Identification of the Ubiquinone-binding Domain in the Disulfide Catalyst Disulfide Bond Protein B*  

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Disulfide bond (Db) formation is catalyzed in the periplasm of prokaryotes by the Dsb proteins. DsbB, a key enzyme in this process, generates disulfides de novo by using the oxidizing power of quinones. To explore the mechanism of this newly described enzymatic activity, we decided to study the ubiquinone-protein interaction and identify the ubiquinone-binding domain in DsbB by cross-linking to photoactivatable quinone analogues. When purified Escherichia coli DsbB was incubated with an azidoubiquinone derivative, 3-azido-2-methyl-5-[3H]methoxy-6-decyl-1,4-benzoquinone ([3H]azido-Q), and illuminated with long wavelength UV light, the decrease in enzymatic activity correlated with the amount of 3-azido-2-methyl-5-methoxy-6-decyl-1,4-benzoquinone (azido-Q) incorporated into the protein. One azido-Q-linked peptide with a retention time of 33.5 min was obtained by high performance liquid chromatography of the V8 digest of [3H]azido-Q-labeled DsbB. This peptide has a partial NH$_2$-terminal amino acid sequence of NH$_2$-HTMLQLY corresponding to residues 91–97. This sequence occurs in the second transmembrane domain of the inner membrane protein DsbB in a loop connecting transmembrane helices 3 and 4. We propose that the quinone-binding site is within or very near to this sequence.

DsbA is a small soluble protein, which contains a thio- 
doxin-fold with a highly unstable disulfide bond. DsbA acts by using its active site disulfide bond to rapidly oxidize 
dissulfides. This oxidation of a target protein results in the reduction of the active site CXXC motif of DsbA (12–15). The inner membrane protein DsbB reoxidizes the active site CXXC motif of DsbA (6, 16).

The question of how DsbB is reoxidized was recently an-
swered (7, 17, 18). Kobayashi and Ito (17) observes that the CXXC motif of DsbB is oxidized in the presence of a functional respiratory chain. This provides the first link between disulfide bond formation and electron transfer chains. By reconstituting the DsbA-DsbB system with purified electron transfer components in vitro, Bader et al. (7) identifies ubiquinone as the first electron acceptor of DsbB. Reduced quinones are then reox-
idized by the terminal oxidases cytochromes $b$ and $o$, which finally transfer electrons to oxygen. The observation that quino-
nes are electron acceptors in DsbB explains why disulfide bonds are formed during anaerobic growth of E. coli. Menaqui-
nones, which are synthesized upon oxygen deletion, are able to oxidize reduced DsbB. Reduced menaquinones are then reox-
idized by anaerobic reductases such as fumarate reductase (7). DsbB generates disulfides de novo by quinone reduction. This novel catalytic activity is apparently the major source of disul-

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<th>Disulfide bond (Db) is crucial for the structure and stability of many proteins. In recent years, much progress has been made in understanding how disulfide bonds are formed during protein folding in cells. Disulfides form in the periplasm of prokaryotes (1) and in the endoplasmic reticulum of eukaryotes (2). A number of Dsb proteins, which catalyze the oxidation, reduction, and isomerization of disulfide bonds in newly exported proteins, has been identified in Escherichia coli (1, 3, 4). DsbA and DsbB are required for oxidative disulfide bond formation (5–7). DsbC, DsbD, and DsbG are responsible for the isomerization of incorrect disulfide bonds in proteins with multiple disulfide bonds (8–11). Together these proteins cooperate to catalyze an important step in protein folding.</th>
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‡ The abbreviations used are: Dsb, disulfide bond; Q, ubiquinone; Q$_m$C$_{0.0}^{+}$, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; HPLC, high performance liquid chromatography; [3H]azido-Q, 3-azido-2-methyl-5-[3H]methoxy-6-decyl-1,4-benzoquinone; 5-[3H]azido-Q, 5-azido-2,3-[3H]dimethoxy-6-decy1-1,4-benzoquinone.

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1649
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RESULTS AND DISCUSSION

Preparation of DsbB and Properties of Azido-Q Derivatives—To analyze the quinone-binding properties of DsbB, we first purified His-tagged DsbB protein from overproducing cells using Ni affinity chromatography (18). These preparations contain 0.5–0.6 mol of bound coenzyme Q₈ (ubiquinone-40)/mol of DsbB protein. When this DsbB preparation is titrated with exogenous Q₈C₈Br or Q₈C₈, no additional Q is observed for binding, suggesting that the vacant Q-binding site(s) has been masked either by the dodecylmaltoside detergent or by phospholipids present in the buffer. The fact that exogenously added Q₈C₈ is unable to displace the Q₈ that copurifies with the DsbB protein suggests that the binding affinity of Q₈C₈ is weaker than that of endogenous Q₈ (18). Because the binding affinity of azido-Q derivatives to the Q-binding sites of several Q-binding proteins has been reported to be significantly weaker than that of Q₈C₈ (19), we concluded that these quinone analogues serve as sufficiently weak electron acceptors for DsbB.

Table I. Electron acceptor activity of azido-Q derivatives for DsbB

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<td>Q₈C₈</td>
<td>275</td>
<td>100%</td>
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<tr>
<td>5-azido-Q</td>
<td>306</td>
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<td>33%</td>
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method successfully for other proteins. When DsbB was incubated with 10-fold molar excess of these [3H]azido-Q derivatives for 1 h at 0 °C in the dark and then illuminated with a long wavelength of UV light for 7 min, the [3H]azido-Q-treated sample showed inactivation and radioactivity uptake by protein, indicating that [3H]azido-Q is suitable for use in identifying the Q-binding domain in DsbB. 3-Azido-2-methyl-5-methoxy-6-decyl-1,4-benzoquinone has been successfully used to identify the Q-binding sites in succinate-Q reductase and cytochrome bc1 complex from several sources (19–21). For the other quinone analogue 5-[3H]azido-Q, we failed to detect radioactive uptake by DsbB after illumination. This analogue was apparently unable to inactive DsbB. There are a number of possible reasons for this failure including 1) a low affinity of this derivative for the Q-binding site in DsbB or 2) intramolecular cyclization between the generated nitrene and its neighboring methoxy or methylene group on the Q molecule during illumination.

**Effect of Azido-Q Concentration on DsbB Activity after Illumination**—When DsbB was incubated with various concentrations of the [3H]azido-Q and illuminated, the activity decreased as the concentration of azido-Q increased (Fig. 1). Maximum inactivation of ~40% was obtained when 10 mol of azido-Q/mol of DsbB was used. Inactivation was not attributed to inhibition of DsbB by photolyzed products of azido-Q, because when azido-Q was photolyzed in the absence of protein and then added to DsbB, no inhibition was observed. Inactivation was also not due to damage of DsbB by the illuminating process, because when the enzyme alone was illuminated, no activity loss was observed. Because DsbB activity is assayed in the presence of excess Q0C10 (25 μM), the extent of inactivation of the azido-Q-treated DsbB after illumination is a measure of the fraction of Q-binding sites covalently linked to azido-Q.

Whether [3H]azido-Q can occupy 40% Q-binding sites in DsbB, one would expect to see a decrease in the activity of an azido-Q-treated sample before illumination. The failure to observe such a decrease is because of the facts that the concentration of Q0C10 in the assay mixture is several orders of magnitude higher than that of azido-Q, and that the azido-Q has a much lower affinity for DsbB than Q0C10. Thus Q0C10 can easily displace azido-Q from the binding sites, and the inferior electron transfer activity of azido-Q is not expressed. After illumination, the covalently linked azido-Q cannot be displaced by Q0C10 as inhibition occurs.

**Correlation between Azido-Q Incorporation and Inactivation of DsbB**—When azido-Q-treated DsbB was illuminated at 0 °C for various lengths of time, activity decreased as illumination
time was increased; maximum inactivation (40%) was reached in 7 min. The amount of azido-Q uptake paralleled the extent of inactivation until the maximum was reached (approximately 0.45 mol of azido-Q uptake/mol of DsbB at 40% inactivation), suggesting that inactivation is the result of binding of azido-Q at the ubiquinone-binding site. Because purified DsbB after detergent exchange with sodium cholate still contains 0.3 mol of endogenous Q, one would ideally expect to observe a maximum uptake of 0.7 mol of azido-Q/mol of DsbB. That we observe lower than ideal uptake is not unexpected, azido-Q has a substantial weaker binding affinity than does Q0C10. Although illumination for longer than 7 min caused no further decrease in activity, azido-Q uptake continued but at a much slower rate, suggesting that this slower incorporation is the result of non-specific binding of azido-Q to protein. The photoinactivation rate of DsbB was affected by the protein, alcohol, and detergent concentrations in the system. Interaction with azido-Q derivative was most effective when the reaction system contained 1–1.2 mg/ml DsbB, 1% sodium cholate, and <1% ethanol.

Because Q0C10 is a better electron acceptor than azido-Q, it is expected that in the presence of Q0C10 during illumination a drastic decrease in inhibition and azido-Q uptake by DsbB would be observed. This is indeed the case in the presence of equal molars of Q0C10 and azido-Q. Less than 5% inactivation was observed.

**Isolation and Characterization of Ubiquinone-binding Peptides of DsbB**—To identify the Q-binding domain in DsbB by isolating and sequencing an azido-Q-linked peptide, it is absolutely necessary that [3H]azido-Q-labeled DsbB be free from contamination with unbound azido-Q and susceptible to proteolytic digestion. Therefore, unbound azido-Q in illuminated [3H]azido-Q-treated DsbB was removed. We performed this method by paper chromatography with a mixture of chloroform:methanol (2:1). Labeled DsbB, devoid of unbound azido-Q, remained at the paper origin. It was eluted from the paper with ammonium bicarbonate buffer, pH 8.0, containing 0.1% SDS and 1 M urea. The inclusion of 0.1% SDS and 1 M urea in the elution buffer unfolds and disaggregates the labeled DsbB, thus increasing proteolytic efficiency.

Fig. 2 shows [3H] radioactivity distribution among the V8 peptides of DsbB separated by HPLC. Most of the radioactivity was found in a fraction with a retention time of 33.5 min. Radioactivity recovery is approximately 60% based on that applied to the HPLC column. The partial NH2-terminal amino acid sequence of this peptide was found to be NH2–HTMQLQLY corresponding to amino acid residues 91–97 of DsbB. Because this peptide has resulted from the V8 digestion, it probably comprises residues His91/Glu112 of DsbB. The Q-binding domain in the proposed structure of DsbB (Fig. 3) is located in the connecting loop between transmembrane helices 3 and 4 on the periplasm side of the membrane. Some radioactivity is also found in a fraction with a retention time of 58.1 min. This fraction is most likely to be the undigested DsbB.

If the Q-binding site in DsbB resembles the one in NADH-quinone acceptor oxidoreductase (22), histidine 91 may form a hydrogen bond with the carbonyl groups of the 1,4-benzoquinone ring, and phenylalanine 106 may provide hydrophobic contact. The three-dimensional structure of NADH-quinone acceptor oxidoreductase from human and mouse, co-crystallized with substrate duroquinone, has been determined at 1.7 Å resolution (22). Duroquinone is hydrogen-bonded to a water molecule that bridges the NE of histidine 161 and the –OH of tyrosine 128. In addition, five aromatic residues of NADH-quinone acceptor oxidoreductase (Trp-105, Phe-106, Phe-178, Tyr-126, and Tyr-128) provide most of the hydrophobic contacts. The sequence alignment shows that amino acid residues involved in Q binding in NADH-quinone acceptor oxidoreductase appear to be conserved in the two periplasmic loops of DsbB. They correspond to Trp-31, Phe-32, Tyr-46, Arg-48, His-91, and Phe-106 of DsbB.

We already have good genetic and biochemical data that indicate Arg-48 residue is involved in quinone binding. DsbB mutants in this Arg-48 residue show a major in vivo defect in their ability to catalyze disulfide bond formation. The purified R48H DsbB protein exhibits an apparent Michaelis-Menten constant Km for ubiquinone seven times greater than that of the wild-type BsbB, suggesting that this highly conserved arginine residue plays an important role in the interaction of DsbB with quinones (23).

More detailed information on Q binding must await determination of the three-dimensional structure of DsbB. The information reported herein provides a basis for future mutagenic studies to identify the essential amino acid residues involved in Q binding.

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**REFERENCES**