-benzoquinone-linked peptide, with a retention time of 41.4 min, was obtained by high performance liquid chromatography of the protease K digest of the labeled subunit. This peptide had a partial NH₂-terminal amino acid sequence of NH₂-VMLAILALV-, which corresponds to amino acid residues 184–193 of NuoM. The secondary structure prediction of NuoM using the Toppred hydroxylation analysis showed that the Q-binding peptide overlapped with a proposed Q-binding motif located in the middle of the transmembrane helix 5 toward the cytoplasmic side of the membrane. Using the PHDhtm hydropathy plot, the labeled peptide is located in the transmembrane helix 4 toward the periplasmic side of the membrane.

The NADH:ubiquinone oxidoreductase (also known as respiratory complex I) is the first segment of the energy-conserving electron transfer chains of mitochondria and many respiratory and photosynthetic bacteria. This complex catalyzes electron transfer from NADH to ubiquinone and concomitantly transfers protons across the membrane to generate a membrane potential and proton gradient for ATP synthesis (1, 2). Whereas the mitochondrial enzyme contains up to 46 different subunits (3), the bacterial enzyme is made up of 13–14 subunits (4) and thus can be considered as the minimal core complex I.

The Escherichia coli NADH-oxidoreductase has been purified to homogeneity by liquid chromatography in the presence of detergent alkyl polyglycosides (5) or dodecyl maltoside (6). The purified complex has a molecular mass of approximately 550 kDa and contains 13 subunits encoded by the nuo genes (7). This bacterial complex can adopt either a conserved L-shaped or horseshoe-shaped quaternary structure made of a peripheral arm and a membrane arm (8). The horseshoe-shaped structure is believed to be the active form. Six subunits, NuoB, NuoCD, NuoE, NuoF, NuoG, and NuoI, constitute the peripheral arm (5). The remaining seven subunits, NuoA, NuoH, NuoJ, NuoL, NuoM, and NuoN, are hydrophobic proteins, which comprise the membrane arm. The peripheral arm can be subdivided into an NADH dehydrogenase domain composed of subunits NuoE, NuoF, and NuoG, which catalyzes the oxidation of NADH, and a connecting domain composed of subunits NuoB, NuoCD, and NuoI. The dehydrogenase domain contains the FMN-binding site and the EPR-detectable FeS clusters, N1a, N1b, N3, and N4 (5), whereas the connecting domain contains the EPR-detectable FeS cluster N2 (5) and the UV-visible-detectable clusters N6a and N6b (9). The subunits of the membrane arm contain no known cofactors, such as flavin or iron-sulfur clusters, and are thought to be involved in quinone binding and proton translocation (10, 11).

The mechanism of electron transfer and its coupling to proton translocation in NADH-Q oxidoreductase is poorly understood. Ubiquinone is the final electron acceptor in NADH-Q oxidoreductase and may take part in electron recycling and/or proton transport processes. Knowledge of ubiquinone binding is essential for mechanistic studies of this complex. However, the number of binding sites for ubiquinone in NADH-Q oxidoreductase is an unsolved question subject to intense controversy (12–14). Up to three sites have been proposed (14). Most suggestions derive from studies involving labeled inhibitor analogues. The Q-binding site has long been thought to be in the membrane domain of the enzyme due to its lipophilic nature. The ND1 in mitochondria (NuoH in E. coli) was identified as a quinone- and rotenone-binding protein by photoaffinity labeling using rotenone analogs (15, 16) and by mutational studies of human mitochondrial DNA (17–19). The results of rotenone binding were used to indicate that ubiquinone binds to the same site. Recently, the inhibitor/quinone-binding site was postulated to be located at the interface between the peripheral and membrane domains of complex I and to involve subunits NuoH and NuoD (20, 21). This suggestion stemmed from identification of a missense mutation in the hydrophilic subunit NuoD of Rhodobacter capsulatus that conferred resist-
Ubiquinone-binding Site

Identification of Endogenous Quinone in E. coli Complex I

Materials—Sodium cholate was obtained from Sigma and re-crystallized from methanol. n-Dodecyl-β-D-maltoside was purchased from AnaTrace. In-stead of dilution mixture, chloroform from ICN, Octyl-Chloroform were of the highest purity commercially available. The ubiquinone derivatives, 2,3-dimethoxy-5-methyl-6-isoprenoyl-1,4-benzoquinone (Q), 2,3-dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinone (Q, Br), azido-Q, 3-azido-2-methyl-5-methoxy[3H]-6-decyl-1,4-benzoquinone (1H-azido-Q), and 5-azido-2,3-dimethoxy-6-decyl-1,4-benzoquinone, were synthesized by methods reported previously (23).

EXPERIMENTAL PROCEDURES

Enzyme Preparations and Assays—E. coli Complex I was prepared and used as described previously (6). Complex I, azido-Q-treated or untreated, was mixed with asolectin at a ratio of 1:20 (by weight) and incubated at 4°C for 15 min before assaying for activity. The reaction mixture (1 ml) contained 50 mM Tris-Cl buffer, pH 7.5, 5 mM NaCl, 0.15% dodecyl maltoside, 100 μM NADH, and 60 μM Q. The reaction was started by addition of an appropriate amount of azido-Q-treated or untreated complex I. The oxidation of NADH was followed by measuring the absorption decrease at 340 nm, using a millimolar extinction coefficient of ε240nm = 6.22 mmol−1 cm−1.

Identification of Endogenous Quinone in E. coli Complex I—Quinones were extracted from purified E. coli complex I with hexane as reported previously (24). The concentration of quinone was determined by the method of Redfern (24). A millimolar extinction coefficient of 12.25 mmol−1 cm−1 was used as the difference in absorption of the oxidized and reduced forms of Q at 275 nm. Quinone identity was determined by matching the retention time of quinone obtained from complex I with those of reference quinones, Q, Q, and Q, in a HPLC system using a Nova-Pak reverse phase column (C18; 3.9 × 150 mm) from Waters, eluting with a linear gradient of methanol from 90% to 100% (v/v) in 20 ml at a flow rate of 0.8 ml/min.

Photoaffinity Labeling of E. coli Complex I with [3H]Azido-Q—The dodecyl maltoside present in purified complex I was replaced with sodium cholate by repeated dilution-concentration using centricon-30 as described previously (25). Complex I, as prepared (specific activity, 0.301 μmol of NADH oxidized/min/mg of protein), was in 50 mM NaCl, 0.15% dodecyl maltoside, 50 mM MES/NaOH, pH 6.0. This complex was diluted with 1% sodium cholate to a protein concentration of 1 mg/ml in 50 mM K+/Na+ phosphate buffer, pH 7.5, and concentrated to 10 mg/ml by centrifugation for 30 min at 3,000 rpm, using a JS-42 rotor in a Beckman centrifuge J6-HC. The concentrated complex I was diluted again with the same buffer containing 1% sodium cholate and concentrated again to 10 mg/ml. This process was repeated eight times. The complex (specific activity, 0.287 μmol of NADH oxidized/min/mg of protein) was then adjusted to a protein concentration of 4 mg/ml in the same buffer containing 1% sodium cholate. 300 μl of this solution was mixed with 5 μl of [3H]azido-Q (9.0 nm in 95% ethanol) and incubated at 0°C for 30 min in the dark. The specific radioactivity of [3H]azido-Q used was 9.7 × 106 cpm/mmol in 95% ethanol and 3.6 × 105 cpm/mmol in the 50 mM K+/Na+ phosphate buffer, pH 7.5, containing 1.0% sodium cholate in the presence of E. coli complex I. This mixture was transferred to a 2-mm light path quartz cuvette that was sealed with paraffin film and mounted on an inclined stage microscope. The assembly was immersed in ice water in a container with a quartz window and illuminated with long-wavelength UV light (Spectroline EN-14, 365-nm wavelength, 23 watts) for 10 min at a distance of 4 cm from the light source. NADH-Q oxidoreductase activity was assayed after reconstitution with asolectin, before and after the illumination.

To determine the amount of [3H]azido-Q incorporated into complex I, illuminated and unilluminated samples were subjected to SDS-PAGE gel and developed with a mixture of chloroform and methanol (2:1, v/v) to remove non-protein-bound [3H]azido-Q. After the paper was air-dried, the origin spot was cut into small pieces and subjected to liquid scintillation counting.

Determination of the Distribution of 3H Radioactivity among the Subunits of E. coli Complex I—The illuminated, [3H]azido-Q-treated sample was digested with 1% SDS and 1% β-mercaptoethanol at 37°C for 2 h before being subjected to SDS-PAGE. The SDS-polyacrylamide gel was prepared according to Schägger and von Jagow (26), but bisacrylamide was substituted for the cleavable cross-linker, N,N′-diallyltartardiamide. Electrophoresis was run at 30 V for 2 h and then at 80 V for another 6 h. After electrophoresis, the gel was stained and dried according to the method of von Jagow (26). The portion containing no protein was also sliced to the same size as that of the protein bands. Gel slices were completely dissolved by incubation in 0.3 ml of 3% periodic acid at room temperature for 1 h. 5 ml of Insta-Gel counting fluid was added, and radioactivity was determined.

Isolation of [3H]Azido-Q-labeled NuoM—Part of the digest (see above) was subjected to preparative SDS-PAGE. A small portion (10%) of the SDS-digested protein solution was treated with fluorescamine (100-fold molar excess) for 10 min at 37°C and placed in the reference wells, located on both edges and the middle of the gel. The SDS-PAGE gel and electrophoresis conditions were as described in the preceding section. The protein bands were visualized by the UV fluorescence in the reference lanes. The SDS-PAGE pattern of the fluorescamine-treated sample was identical to that of the untreated sample, as established by Coomassie Blue staining. The NuoM protein band was excised from the SDS-PAGE gel. The protein was eluted from the combined gel slices with an electro-eluter from Bio-Rad.

Protease K Digestion of [3H]Azido-Q-labeled NuoM—Purified [3H]azido-Q-labeled NuoM obtained by electro-elution was subjected to a repeated dilution and concentration process using centricon-30 with a dilution buffer of 30 mM Tris-Cl, pH 7.5, to remove SDS. The final protein concentration was about 1 mg/ml, with the SDS concentration around 0.5%. Protein was then digested with protease K at 37°C for 6 h using a protease K/NuoM ratio of 1:50 (w/w).

Isolation of Ubiquinone-binding Peptides—Aliquots (100 μl) of the protease K-digested NuoM were separated by HPLC on a Supersil LC-308 column (C8; 5-μm particles; 300 A pores; inner diameter, 4.6 mm; length, 25 cm) using a gradient formed from 0.1% trifluoroacetic acid and 90% acetonitrile containing 0.1% trifluoroacetic acid with a flow rate of 0.8 ml/min. 0.8-ml fractions were collected. For each fraction, the absorbance (from 290 to 400 nm) was recorded with a Waters 996 Diode Array Detector, and radioactivity was measured. Peaks with high specific radioactivity were collected, dried, and subjected to peptide sequence analysis.

Amino Acid Sequence Determination—Amino acid sequence analyses were done at the Molecular Biology Resource Facility, Saint Francis Hospital of Tulsa Medical Research Institute, University of Oklahoma Health Sciences Center, under the supervision of Dr. M. R. Judson.

Effect of Cofactors on the Labeling—Aliquots of 0.94 nmol of NADH-Q oxidoreductase from E. coli in 50 mM K+/Na+ phosphate buffer, pH 7.5, containing 1.0% sodium cholate were incubated with [3H]azido-Q (18.7 nmol) for 30 min at 0°C in the dark, and then NADH, NAD, or ATP (400 μmol, final concentration) plus 1 μmol sodium pyruvate were added for activation, and the incubation was continued for 30 min. Residual NADH was oxidized by L-lactate dehydrogenase (type II, from rabbit muscles) to NAD before photoradiation because it would quench the UV light responsible for photoactivation of [3H]azido-Q. Then, the mixtures were illuminated under UV light for 10 min at 0°C as described above.

The abbreviations used are: azido-Q, 3-azido-2-methyl-5-methoxy-6-decyl-1,4-benzoquinone; Q, 2,3-dimethoxy-5-methyl-6-isoprenoyl-1,4-benzoquinone; Q, Br, 2,3-dimethoxy-5-methyl-6-(1-bromodecyl)-1,4-benzoquinone; Q, HPLC, high performance liquid chromatography; [3H]azido-Q, 3-azido-2-methyl-5-methoxy[3H]-6-decyl-1,4-benzoquinone; MES, 2-morpholinoethanesulfonic acid.
Preparation of NADH-Q Oxidoreductase—NADH-Q oxidoreductase, prepared according to the procedure described previously (6), contains 0.5 mol of bound coenzyme Q-8 (ubiquinone-40)/mol of protein. When this preparation is titrated with exogenous Q_10Br, no Q binding is observed, suggesting that the vacant Q-binding site(s) are masked by the detergent decyl maltoside or phospholipids and that the binding affinity of Q_10Br is weaker than that of endogenous Q, phospholipid, and detergent used. Because the binding affinity of azido-Q derivatives to the Q-binding sites of several Q-binding proteins was reported to be weaker than that of 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone or Q_10Br (23, 27–29), study of the Q/protein interaction in NADH-Q oxidoreductase, using azido-Q derivatives, requires prior removal of endogenous Q from the complex and an unmasking of the Q-binding sites.

Preparation of NADH-Q oxidoreductase by partial purification was performed as described previously. The sample was then diluted and centrifuged as described previously (6), containing the indicated concentrations of azido-Q derivative, and was then mixed with the enzyme, no inhibition is observed. Inactivation is also not due to protein damage by UV radiation because when the enzyme alone is illuminated, no activity loss is observed. Because the activation of the azido-Q-treated NADH-Q oxidoreductase, after illumination, is assayed in the presence of excess Q_8 (60 μM), the extent of inactivation should be proportional to the fraction of the Q-binding sites covalently linked to azido-Q.

Correlation between Azido-Q Incorporation and Inactivation of NADH-Q Oxidoreductase—To further confirm that the inactivation observed results from covalent linkage of azido-Q to protein in the complex, azido-Q uptake and the extent of inactivation were determined for different periods of illumination. As shown in Fig. 3, when the complex is treated with a 20-fold molar excess of azido-Q and illuminated for different time periods, activity decreases as illumination time increases; maximum inactivation (40%) is reached at 10 min. Moreover, the amount of azido-Q incorporated into protein parallels the extent of inactivation, until the maximum is reached, suggesting that inactivation results from binding of azido-Q to the Q-binding site. Although illumination for longer than 10 min causes no further decrease in activity, azido-Q uptake continues, but at a slower rate, indicating that this incorporation is due to nonspecific binding of azido-Q to protein. It should be mentioned that a control sample containing the same amount of ethanol, illuminated under identical conditions, shows little (<5%) activity loss over the time periods studied.

Identification of Q-binding Subunit in NADH-Q Oxidoreductase by Photoaffinity Labeling with [3H]Azido-Q Derivatives—Because the uptake of azido-Q derivative by NADH-Q oxidoreductase was identified by partial NH_2-terminal amino acid sequence analysis as a proteolytic digestion product of NADH-Q oxidoreductase, after illumination, is assayed in the presence of a 20-fold molar excess of azido-Q and illuminated for different time periods, activity decreases as illumination time increases; maximum inactivation (40%) is reached at 10 min. Moreover, the amount of azido-Q incorporated into protein parallels the extent of inactivation, until the maximum is reached, suggesting that inactivation results from binding of azido-Q to the Q-binding site. Although illumination for longer than 10 min causes no further decrease in activity, azido-Q uptake continues, but at a slower rate, indicating that this incorporation is due to nonspecific binding of azido-Q to protein. It should be mentioned that a control sample containing the same amount of ethanol, illuminated under identical conditions, shows little (<5%) activity loss over the time periods studied.

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doreductase upon illumination is correlated to the enzymatic inactivation, it is reasonable to assume that the azido-Q derivative is bound specifically to the Q-binding site(s). Thus, the distribution of the covalently bound azido-Q among subunits of NADH-Q oxidoreductase after SDS-PAGE indicates the specific Q-binding protein in this enzyme complex. Fig. 4 shows the \(^3H\) radioactivity distribution among subunits of NADH-Q oxidoreductase. The advantage of using the acrylamide/N,N\(^\prime\)-diallytartardiamide gel system, rather than the commonly used acrylamide/bisacrylamide system, is that the gel slices can be completely dissolved in 3% periodic acid, and this solution can be used directly for radioactivity determination. The acrylamide/N,N\(^\prime\)-diallytartardiamide gel system has been used to identify Q-binding proteins in a bacterial reaction center (31) and in mitochondrial ubiquinol-cytochrome c reductase (23). The electrophoretic pattern of illuminated, azido-Q-treated NADH-Q oxidoreductase obtained with the acrylamide/N,N\(^\prime\)-diallytartardiamide gel system is similar to that obtained from the acrylamide/bisacrylamide gel system; 11 major protein bands are observed in each (by Coomassie Blue staining). Radioactivity is found in protein band 5, suggesting that this subunit provides the Q-binding site. No radioactivity is found in slices from a gel loaded with illuminated buffer containing \[^{3}H\]azido-Q and 1% sodium cholate. Because the amount of radioactivity in protein band 5 was directly proportional to the extent of inactivation of the oxidoreductase, participation of this protein in Q binding is established.

This radioactive protein band in SDS-PAGE of the illuminated \[^{3}H\]azido-Q-treated NADH-Q oxidoreductase is identified as NuoM, based on the identification of two NuoM peptides in the protease K digest of the labeled protein. Peptide peaks with retention times of 29.9 and 45.8 min obtained from HPLC separation of protease K-digested labeled protein have the partial NH\(_2\)-terminal amino acid sequence of NH\(_2\)-SAAGLFI- and NH\(_2\)-LPDAH- corresponding to residues 351–357 and 244–248 of NuoM subunit, respectively.

The identification of NuoM as the ubiquinone-binding subunit of NADH-Q oxidoreductase is consistent with the report (32) that human complex I lacking the mtDNA-encoded subunit ND4, due to a frameshift mutation in the gene, has no NADH-Q\(_1\) oxidoreductase activity but has normal NADH: Fe(CN)\(_6\) oxidoreductase activity. ND4 of human complex I is the counterpart of NuoM of \(E.\) coli enzyme (33).

**Ubiquinone-binding Site**

**Fig. 3.** Effect of illumination time on azido-Q uptake and inactivation of NADH-Q oxidoreductase. The NADH-Q oxidoreductase (1 mg/ml) in 50 mM K\(^+\)/Na\(^+\) phosphate buffer, pH 7.5, containing 1% sodium cholate was incubated with azido-Q in ethanol (○ and △) or ethanol only (●) for 30 min at 0 °C in the dark. The samples were then illuminated with long-wavelength UV light for the indicated times at 0 °C. The determination of activity (○ and △) and radioactivity (△) was performed as described under "Experimental Procedures."

**Fig. 4.** \(^3H\) radioactivity distribution among subunits of NADH-Q oxidoreductase. Purified NADH-Q oxidoreductase was treated with a 20-fold molar excess of \[^{3}H\]azido-Q in the dark for 30 min, illuminated for 10 min at 0 °C, and digested with 1% SDS and 1% \(\beta\)-mercaptoethanol at 37 °C for 2 h before being applied to a SDS-PAGE gel. The electrophoretic conditions are described under "Experimental Procedures." Protein bands were visualized by Coomassie Brilliant Blue, after staining and de-staining, and sliced. The portion containing no protein was also sliced to the same size as that of the protein bands. The gel slices were dissolved with 3% periodic acid and mixed with 5 ml of Insta-Gel, and the radioactivity was determined.

**Fig. 5.** \(^3H\) radioactivity distribution in an HPLC chromatogram of a protease K digest of \[^{3}H\]azido-Q-labeled NuoM protein. The labeled protein (1 mg/ml, \(1 \times 10^5\) cpm/mg) was digested, fractionated, and assayed for radioactivity as described under "Experimental Procedures."
elution, and repeated dilution/concentration with centrifprep-30. The SDS-PAGE step removes non-protein-bound azido-Q adducts. The SDS concentration in the final purification step is about 0.5%, whereas the concentration of [3H]azido-Q-labeled NuoM is about 1 mg/ml. Isolated [3H]azido-Q-labeled NuoM shows only one band, in SDS-PAGE, which corresponds to the fifth subunit of NADH-Q oxidoreductase (data not shown). About 40% of the NuoM protein present in NADH-Q oxidoreductase is recovered in the final purification step, assuming a molecular mass of 535 kDa for the E. coli NADH-Q oxidoreductase and that it contains 1 mol of NuoM/mol of enzyme. This low yield of NuoM is probably due to our very small slicing of the NuoM band, in order to avoid contamination with neighboring proteins.

When SDS present in purified [3H]azido-Q-labeled NuoM was removed by the commonly used cold acetone precipitation method, the resulting protein is highly aggregated and resistant to proteolytic enzyme digestion. Inclusion of 0.1% SDS and 2 M urea in the digestion mixture does not increase proteolysis. Because the SDS-free, [3H]azido-Q-labeled NuoM is not digested by proteolytic enzymes, we needed a protease that is active when SDS concentration is higher than 0.5%. Of the commercially available proteolytic enzymes, only protease K was reported to be active in 0.5% SDS and 1 M urea; therefore,

Table I

| Additions | Relative amount of radioactivity incorporated by protein |
|-----------|--------------------------------------------------------|
| Control   | 100%                                                   |
| NADH (400 mM) | 99                                                   |
| NAD (400 mM)  | 98                                                   |
| ATP (400 mM)   | 99                                                   |

* 100% indicates 5,626 cpm/nmol of protein.

![Diagram](https://example.com/diagram.png)

**Fig. 6. Putative Q-binding domain in the proposed structure of NuoM.** The proposed secondary structure of NuoM of E. coli NADH-Q oxidoreductase was constructed from the hydropathy plots of its amino acid sequence using Toppred hydropathy analysis (a) and the PHDhtm program (b). The shaded area indicates the Q-binding peptide identified in this report. The Q-binding motif (L-X3-H-X3-T) in complex I predicted by Fisher and Rich (35) is shown by squares.
Experimental Procedures.

To obtain the optimal digestion time, 100 μM H9262 digestion at 37 °C of the proposed Q-binding motif (L-X-T) was analyzed (34) of NuoM, this Q-binding peptide overlaps with a substantial amount of endogenous Q (0.3 mol/mol of protein) in inhibition of the azido-Q-treated sample is less than 50%, and it rules out the possibility of more than one Q-binding site because the Q-binding peptide is identified in this study, one cannot rule out binding in NADH-Q oxidoreductase (15, 16). Although only one Q-binding site was identified in this study differs from that identified for rotenone in transmembrane helix 4, toward the periplasmic side of the membrane. It should be noted that the Q-binding domain identified in transmembrane helix 5 toward the cytoplasmic side of the membrane (see Fig. 6a). If the PHDhtm hydropathy plot (36) is used (see Fig. 6b), the Q-binding peptide is located in transmembrane helix 4, toward the periplasmic side of the membrane. This should be noted that the Q-binding domain identified in this study differs from that identified for rotenone binding in NADH-Q oxidoreductase (15, 16). Although only one Q-binding peptide is identified in the this study, one cannot rule out the possibility of more than one Q-binding site because inhibition of the azido-Q-treated sample is less than 50%, and a substantial amount of endogenous Qₐ (0.3 mol/mol of protein) remains in the Q-deficient complex. The residual Qₐ would render a portion of the Q-binding site or a different Q-binding site inaccessible to azido-Q.

Recently, Nakamura-Ogiso et al. (37) have identified subunit ND5 (NuoL in E. coli) as a Q-binding site based on the pho-

toaffinity labeling study of submitochondrial particles using an analog of fenpyroximate, a specific inhibitor of complex I. Although the assumption that this inhibitor binds directly at the Q-binding site is difficult to establish, especially with inhibitors whose chemical structures have little resemblance to Q, identification of ND5 as one of the subunits possibly involved in quinone binding is very interesting because ND5 also contains a Q-binding motif, A-X₃-H-X₇-T. ND5 is composed of 14 transmembrane helices in the Toppred hydropathy plot; the Q-binding motif is located at the connecting loop of transmembrane helices 8 and 9 on the periplasmic side of the membrane. In the similar plot, the Q-binding motif of NuoM, L-X₇-H-X₇-T, is located in the end of transmembrane helix 5 toward the cytoplasmic side of the membrane. This would indicate that the Q-binding site in ND5 is not a part of the Q-binding site located in ND4. On the other hand, if PHDhtm hydropathy plots are compared, then the Q-binding motif in ND5 could be a part of the Q-binding site in ND4 because the motif of ND5 is located on the end of transmembrane helix 9 toward periplasmic side of the membrane, which is spatially similar to the Q-binding motif of NuoM located at the connecting loop of transmembrane helices 4 and 5 on the periplasmic side of the membrane. In this context, it is noteworthy to mention that ND4 and ND5 have evolved from a common ancestor (38) and might share related but not identical functions in NADH-Q oxidoreductase (11).

Effects of Cofactors on the Labeling of NuoM by [3H]Azido-Q—Table I shows the effects of 400 mM NADH, NAD, and ATP on [3H]Azido-Q labeling of NuoM. NAD and ATP had essentially no effect, which is consistent with the data reported recently by Nakamura-Ogiso et al. (37) during photoaffinity labeling of inhibitor/quinone probes in complex I when isolated submitochondrial particles were used. [3H]Azido-Q labeling of NuoM subunit was not influenced by prior NADH incubation, even though NADH has been reported to stimulate the labeling of inhibitor/quinone probes in mitochondrial complex I (37, 39). Similar data were also obtained with a [3H]pyridaben analog labeling of Nqo6 (PSST in mitochondria) subunit from Paracoccus denitrificans (37). Activation of NADH-Q oxidoreductase by NADH has been demonstrated solely for the mitochondrial enzyme, but not for the bacterial enzyme. The E. coli NADH-Q
The binding site(s) of FAD is not influenced by addition of inhibitors, indicating that the binding site(s) may not be identical to the quinone binding site(s).

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