Mass Spectrometric Analysis of the Ubiquinol-binding Site in Cytochrome bd from Escherichia coli

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Cytochrome bd (CydAB) is a heterodimeric ubiquinol oxidase in the aerobic respiratory chain of Escherichia coli. For understanding the unique catalytic mechanism of the quinol oxidation, mass spectrometry was used to identify amino acid residue(s) that can be labeled with a reduced form of 2-azido-3-methoxy-5-methyl-6-geranyl-1,4-benzoquinone or 2-methoxy-3-azido-5-methyl-6-geranyl-1,4-benzoquinone. Matrix-assisted laser desorption ionization quadrupole time-of-flight mass spectrometry demonstrated that the photo inactivation of ubiquinol-1 oxidase activity was accompanied by the labeling of subunit I with both azidoquinols. The cross-linked domain was identified by reverse-phase high performance liquid chromatography of subunit I peptides produced by in-gel double digestion with lysyl endopeptidase and endoproteinase Asp-N. Electro spray ionization quadrupole time-of-flight mass spectrometry determined the amino acid sequence of the peptide (1047.5) to be Glu278–Lys283, where a photoproduct of azido-Q2 was linked directly that the N-terminal region of periplasmic loop VI/VII (Q-loop) is a part of the quinol oxidation site and indicates that the 2- and 3-methoxy groups of the quinone ring are in the close vicinity of I-Glu280.

Cytochrome bd (CydAB) is a heterodimeric ubiquinol oxidase in the aerobic respiratory chain of Escherichia coli and is predominantly expressed under microaerophilic growth conditions (see Refs. 1–3 for reviews). It catalyzes dioxygen reduction with two molecules of ubiquinol-8 (Q8H2), leading to the release of four protons from quinols to the periplasm. Through a putative proton channel, four protons used for dioxygen reduction are taken up from the cytoplasm and delivered to the dioxygen reduction site at the periplasmic side of the cytoplasmic membrane (4). During dioxygen reduction, cytochrome bd generates an electrochemical proton gradient (ΔpH and membrane potential) across the membrane through apparent transmembrane movement of four chemical protons (5–7). In contrast to cytochrome bo, an alternative oxidase under highly aerated growth conditions, cytochrome bd has no proton pumping activity and does not belong to the heme-copper terminal oxidase superfamily.

On the basis of spectroscopic and ligand binding studies, three distinct redox metal centers have been identified as heme b558, heme b569, and heme d (see Ref. 8 for a review). Unlike cytochrome bo, cytochrome bd does not contain a tightly bound Qn. Heme b558 is a low spin protoporphyrin IX and is ligated by 1-His166 (helix V) and 1-Met195 (helix VII) of subunit I (CydA) (9). Heme b569 is a high spin protoporphyrin IX bound to 1-His19 (helix I) of subunit I (9) and mediates electron transfer from heme b558 to heme d, where dioxygen is reduced to water (10–13). Heme d is a high spin chlorin bound to an unidentified nitrogenous ligand (14–16) and forms a di-heme binuclear center with heme b569 (16, 17). Topological analysis suggests that all of the hemes are located at the periplasmic end of transmembrane helices (4).

In loop VI/VII (Q-loop) of subunit I, binding of monoclonal antibodies to 253KLAIAEEWET262 (18, 19) and proteolytic cleavage with trypsin at I-Tyr290 or chymotrypsin at I-Arg298 (20, 21) suppressed ubiquinol oxidase activity. Photoaffinity labeling studies with 2-methyl-3-azido-5-methoxy-6-(3,7-dimethyl-1H)octyl)-1,4-benzoquinone ([1H]3-azido-2-methyl-5-methoxy-BQn) indicate the presence of the quinol oxidation site in subunit I (22). These results suggest that periplasmic loop VI/VII in subunit I is involved in the ubiquinol oxidation site. Inhibitor binding studies indicated the close proximity of the quinol oxidation site to heme b558 (23, 24).

Recently, the x-ray structure of the E. coli cytochrome bo has been solved at 3.5-Å resolution, but the structure of the quinol oxidation site remains unknown (25). A low affinity for substrates and the difficulty in anaerobiologically keeping substrates as reduced forms make it difficult to crystallize a quinol-bound form. Currently, no atomic structure for cytochrome bd is available; thus photoaffinity cross-linking with photosensitive substrate analogs is an alternative approach to identify the substrate-binding site. We have developed an efficient method for synthesis of 2-azido-3-methoxy-5-methyl-6-geranyl-1,4-benzoquinone (2-azido-Qn), and 2-methoxy-3-azido-5-methyl-6-geranyl-1,4-benzoquinone (3-azido-Qn) (26), which are more closely related to native ubiquinone...
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**FIGURE 1. Time course of photoinactivation of cytochrome bd with 2-azido- and 3-azido-Q2H2.** The enzyme (10 μM in 50 mM potassium phosphate (pH 7.4) containing 0.1% sucrose monolaurate) was incubated on ice for 10 min in a quartz cuvette and illuminated with long wavelength UV light. The samples are controls without illumination ( ), with illumination in the absence of substrate ( ), 40 μM 2-azido-Q2H2 ( ), and 40 μM 3-azido-Q2H2 ( ).

(2,3-dimethoxy-5-methyl-6-polypropenyl-1,4-benzoquinone) than azidoquinones used for previous studies (3-azido-2-methyl-5-methoxy-BQ, for cytochrome bd(22), 2-methyl-3-azido-5-methoxy-6-geranyl-1,4-benzoquinone (3-azido-2-methyl-5-methoxy-BQ2) for bovine Complex III (27), and the E. coli cytochrome bo (28, 29) and the E. coli Complex II (30) and 2-methyl-3-azido-5-methoxy-6-n-decyl-1,4-benzoquinone (3-azido-2-methyl-5-methoxy-dBQ) for bovine Complex II (31, 32) and Complex I (33).

Here we report the azido-Q2H2-labeled site in cytochrome bd, determined by mass spectrometry (MS). Reduced forms of 2-azido- and 3-azido-Q2 serve as efficient electron donors to cytochrome bd, and UV illumination in the presence of azido-Q2H2 inactivated the quinol oxidase activity. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS identified subunit I as the azido-Q2-labeled site. In-gel digestion followed by electrospray ionization tandem mass spectrometry (ESI MS/MS) revealed the cross-linked amino acid residue for the first time. The oxidized form of a photoproduct of azido-Q2 (azido-Q2*) was covalently linked to the carboxylic side chain of I-Glu280 in the hexapeptide EEETNK283, that is present in the Q-loop. This study demonstrated directly that the N-terminal region of periplasmic loop VI/VII (Q-loop) is a part of the quinol oxidation site and indicates that the 2- and 3-methoxy groups of the quinone ring are in the close vicinity of I-Glu280 in loop VI/VII.

**EXPERIMENTAL PROCEDURES**

**Purification of Cytochrome bd and Quinol Oxidase Assay**—The enzyme was isolated from the cytochrome bd-overproducing strain GR84N/pNG2 (34), as described previously (14). The concentration of the enzyme was calculated from the Soret absorption of the air-oxidized form by using an extinction coefficient of 223,000 M⁻¹ cm⁻¹ (35). The purified enzyme in 50 mM potassium phosphate (pH 6.8) containing 0.1% sucrose monolaurate (Mitsubishi-Kagaku Foods Co., Tokyo) was stored at −80°C until use.

The enzyme activity was determined at 25°C with a JASCO V-550 UV-visible spectrophotometer, as described previously (36). The reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 7.3), 0.1% sucrose monolaurate, and 40 mM cytochrome bd. The reaction was started by the addition of a reduced form of Q1, a kind gift from Eisai Co. (Tokyo, Japan), at a final concentration of 0.14 mM.

**Photoaffinity Labeling of Cytochrome bd—2-Azido-Q2 and 3-azido-Q2 were synthesized as previously described (26). Cytochrome bd (10 μM in 0.5 ml of 50 mM potassium phosphate (pH 7.4) containing 0.1% sucrose monolaurate) was placed in a quartz cuvette and incubated on ice for 10 min. A 4-fold molar excess of reduced azido-Q2 was added to the enzyme solution and subjected to illumination on ice with long wavelength UV light (Black-Ray model B-100 A; UVP, Upland, CA; 365 nm) at a distance of 5 cm from the light source. For the competition with azido-Q2H2, 2-n-heptyl-hydroxyquinoline-N-oxide (HQNO; Sigma-Aldrich) was added at a final concentration of 1 mM. Q1H2 oxidase activity was measured before and after the illumination.

**In-gel Digestion of Subunit I**—Samples containing 20 μg of proteins were subjected to 10% SDS-PAGE as described by Laemmli (37), and protein bands were stained with Coomassie Brilliant Blue R-250. In-gel digestion was performed by the method of Hellman et al. (38). Gel pieces containing subunit I band were washed three times with 1 ml of 70% (v/v) acetoni tri le, dried in a vacuum centrifuge, and rehydrated with 0.1 ml of the digestion buffer. The enzymatic cleavage was allowed to proceed at 37°C for 18 h at a protease-to-protein ratio of 1:200. The digestion buffers used for lysyl endopeptidase (Lep; Wako Pure Chemical Industries, Osaka) and endoprotease Asp-N (Roche Applied Science) were 50 mM Tris-HCl (pH 9.0) and 20 mM sodium borate (pH 7.0), respectively. Digestion was terminated by the addition of 1 μl of acetic acid.

**FIGURE 2. MALDI-TOF mass spectra of subunits I and II of cytochrome bd without (A) and with UV illumination in the presence of 2-azido-Q2H2 (B) and 3-azido-Q2H2 (C).** The insets show the mass range for subunit I.

**FIGURE 3. Reverse-phase HPLC chromatograms of in-gel double-digested subunit I with Lep and Asp-N.** Subunit I was digested by Lep and Asp-N. The samples were without (A) and with illumination in the presence of 2-azido-Q2H2 ( ), and 3-azido-Q2H2 ( ).
In-gel CNBr cleavage was performed in 0.1 ml of 70% trifluoroacetic acid at a CNBr-to-protein ratio of 1:10. The gel pieces were first incubated with Lep, washed three times with 1 ml of 70% (v/v) acetonitrile, and then treated with Asp-N or CNBr as described above. The peptides were extracted twice from the gel pieces with 100 μl of 60% (v/v) acetonitrile and concentrated with ZipTip μ-C18 (Millipore) to 20 μl in 60% acetonitrile containing 0.1% trifluoroacetic acid.

MALDI-TOF MS—The samples were analyzed on an AXIMA-CFR plus (Shimadzu Co., Kyoto) TOF mass spectrometer in a linear (for proteins) or reflection (for peptides) mode with positive ion detection. The matrix solution was 50% acetonitrile saturated with -cyano-4-hydroxycinnamic acid (Sigma-Aldrich). One-μl of desalted proteins or peptides were mixed with an equal amount of the matrix solution on the target plate and dried. A nitrogen laser at 337 nm was used to desorb solute molecules from the sample plate. The coarse laser energy was set to 50% with fine adjustment for each sample, and 1–100 laser shots were accumulated for each spectrum. A voltage of 20 kV was established in the source region, and the microchannel detector was set at 2.8 kV. MS spectra were calibrated externally with rabbit muscle aldolase and bovine serum albumin (molecular masses were 39,212 and 66,430 Da, respectively) for proteins and human bradykinin fragment, human angiotensin II, P3,R, human ACTH fragment 18–39, and bovine insulin oxidized B chain (molecular masses were 757.40, 1,046.54, 1,533.86, 2,465.20, and 3,494.65 Da, respectively) for peptides. Data analysis was carried out with Kompact (Shimadzu Co.).

Reverse-phase HPLC—Subunit I peptides (100 μg) produced by double digestion with Lep and Asp-N were separated by reverse-phase HPLC on a Develosil 300C8-HG-5 column (4.6-mm inner diameter × 15 cm; Nomura-Kagaku Co.) using a gradient formed from 0.1% acetic acid and 30% acetonitrile containing 0.1% acetic acid with a flow rate of 1 ml/min. Elution profiles of peptides were monitored at 212 nm with a SPD-M10A, Shimadzu photodiode array detector, and 0.5-ml fractions were collected. The fractions were dried up with a vacuum centrifuge, and the peptides were dissolved in 30 μl of 0.1% formic acid.

ESI MS—The samples were applied to a C18 column (0.1-mm inner diameter × 25 cm; GL Science) coupled to ESI source and analyzed on a Q-TOF2 (Micromass) quadrupole TOF mass spectrometer. The analysis was carried out in the positive ion detection mode with a capillary voltage of 2.8 kV and a sampling cone voltage of 40 V. For MS experiments, the quadrupole analyzer was used in wide band pass mode, and the microchannel plate detector was set at 2.7 kV.

For MS/MS experiments the quadrupole (first) analyzer was used to select sequentially the peaks of interest in the m/z spectrum, allowing one particular precursor or parent mass to proceed through the collision cell into the TOF (second) analyzer. Argon gas was admitted into the collision cell so that the pressure in that region increased by a factor of 10, and the collision energy was 10 eV. The resulting fragment ions were analyzed, and MS/MS spectra acquired over the appropriate m/z range and were deconvoluted with MaxEnt3 (Micromass). Data processing was achieved manually with the aid of the PepSeq program of MassLynx package (Micromass). The nomenclature used for fragment ions was N-terminal C=O-containing fragments (b type) and C-terminal N-H-containing fragments (y type), which were derived by the cleavage at the middle of peptide bonds (39).
**RESULTS**

*Photo Inactivation of Cytochrome bd with Azido-Q2H2*—Electron-donating activities of 2-azido- and 3-azido-Q2H2 to cytochrome bd are 97 and 94%, respectively, of that of Q2H2 at 0.04 mM, and thus they are suitable for probing the structure of the quinol oxidation site. After 15 min of illumination with UV light in the presence and absence of 0.04 mM 3-azido-Q2H2, Q1H2 oxidase activity was decreased to 28 and 68%, respectively, of the original activity without illumination (Fig. 1). Monophasic decay of the oxidase activity indicates the labeling of the quinol oxidation site with a single azido-Q2H2 molecule (data not shown). UV illumination in the presence of 0.04 mM 2-azido-Q2H2 resulted in a 40% loss of the quinol oxidase activity. Inhibition levels are comparable with 35–50% reported for reactions of azidoquinones with cytochrome bd (22), cytochrome bo (28), succinate dehydrogenase (30), and DsbB (56). A difference in the inactivation levels between 2-azido- and 3-azido-Q2H2 may be related to interactions of their photoproducts with the protein. The incomplete inactivation with azido-Q2H2 may be partly due to the intramolecular reaction with the nearby methoxy group on the quinone ring. These results indicate that the photoaffinity labeling of azido-Q2H2 to the quinol-binding site inactivates the quinol oxidase activity of cytochrome bd.

Our azido-Q2 derivatives are not radioactive; thus labeling profiles were examined by mass spectrometry. In MALDI-TOF mass spectra of the purified cytochrome bd, subunits I and II were identified as singly protonated ions at \( m/z \) 58,160 ± 60 and 42,410 ± 70 (n = 5) (Fig. 2), respectively, which are comparable with the calculated molecular
masses of 58,205 and 42,460 Da, respectively, from the deduced amino acid sequence (40). Upon UV illumination in the presence of 2-azido- or 3-azido-Q2H2, a part of subunit I molecules increased molecular mass, indicated by the presence of a shoulder peak at a higher m/z region (Fig. 2). In contrast, subunit II remained as a sharp peak, and nonspecific binding to subunit II (22) did not occur for our azido-Q2H2 derivatives. These observations indicate that the quinol-binding site of cytochrome bd is located within subunit I and that the labeling of subunit I with azido-Q2H2 resulted in the inactivation of the quinol oxidase activity.

Identification of the Azido-Q2-cross-linked Peptide—For the identification of the azido-Q2-cross-linked site by MS, subunit I was separated from subunit II and free substrates and its photoproducts by 10% SDS-PAGE and subjected to in-gel digestion with endoproteinase Lep and/or Asp-N or in-gel cleavage with CNBr. In combination with proteolytic and chemical cleavage, peptides corresponding to 89.1% of the total sequence and 99.4% of the Q-loop were recovered and identified (supplemental Table S1). The coverage was comparable with 79% for subunit II of the E. coli P/Q by MALDI MS (29), 83% for the E. coli H+/galactose symporter GalP by ESI MS (41), and 97% for the E. coli H+/lactose symporter LacY by ESI MS (42).

In the Lep/Asp-N double digest of subunit I labeled with 2-azido- or 3-azido-Q2H2, we reproducibly identified a new peak at 29.5 min in reverse-phase HPLC (Fig. 3, B and C) and at m/z 1047.5 in MALDI-TOF mass spectra (Fig. 4B). Photoaffinity labeling with 3-azido-Q2H2 in the presence of 1 mM HQNO, a competitive inhibitor (I_{50,7/19262} M), eliminated the m/z 1047.5 peak (Fig. 4C), confirming the attachment of the photoproduct to this peptide.

Identification of Azido-Q2-labeled Amino Acid Residue—The Lep/Asp-N double-digested peptide with m/z 1047.5 was subjected to ESI-Q-TOF tandem mass spectrometry (MS/MS). Doubly charged N-terminal fragment ions (b-series) and C-terminal fragment ions (y-series) thus yielded identified the amino acid sequence of this peptide to be the hexapeptide 728EEETNK 283 (monoisotopic mass of 748.32) and demonstrated that a photoproduct (300.36 Da) of 2-azido- (data not shown) and 3-azido-Q2H2 (Fig. 5) is covalently linked to the carboxylic side chain of I-Glu 280 (m/z 128.03) via the COO-NH linkage (Fig. 6D). The b’ series (b3’, b4’, and b5’), which lost the cross-linked Azido-Q2*, confirmed this assignment. Our result demonstrated that the 2- and 3-substituents of the quinone ring are in the vicinity of I-Glu 280 in the...
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periplasmic loop VI/VII of subunit I. The observed mass for the photoproduct indicates a loss of four hydrogen from the expected structure (304.39 Da) (Fig. 6C), because of the auto-oxidation of the reduced photoproduct during the preparation for the photoaffinity labeling.

**DISCUSSION**

Cytochrome bd has been isolated as ubiquinol oxidase from γ-proteobacteria including *E. coli* (5, 43–48) and as menaquinol oxidase from Gram-positive bacteria (49–51). Steady state kinetics in ubiquinol oxidases has been interpreted as a simple Michaelis-Menten type. The $K_m$ values estimated for Q$_1$H$_2$ are usually ~0.1–0.2 mM (5, 22, 43, 45, 47, 48, 52), and the $K_m$ value for Q$_2$H$_2$ has been reported to be 0.05 mM for the *E. coli* enzyme (22). Because of auto-oxidation of low potential quinols, the $K_m$ values are not reported for menaquinol oxidases. Kinetic studies, electron paramagnetic resonance studies on semiquinone anion (53), and inhibitor binding studies (54) are consistent with the presence of a single quinol/quinone-binding site in cytochrome bd.

**Identification of Quinone/Quinol-binding Site with Azidoquinones**

To identify the quinol oxidase site in cytochrome bd, we have improved the method for synthesis of 2-azido- and 3-azido-Q$_2$ (26) and carried out photoaffinity cross-linking studies with the reduced forms. MALDI-TOF MS showed that photo inactivation of the Q$_1$H$_2$ oxidase activity in the presence of azido-Q$_2$H$_2$ was accompanied with the labeling of I-Tyr$_{290}$ or I-Arg$_{298}$ would lose the interactions between the internal repeats (i.e. I-Arg$_{298}$ would lose the interactions between the internal repeats). The sequence 210EQQHEQ220 in the second repeat may be related to the proposed quinone-binding motif (L$_X$H$_X$(T/S/L)$_X$) (55). Similar motifs can be found in the azidoquinone-binding peptides of Complex I (192LTVFHVNT200 in NuoM (23)), Complex II (116SOLIQHSVQ214 in bovine Qps1 (31), 26ASILHRVSG34 in the *E. coli* SdhC (30), and periplasmic disulfide bond protein (87LTYEHTMLQ95 in DsbB (56)). The atomic structure of the *E. coli* Complex II (57) revealed that C-Ile$^{252}$ and the Cβ atom of C-Ser$^{252}$ participate in the hydrophobic quinone-binding pocket and that the C-Arg$_{189}$ side chain is within 4 Å of the 2-methoxy group. Substitutions of C-Ser$_{257}$ and C-Arg$_{271}$ abolished the succinate-ubiquinone reductase activity and the labeling with 3-azido-2-methyl-5-methoxy-dBQ$_2$ (30). Thus photoaffinity cross-linking with azidoquinones has proved to be useful for probing the quinol/quinone-binding site in the respiratory complexes.

Cytochrome bd is widely distributed as the terminal oxidase from Archaea to Eubacteria and can be divided into two groups with or without the C-terminal third of Q-loop (58). Alternatively, there are three groups with different substrate specificity: ubiquinol (α-, β-, and γ-proteobacteria), menaquinol (other bacteria), or plastoquinol (cyanobacteria). Amino acid residues, which define the substrate specificity, remain unknown. I-Glu$^{280}$ is not conserved even in γ-proteobacteria, but we can find acidic residues at approximately the equivalent position, ~10 amino acid residues upstream of the conserved I-Pro$^{289}$ (Fig. 8). Our photoaffinity cross-linking studies with azido-Q$_2$H$_2$ directly demonstrated that I-Glu$^{280}$ in the first internal repeat is a part of the quinol-binding pocket of the *E. coli* cytochrome bd. A hydrophilic segment containing I-Glu$^{280}$ can be predicted to be coil by Jpred (Pro$^{289}$–The$^{295}$) (59), PROF (Ile$^{273}$–Glu$^{280}$) (60), and the New Joint method (Gly$^{273}$–Asn$^{285}$) (61) between two short β-strands, allowing the accommodation of both a bulky azido or methoxy group and a reactive nitrene group on
the quinone ring. In the periplasmic region of subunit I, I-Glu$^{249}$, I-Lys$^{252}$, and I-Glu$^{257}$ at the N terminus of loop VI/VII and I-Arg$^{348}$ at the N terminus of loop VIII/IX are conserved charged and/or hydrophilic residues and could directly provide hydrogen bonds to the hydroxyl and/or oxo group of the quinone ring. Our recent kinetic studies on the loop VI/VII mutants provided the supporting evidence for the involvement of I-Glu$^{249}$, I-Lys$^{252}$, I-Glu$^{257}$, and I-Glu$^{267}$ in the binding and oxidation of ubiquinols (62). In the E. coli cytochrome bd (25), a member of the heme-copper terminal quinol oxidases, a ubiquinone-binding motif (R$_{X}$D plus HX$_{C}$Q) has been proposed for the high affinity quinone-binding site, where Asp$^{75}$ in helix I and His$^{98}$ in helix II in subunit I serve as ligands for the quinone carbonyl groups. Thus quinol/quinone-binding motifs in bacterial quinol oxidases are different from L$_{X}$H$_{X}$T/(T/S/L) proposed by Fischer and Rich (55).

**Mechanism of Photoaffinity Labeling with Azidoquinones**—Upon photolysis of an oxidized form of 3-azido-Q$_{2}$ (329.4 Da; Fig. 6C) and no signal was assigned to solvent insertion adduct. $^{1}H$ NMR spectra also indicated that the number of total protons is identical for 3-azido-Q$_{2}$, and no signal was assigned to solvent insertion adduct. $^{1}H$ NMR spectra showed resonance signals of the methoxy and/or oxogroup of the quinone ring. In the periplasmic region of subunit I, I-Gln$^{249}$, I-Lys$^{252}$, I-Glu$^{257}$, and I-Glu$^{280}$ at the N terminus of loop VI/VII, I-Glu$^{280}$ in the first internal repeat has been demonstrated to be a part of the binding site for the 2- and 3-methoxy groups of the quinone ring. Mutation (62) and x-ray crystallographic studies would provide further insights into the understanding of the catalytic mechanism for the quinol oxidation in bd-type terminal oxidases.

**Acknowledgments**—We thank R. B. Gennis (University of Illinois) for the E. coli strain GR84N/pNG2, Y. Iwafune, H. Kawasaki, and H. Hirano (Yokohama City University) for ESI-Q-TOF MS/MS analysis, A. Hirahisi (Toyohashi University of Technology) for valuable comments on the distribution of quinones in bacteria, and Eisai Co. (Tokyo, Japan) for Q$_{2}$.

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16.马来D-TOF MS studies on the E. coli cytochrome bd, Tsatsos et al. (29) identified the trypptic fragment at m/z 1931.9 as the cross-linked product of Val$^{166}$-Arg$^{278}$ (1597.7 Da) of subunit II with 3-azido-2-methyl-5-methoxy-BOQ$_{2}$ (329.4 Da). Because of the release of N$_{2}$ from azido-BOQ$_{2}$, a mass of the cross-linked peptide would be 1900.1 Da. Gong et al. (33) labeled the E. coli Complex I with 3-azido-2-methyl-5-methoxy-dBOQ (341.5 Da) and identified the cross-linked peptide at m/z 2929.4 to be Val$^{184}$-Asn$^{206}$ (2593.1 Da) of NuoM. However, the observed mass was 22 Da larger than that for the peptide plus the protonated photoproduct (2907.6 Da). The cause of such large discrepancies in assignments is not known.

In photoaffinity labeling of cytochrome bd with 3-azido-Q$_{2}$H$_{2}$, the protonated photoprodovt covalently linked to I-Glu$^{267}$ showed m/z 300.4 (Fig. 5), which is m/z 4.0 smaller than that expected (Fig. 6C). Auto-oxidation of the primary photoproduct during the isolation of peptides would yield a loss of two hydrogens from both the quinone ring and the isoprene moiety (Fig. 6D). The presence of [M + H]$^{+}$ ions for the cleaved azido-Q$_{2}^{+}$ and its oxidized isoprene unit at m/z 301.19 (301.37 expected) and 136.09 (136.23 expected), respectively, in the ESI-Q-TOF MS/MS spectrum (Fig. 5) supports the proposed structure for the cross-linked photoprodod (Fig. 6D).

In conclusion, photoaffinity labeling of the E. coli cytochrome bd, followed by MS analysis of peptides, revealed a new feature of the quinol oxidation site in loop VI/VII of subunit I. Although distal to the strictly conserved I-Glu$^{249}$, I-Lys$^{252}$, and I-Glu$^{257}$ at the N terminus of loop VI/VII, I-Glu$^{267}$ in the first internal repeat has been demonstrated to be a part of the binding site for the 2- and 3-methoxy groups of the quinone ring. Mutation (62) and x-ray crystallographic studies would provide further insights into the understanding of the catalytic mechanism for the quinol oxidation in bd-type terminal oxidases.
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