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

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Disulfide bond (Dsb) 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-[^{3}H]methoxy-6-decyl-1,4-benzoquinone ([^{3}H]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 [^{3}H]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 periplasmic 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.

Disulfide bond (Dsb)[1] formation 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.

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

The question of how DsbB is reoxidized was recently answered (7, 17, 18). Kobayashi and Ito (17) observes that the C*XX*C 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 reoxidized by the terminal oxidases cytochromes *bd* and *bo*, which finally transfer electrons to oxygen. The observation that quinones are electron acceptors in DsbB explains why disulfide bonds are formed during anaerobic growth of *E. coli*. Menaquinones, which are synthesized upon oxygen deletion, are able to oxidize reduced DsbB. Reduced menaquinones are then reoxidized 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 disulfides in prokaryotes.

Recently, Bader *et al*. (18) discovered a novel ubiquinone reduction activity in DsbB and shows that DsbB contains a single high affinity quinone-binding site. To understand the DsbB-quinone interaction in and the mechanism of Q reduction by DsbB requires knowledge of the Q-binding site. The availability of azido-Q derivatives in our laboratory (19) encouraged us to study the quinone:protein interaction and identify the quinone-binding domain in DsbB by photoaffinity labeling. Herein we report the conditions for photoaffinity labeling of DsbB with azido-Q derivatives and the identification of a Q-binding peptide.

EXPERIMENTAL PROCEDURES

Materials—Sodium cholate was obtained from Sigma and recrystallized from methanol. *N*-Dodecyl-β-D-maltoside was from Anatrace. Insta-Gel liquid scintillation mixture was from Packard Instruments. Other chemicals were of the highest purity commercially available.

The ubiquinone derivatives 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (Q_0C_{10}), 3-azido-2-methyl-5-methoxy-6-decyl-1,4-benzoquinone (azido-Q), 3-azido-2-methyl-5-[^{3}H]methoxy-6-decyl-1,4-benzoquinone ([^{3}H]azido-Q), 5-azido-2,3-dimethoxy-6-decyl-1,4-benzoquinone (5-azido-Q), and 5-azido-2,3-[^{3}H]dimethoxy-6-decyl-1,4-benzoquinone (5-[^{3}H]azido-Q) were synthesized by methods reported previously (19).

Enzyme Preparations and Assays—DsbA (16) and DsbB (18) were purified essentially as described above. Reduced DsbA was prepared by incubation of 10 mg/ml purified protein in 50 mM sodium phosphate, pH 6.2, containing 100 mM NaCl and 0.1% *N*-dodecylmaltoside with 10 mM dithiothreitol for 20 min on ice. Excess dithiothreitol was removed by a 2-ml Pierce desalting column preequilibrated with 50 mM sodium phos-

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[1] The abbreviations used are: Dsb, disulfide bond; Q, ubiquinone; Q_0C_{10}, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; HPLC, high performance liquid chromatography; [^{3}H]azido-Q, 3-azido-2-methyl-5-[^{3}H]methoxy-6-decyl-1,4-benzoquinone; 5-[^{3}H]azido-Q, 5-azido-2,3-[^{3}H]dimethoxy-6-decyl-1,4-benzoquinone.

phate, pH 6.2, containing 100 mM NaCl and 0.1% *N*-dodecylmaltoside. The DsbB concentration was determined after reduction of protein-bound quinone with $NaBH_4$ using a millimolar extinction coefficient of $E_{276} = 46.5\ mM^{-1}\ cm^{-1}$ (16).

DsbB activity was assayed by measuring its ability to catalyze ubiquinone reduction by reduced DsbA at room temperature in a Shimadzu UV-2101 PC. The reaction mixture (1 ml) contained 50 mM sodium phosphate buffer, pH 6.0, 300 mM NaCl, 0.1% dodecylmaltoside, 5.2 nmol of reduced DsbA, and 25 nmol of Q_0C_{10}. The reaction was started by the addition of an appropriate amount of azido-Q treated-DsbB or untreated-DsbB. The reduction of Q_0C_{10} was followed by measuring the absorption decrease at 275 nm using a millimolar extinction coefficient of $E_{275} = 12.25\ mM^{-1}\ cm^{-1}$ (19). When 5-azido-Q or azido-Q was used as the electron acceptor, the absorption decrease at 306 and 315 nm, respectively, was followed using the same extinction coefficient of Q_0C_{10} (19).

Photoaffinity Labeling of DsbB with [^{3}H]Azido-Q—DsbB as prepared was in 0.1% *N*-dodecylmaltoside, which masks the quinone-binding site (18). DsbB was diluted with 1% sodium cholate to a protein concentration of 1 mg/ml in 50 mM K^+/Na^+ phosphate buffer, pH 7.4 and then concentrated to 10 mg/ml by ultrafiltration. The concentrated DsbB was diluted again with 1% sodium cholate in the same buffer and concentrated by ultrafiltration again to 10 mg/ml. This dilution and concentration process was repeated five times. They serve the purposes of replacing *N*-dodecylmaltoside with sodium cholate and partially depleting endogenous Q_8, thus facilitating the interaction between DsbB and azido-Q.

400 μl of DsbB (2.6 mg/ml) in 50 mM K^+/Na^+ phosphate buffer, pH 7.4, containing 1% of sodium cholate was incubated with 16 μl of [^{3}H]azido-Q (26 mM in 95% ethanol) at 0 °C for 1 h in the dark. The specific radioactivity of [^{3}H]azido-Q used was 1.6×10^4 cpm/nmol in 95% ethanol and 6×10^3 cpm/nmol in the 50 mM K^+/Na^+ phosphate buffer, pH 7.4, containing 1.0% sodium cholate in the presence of DsbB. This mixture was transferred to a 2-mm light path quartz cuvette. The cuvette was sealed with paraffin film and mounted on an illuminating apparatus. The assembly was immersed in ice water in a container with a quartz window and then illuminated with long wavelength UV light (Spectroline EN-14, 365 nm long wavelength, 23 watts) for 7 min at a distance of 4 cm from the light source. DsbB activity was assayed before and after the illumination.

To determine the amount of [^{3}H]azido-Q incorporated into DsbB, illuminated samples were spotted on Whatman filter paper and developed with a mixture of chloroform and methanol (v/v) (2:1) to remove non-protein bound [^{3}H]azido-Q. After the paper was air-dried, the origin spot was cut into small pieces and subjected to liquid scintillation counting.

V8 Digestion of [^{3}H]Azido-Q-labeled DsbB—The UV-illuminated [^{3}H]azido-Q-treated DsbB was applied as a strip to Whatman filter paper. After non-protein bound Q was removed as described above, the strip containing the protein was cut out and eluted with ammonium bicarbonate buffer, pH 8.0, containing 1.0 M urea and 0.1% SDS. The eluted protein was digested with protease V8 at 37 °C for 40 h using a V8:DsbB ratio of 1:50 (w/w).

Isolation of Ubiquinone-binding Peptides—The V8 digest of [^{3}H] azido-Q-labeled DsbB was mixed with an equal volume of 90% acetonitrile containing 0.1% trifluoroacetic acid, incubated at 37 °C for 30 min, and then centrifuged at 13,000 rpm (Eppendorf Centrifuge 5415D) for 10 min to remove any precipitate. 100-microliter aliquots of the supernatant solution were separated by high performance liquid chromatography (HPLC) on a Supelcosil™-LC-308 column (C8, 5 μm-particles, 300-Å pores, 4.6 mm inner diameter, 25-cm length) using a gradient formed from 0.1% trifluoroacetic acid and 90% acetonitrile containing 0.1% trifluoroacetic acid with a flow rate of 1 ml/min. 1-ml fractions were collected. The absorbance from 160 to 400 nm of each fraction was recorded with a Waters 996 diode array detector. The radioactivity of each fraction 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. Ken Jackson.

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 Q_8 (ubiquinone-40)/mol of DsbB protein. When this DsbB preparation is titrated with exogenous $Q_0C_{10}Br$ or Q_0C_{10}, 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 Q_0C_{10} is unable to displace the Q_8 that copurifies with the DsbB protein suggests that the binding affinity of Q_0C_{10} is weaker than that of endogenous Q_8 (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 Q_0C_{10} (19, 21, 22), a study of the ubiquinone:protein interaction in DsbB using azido-Q derivatives requires prior removal of endogenous Q from the enzyme preparation and the unmasking of the Q-binding sites. We previously showed that we could partially remove endogenous Q_8 (from 0.5–0.6 mol/mol down to 0.3 mol/mol protein) by a repetitious dilution ultrafiltration procedure in which the dodecylmaltoside detergent was exchanged with sodium cholate. This procedure also serves to unmasks the Q-binding site. That unmasking occurs during detergent exchange is evident from the binding of 0.7 mol of $Q_0C_{10}Br$/mol of DsbB, which had undergone detergent exchange (18). Purified DsbB after detergent exchange proved to be an excellent preparation for studying ubiquinone:protein interactions and for identifying the Q-binding site with azido-Q derivatives.

TABLE I
Electron acceptor activity of azido-Q derivatives for DsbB

Azido-Q derivatives	Wavelength	Activity[a]
	nm	*%*
Q_0C_{10}	275	100[b]
5-azido-Q	306	35
5-[^{3}H]azido-Q	306	33
azido-Q	315	17
[^{3}H]azido-Q	315	17

[a] Assay conditions were as described in "Experimental Procedures" with the exception that the indicted Q derivatives were used, and Q reduction was monitored at indicated wavelengths.

[b] 100% activity represents 8.95 μmol Q reduced/mg protein of the sodium cholate replaced DsbB/min. Because quinone derivatives used were in well excess, DsbB as prepared or its Q-deficient preparation gave the same activity.

Two azido-Q derivatives, azido-Q and 5-azido-Q, were synthesized as described previously (19) and tested for their suitability for identifying Q-binding sites in DsbB. Both derivatives exhibit substantial electron acceptor activity with DsbB (see Table I). The maximal activity observed for these two Q analogues 5-azido-Q and azido-Q was 35 and 17%, respectively, compared with the activity observed with Q_0C_{10}. Therefore, we concluded that these quinone analogues served as sufficiently physiological substrates to allow us to use them to study the Q-binding properties of DsbB.

[^{3}H]Azido-Q and 5-[^{3}H]azido-Q were prepared by methylation of the hydroxyl groups on 5-H-2,3-dihydroxy-6-decyl-1,4-benzoquinone and 3-H-2-methyl-5-hydroxy-6-decyl-1,4-benzoquinone, respectively, with $C[^3H]_3I$ before nucleophilic substitution with NaN_3. These two [^{3}H]azido-Q derivatives have the same electron acceptor activity for DsbB as their unlabeled isomers, suggesting that they can serve as reasonably physiological substrates. We then tested both of these derivatives for their ability to inactivate DsbB after photo cross-linking. Our rationale being that if inactivation occurs upon cross-linking, this would indicate at least that the cross-linking interferes with an important part of DsbB activity, possibly by reacting directly to the quinone-binding site in DsbB. We and others (19) and have previously used this

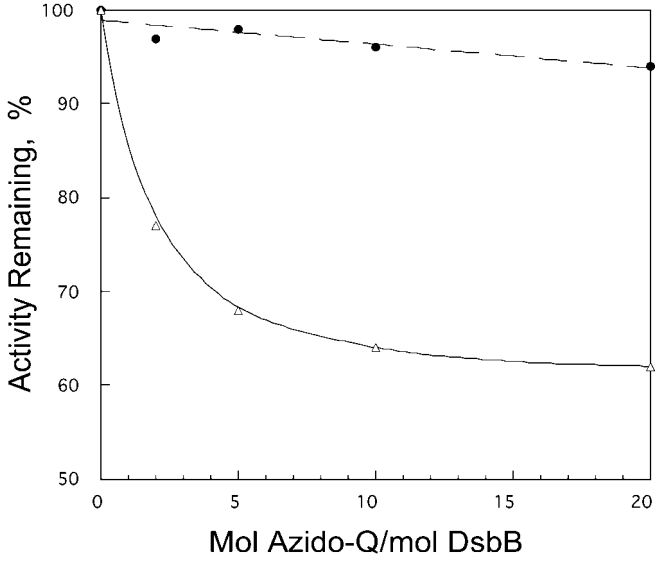

FIG. 1. **Effect of azido-Q concentration on DsbB activity after illumination.** Aliquots (50 μl) of sodium cholate-extracted Q-deficient DsbB, (0.4 mg/ml) in 50 mM phosphate buffer, pH 7.4, containing 1% sodium cholate were mixed with 1 μl of an alcoholic solution of azido-Q derivative (concentrations indicated) in the dark. After incubation at 0 °C for 1 h, the samples were illuminated for 7 min at 0 °C. DsbB activity was assayed before (*solid circles*) and after (*open triangles*) illumination using Q_0C_{10} as electron acceptor. Activity of DsbB without treatment with azido-Q and without illumination was used at 100% (8.95 μmol Q-reduced/min/mg protein).

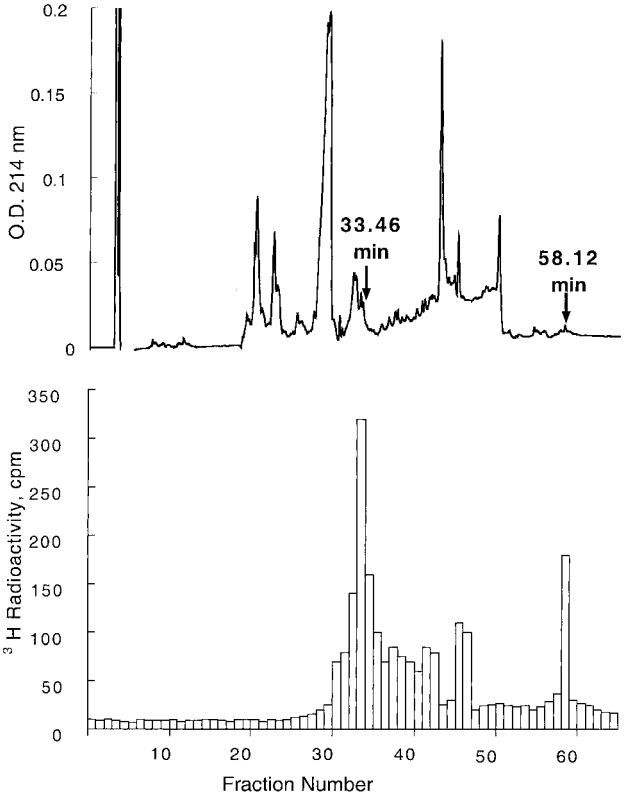

FIG. 2. **^{3}H radioactivity distribution on HPLC chromatogram of V8-digested, [^{3}H]azido-Q-labeled DsbB.** The [^{3}H]azido-Q-labeled DsbB (1 mg/ml, 1.2×10^5 cpm/mg) was digested with V8, and 100-μl aliquots were subjected to HPLC separation as described under "Experimental Procedures." 100-μl aliquots were withdrawn from each fraction for radioactivity determination.

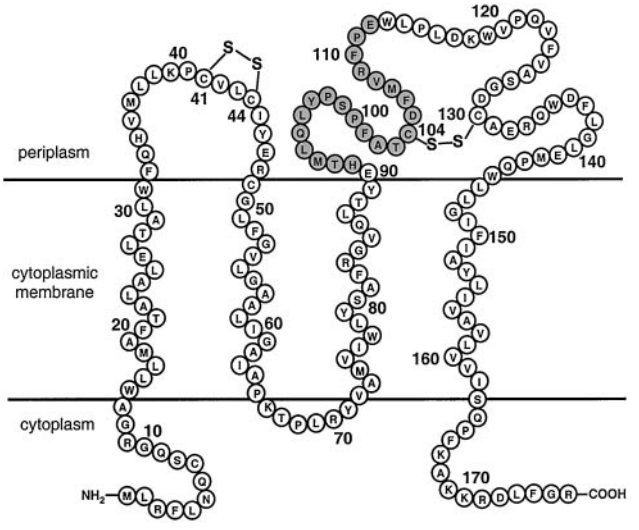

FIG. 3. **Proposed secondary structure of DsbB.** The *shaded* area is the Q-binding peptide identified in this report.

method successfully for other proteins. When DsbB was incubated with 10-fold molar excess of these [^{3}H]azido-Q derivatives for 1 h at 0 °C in the dark and then illumination with a long wavelength of UV light for 7 min, the [^{3}H]azido-Q-treated sample showed inactivation and radioactivity uptake by protein, indicating that [^{3}H]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 bc_1 complex from several sources (19–21). For the other quinone analogue 5-[^{3}H]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 [^{3}H]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 Q_0C_{10} (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 [^{3}H]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 Q_0C_{10} 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 Q_0C_{10}. Thus Q_0C_{10} 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 Q_0C_{10} 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 Q_0C_{10}. 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 nonspecific 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 Q_0C_{10} is a better electron acceptor than azido-Q, it is expected that in the presence of Q_0C_{10} during illumination a drastic decrease in inhibition and azido-Q uptake by DsbB would be observed. This indeed is the case in the presence of equal molars of Q_0C_{10} 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 [^{3}H]azido-Q-labeled DsbB be free from contamination with unbound azido-Q and susceptible to proteolytic digestion. Therefore, unbound azido-Q in illuminated [^{3}H]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 ^{3}H 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 NH_2-terminal amino acid sequence of this peptide was found to be NH_2-HTMLQLY corresponding to amino acid residues 91–97 of DsbB. Because this peptide has resulted from the V8 digestion, it probably comprises residues His^{91}/Glu^{112} 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 Nϵ 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 K_m 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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