Disulfide bond formation protein B (DsbBS-S,S-S) is an inner membrane protein in *Escherichia coli* that has two disulfide bonds (S-S, S-S) that play a role in oxidation of a pair of cysteine residues (SH, SH) in disulfide bond formation protein A (DsbA-SH,SH). The oxidized DsbA-S-, with one disulfide bond (S-S), can oxidize proteins with SH groups for maturation of a folding preprotein. Here, we have described the transient kinetics of the oxidation reaction between DsbA-SH,SH and DsbBS-S,S-S. We immobilized DsbBS-S,S-S embedded in lipid bilayers on the surface of a 27-MHz quartz crystal microbalance (QCM) device to detect both formation and degradation of the reaction intermediate (DsbA-DsbB), formed via intermolecular disulfide bonds, as a mass change in real time. The obtained kinetic parameters (intermediate formation, reverse, and oxidation rate constants (k_f, k_r, and k_cat, respectively) indicated that the two pairs of cysteine residues in DsbBS-S,S-S were more important for the stability of the DsbA-DsbB intermediate than ubiquinone, an electron acceptor for DsbBS-S,S-S. Our data suggested that the reaction pathway of almost all DsbA-SH,SH oxidation processes would proceed through this stable intermediate, avoiding the requirement for ubiquinone.

Transport reactions that occur inside a cell, such as electron transport reactions, are catalyzed by specific enzymes (1). The mechanism of protein interaction-based transport systems is different from that of electron transport reactions of general chemical compounds. The disulfide bond formation (Dsb) system is a catalytic system that accelerates the oxidative formation of protein disulfide bonds in *Escherichia coli* and involves a number of protein factors, including the Dsb proteins DsbA, DsbB, DsbC, DsbD, and DsbG (2–4). Together, DsbA and DsbB function as a disulfide-introducing unit in the bacterial periplasmic space. DsbA belongs to the thioredoxin superfamily and contains a disulfide bond in the thioredoxin domain (Cys-30–X–X–Cys-33) that is known to act as an acceptor of two electrons (and two protons) from a preprotein containing cysteine residues during the transfer of the disulfide bond from the oxidized DsbA-S,S to the preprotein (Fig. 1) (2–5). To repeat this disulfide bond-introducing reaction in the next catalytic cycle, re-oxidation of DsbA-SH,SH is required. DsbBS-S,S-S is an inner membrane protein in *E. coli* that has four transmembrane segments containing four cysteine residues that form two disulfide bonds (Cys-41 with Cys-44, and Cys-104 with Cys-130) within the periplasmic space. Reduced DsbA-SH,SH is reoxidized by the transfer of two electrons (and two protons) to the two thiol groups of the disulfide bonds on DsbBS-S,S-S, resulting in disulfide exchange. Finally, the two electrons (and two protons) are transferred to lipophilic ubiquinone in the inner membrane, where DsbB functions as a quinone reductase, catalyzing the conversion of ubiquinone to ubiquinol (3).

The mechanism and direction of electron transfer between chemical compounds can be explained in terms of its oxidation-reduction potential. In the case of electron transport based on protein factors, such as the DsbA-DsbB system, protein-protein interactions and intermediate complexes are also important for understanding the mechanism of the electron transport reaction. Various methods have been used to investigate the electron transport mechanisms associated with thiol-disulfide exchange in the DsbA-DsbB system. Nonreducing SDS-PAGE analysis has been used to estimate the amounts of...
Reduced DsbA$_{SH,SH}$ and oxidized DsbA$_{S,S}$ after modification by a thiol-reacting alkylating agent (6–8). Previous investigations also used x-ray crystal structure analysis to determine the structures of DsbB and the intermediate DsbB-DsbA complex (inactivated with mutation); this analysis revealed the positional relationship of cysteine residues and ubiquinone within the complex (9, 10). Snapshots of nuclear magnetic resonance solution structures of DsbB and its variant have proven useful for understanding electron movement from DsbA to ubiquinone (11). Moreover, fluorescence measurements have enabled the observation of the oxidation reaction of DsbA over time on the basis of the change in the tryptophan fluorescence of DsbA (12). Stopped-flow absorbance measurements have also been conducted to monitor DsbB-ubiquinone complexes in the process of electron transfer from DsbA to ubiquinone (13).

Based on these previous studies, two potential models of the reaction pathway have been proposed (Fig. 1) (3). In the first step the interprotein disulfide bond between Cys-30(DsbA) and Cys-104(DsbB) is formed by the nucleophilic attack of Cys-30 from DsbA$_{SH,SH}$ on the Cys-104–Cys-130 disulfide bond of DsbB$_{S,S,S}$. Then, Cys-33(DsbA) intramolecularly attacks Cys-30, and oxidized DsbA$_{S,S}$ is released from the reduced DsbB$_{S,S,SH,SH}$ (pathway I, referred to as the rapid pathway) (6, 7). In this pathway, ubiquinone reduction occurs in the reduced DsbB$_{S,H,S,SH}$–ubiquinone complex, independent of DsbA. In the alternative model, after the formation of the Cys-30(DsbA)–Cys-104(DsbB) complex, the newly reduced Cys-130(DsbB) immediately attacks Cys-41(DsbB) in an intramolecular isomerization reaction to form the interloop disulfide bond Cys-41–Cys-130 in DsbB (the stable intermediate). The consequent thiolate of Cys-44(DsbB) interacts with ubiquinone in DsbB to form the electron transfer complex (7, 13, 14). Then, DsbA in the complex is oxidized by the intramolecular attack of Cys-33 on Cys-30 of the Cys-30–Cys-130 disulfide bond and is released (pathway II, referred to as the slow pathway) (8, 13). These reaction models are still currently under discussion (3). Although the structure of the DsbB-DsbA complex is known, the reaction pathway has not yet been elucidated because it involves an intermediate structure that is at the branching point of the two proposed reaction pathways. Thus, identification of the reaction pathway requires quantitative kinetic studies of the behavior of intermediates as key molecules.

Quartz crystal microbalance (QCM) devices can measure nanogram-scale mass changes based on the altered resonance frequency ($\Delta f$) of a quartz-plate oscillator, which indicates mass changes on the plate (15–17). Oscillation of QCMs in aqueous solutions has been applied to nonlabeling biosensors that can detect biomolecular interactions as mass changes on the sensor surface in real time (18). We previously used this technique to detect various biomolecular interactions, such as DNA-DNA, DNA-peptide, and peptide-peptide interactions using a ligand-immobilized quartz plate (19–21). Recently, enzyme reactions, such as DNA polymerase reactions (22), DNA cleavage reactions (23), and protease reactions (24, 25), were carried out on substrate-immobilized QCM plates, allowing the formation and decomposition of the enzyme-substrate complex (intermediate) to be monitored as a detectable mass changes.
change. In addition, transient kinetic analysis could be conducted on the basis of the behavior of the intermediate (24, 25).

In this paper we have described the immobilization of a membrane protein, DsbB, embedded in supported lipid bilayers on a QCM plate and the detection of an intermediate complex between DsbA$_{SH,SH}$ and DsbB$_{S-S,S-S}$ according to mass changes on the QCM device (Fig. 2). A QCM with a sensor surface consisting of a planar gold electrode was used for immobilization of DsbB membrane proteins in supported lipid bilayers to obtain kinetic parameters under nearly native conditions rather than in surfactant-solubilized DsbB in bulk solutions. Three kinetic parameters were measured, the formation and reverse rate constants ($k_f$ and $k_r$, respectively) of the intermolecular disulfide bond in the DsbA-DsbB intermediate and the release rate constant ($k_{cat}$) of oxidized DsbA$_{SH,SH}$ from reduced DsbB$_{S-S,S-S}$,SH,SH in the membrane. These kinetic parameters were compared with those of the mutated variants DsbA(C33A)$_{SH,SH}$, DsbA(C30A)$_{SH,SH}$, and DsbB(C41A,C44A)$_{S-S,S-S}$ in the presence or absence of ubiquinone. Based on the stability of the DsbA-DsbB intermediate estimated by the obtained kinetic parameters, we concluded that DsbB$_{S-S,S-S}$ mainly oxidized DsbA$_{SH,SH}$ in a ubiquinone-independent manner through a stable intermediate in which delocalization of the transferred electron on cysteine residues of DsbB was important (modified pathway II).

**EXPERIMENTAL PROCEDURES**

**Materials**—PCR primer oligonucleotides were purchased from Operon Biotechnologies (Tokyo, Japan). The pET22b(+) vector for protein expression in *E. coli* was purchased from TaKaRa Bio Inc. (Shiga, Japan). PinPoint Xa-1 vector was obtained from Promega KK (Tokyo, Japan). n-Dodecyl-$\beta$-d-maltoside (DDM) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide were purchased from Dojindo Laboratories (Kumamoto, Japan). Isopropyl-$\beta$-d-thiogalactopyranoside, N-hydroxysuccinimide, phenylmethylsulfonyl fluoride (PMSF), and ubiquinone-10 were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). NeutrAvidin, Zeba Desalt Spin Column, and tris(2-carboxyethyl)phosphine were obtained from Thermo Fisher Scientific Inc. (Yokohama, Japan). The *E. coli* total lipid extract was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). 4-Acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) was purchased from Invitrogen. Unless otherwise noted, all other reagents were purchased from Nacalai Tesque Co. (Kyoto, Japan) and used without further purification.
Oxidation Reaction of DsbA and DsbB on QCM

Preparation of DsbASHL and Its Variants—A 627-bp fragment encoding DsbA (EC 5.3.4.1) was amplified from the genomic DNA of E. coli JM109 (K12 wild-type strain) by PCR with the primers 5'-GGAGATACATATGAAAAAGATT-GGCTGGCCGTG-3' and 5'-GAGCTGCAATTTTCTTTTCTCGGAGCAGATTTTACC-3' and cloned into pET22b(+) through the restriction sites NdeI and EcoRI. Two vectors for variant expression of DsbA(C33A)SH and DsbA(C30A)SH were prepared using the QuikChange Site-directed Mutagenesis kit (Agilent Technologies Japan, Tokyo, Japan). The constructed vectors were verified by dideoxy sequencing.

DsbASHL - DsbA(C33A)SH and DsbA(C30A)SH proteins were expressed and purified according to a previously reported method, with minor modifications (26). E. coli JM109 (DE3) harboring each expression vector was grown in 1 liter of LB broth with ampicillin at 37 °C to an OD600 of 1.0 following by additional incubation for 16 h. The cells were harvested and then suspended in 20 ml of a buffer (200 mM boric acid-NaOH, pH 8.0, 160 mM NaCl, 5 mM EDTA) to allow conversion of the cells into spheroplasts. The suspension was stirred on ice for 2 h and centrifuged (48,000 × g, 30 min, 4 °C) to remove the spheroplasts, and the collected supernatant containing the periplasmic contents was repeatedly dialyzed against 2 liters of Buffer A (10 mM MOPS-NaOH, pH 7.0). The solution was added onto a 5-ml DEAE FF anion exchange column (GE Healthcare), and target proteins were obtained by elution with a linear gradient of 0–500 mM NaCl in Buffer A. Typically, 10 mg of purified DsbA per 1 liter of LB broth was obtained and stored at −80 °C until use.

Preparation of Biotin-BCCP-DsbBSSSS and Its Variant—An amplified fragment encoding DsbB (EC 1.8.4.2) was obtained in the same manner as DsbASHL by PCR with the primers 5'-GGAGATACATATGTTGCCATTTTGAACCAATGTTCACAAGGC-3' and 5'-GAGCTGCAATTTTCTTTTCTCGGAGCAGATCAGTTTTTTCG-3' and cloned into pET22b(+) through the restriction sites Ndel and EcoRI. The codons for the two nonessential cysteine residues, Cys-8 and Cys-49, were then replaced by alanine and valine codons, respectively, using the QuikChange method to prevent disulfide-linked oligomerization (7, 8). A fragment encoding the biotin carboxyl carrier protein (BCCP) tag was PCR-amplified from Pinpoint Xa vector and then inserted into the XhoI-HindIII digested vector coding the improved DsbB by using In-Fusion Advantage PCR Cloning Kit (Takara Bio Inc., Shiga, Japan). A vector for the double-mutated DsbB(C41A,C44A)SSS protein was prepared by the QuikChange method.

Biotin-BCCP-DsbBSSS and biotin-BCCP-DsbB(C41A, C44A)SSS proteins were also expressed and purified as described previously (12, 27). E. coli C41 (DE3) harboring each expression vector was grown in 3 liters of LB broth with ampicillin and biotin at 37 °C, and then 15 μm isopropyl-β-D-thio-galactopyranoside was added into the medium for protein induction at an A600 of 0.6 followed by the additional incubation for 4 h. The cells were harvested and suspended in a buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM PMSF) and then passed through a French press. After precentrifugation for 30 min at 10,000 × g to remove untreated cells, the cell lysate was centrifuged for 1 h at 100,000 × g to collect membrane fractions as a pellet. The membrane pellet was homogenized in a Dounce homogenizer and passed through a syringe attached to a needle (0.7 × 50 mm). After the DDM solution was added to the suspension at a final concentration of 1%, the suspension was ultracentrifuged (100,000 × g, 30 min) to remove insoluble membrane fractions. The supernatant was loaded on a 5-ml nickel-nitriitoltriacetic acid column (GE Healthcare) equilibrated with a buffer (50 mM phosphate, pH 8.0, 300 mM NaCl, 0.1% DDM), and the target protein was eluted from the column by a 0–0.5 M imidazole gradient at pH 6.0. The collected protein was additionally purified on a gel filtration column (Superdex 200 pg, GE Healthcare) in Buffer B (50 mM phosphate, pH 6.0, 300 mM NaCl, 0.1% DDM) and stored at −80 °C until use.

Setup of 27-MHz QCM and Immobilization of Biotin-BCCP-DsbB on a NeutrAvidin-modified QCM Plate—An AFFINITY Q4 system used as the QCM instrument (Initium Co. Ltd., Tokyo, Japan) is shown in Fig. 2A. The QCM instrument has four 500-μl cells equipped with a 27-MHz QCM plate (60-μm-thick with 8.7-mm-diameter AT-cut shear-mode quartz plate and gold electrode with a 5.7 mm² area) at the bottom of each cell, a stirring bar, and a temperature controlling system (23–25). NeutrAvidin (60 kDa) was immobilized covalently on the QCM plate as described previously (19, 20, 23). Briefly, 3,3'-dithiodipropionic acid was immobilized on a cleaned bare gold electrode, and then carboxylic acids were activated as N-hydroxysuccinimimidyl esters on the surface using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. NeutrAvidin was reacted with the activated esters by mounting aqueous solutions on the QCM plate. The binding behaviors of biotin-BCCP-DsbBSSSS to the NeutrAvidin-immobilized QCM were followed in DDM(+) buffer (50 mM HEPES-NaOH, pH 7.5, 100 mM NaCl, 0.1% DDM) at 25 °C as frequency changes (Δfwater) with time. When the Δfwater value corresponding to an amount of an anchored biotin-BCCP-DsbBSSSS solubilized with DDM reached ~2000 Hz (a predetermined value), 10 μM free biotin was added to regulate the immobilization amount. At ~2000 Hz, the amount of biotin-BCCP-DsbBSSSS solubilized with DDM was estimated to be 560 ng/cm² (16 pmol cm⁻²) based on a calibration value of 0.28 ng/cm² Hz⁻¹ (28–30). The relationship between frequency changes and mass changes for calibration is described elsewhere (28–30). The coverage of immobilized biotin-BCCP-DsbBSSSS was calculated to be ~68% with 7.1 nm² molecule⁻¹ of DsbBSSSS area on the QCM electrode (5.7 mm²).

Preparation of DsbB Embedded in Lipid Bilayers on a QCM Plate—The procedure for DsbB preparation is illustrated in Fig. 2B. A 200-μl chloroform solution of total lipid extracts from E. coli (10 mg/ml) was evaporated in a recovery flask under light shielding. In the preparation of lipid bilayers containing excess ubiquinone-10, a mixed solution of the above-mentioned lipid extracts and a 200-μl chloroform solution of ubiquinone-10 (1.45 mg/ml, corresponding to 11 mol%) was used. A mixed micelle solution of total lipids from E. coli and DDM was prepared by the addition of 398 μl of DDM(+) buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl, and 0.44% DDM) into the flask. After immobilization of biotin-BCCP-DsbBSSSS in 500 μl of DDM(+) buffer onto a NeutrAvidin-modified QCM plate by
the above-mentioned procedure, 450 μl of DDM(+) buffer was replaced by 20 μl of the mixed micelle solution and 430 μl of DDM(−) buffer (50 mM HEPE- NaOH, pH 7.5, 100 mM NaCl), resulting in a final DDM concentration of 0.028%, which was slightly higher than the critical micelle concentration of DDM (0.01%). After incubating for 1 h to form a lipid bilayer around the immobilized DsbB_{S,S,S}. The remaining DDM was removed from the QCM cell by replenishing the DDM(−) buffer several times. Finally, the QCM cell was filled with 500 μl of a reaction buffer (50 mM citrate, pH 6.0, with 0.1% DDM) for 3 min at room temperature.

We followed the formation and degradation of the DsbA-DsbB intermediate on the QCM as frequency (mass) changes over time (curve a in Fig. 3A). Then we excluded nonspecific binding (curve b in Fig. 3A) from the ladle-shaped curve before curve-fitting. The formation and reverse rate constants (k_1 and k_−1, respectively) of the intermediate and the release rate constant (k_cat) were obtained from curve fittings on the basis of Equation 3. More detailed information about transient kinetic analysis on a QCM has been described in previous studies (24, 25).

The DsbA(C33A)_{SH} variant could form the DsbA-DsbB intermediate with DsbB_{S,S,S} but could not proceed to the oxidation step. The formation of an intermediate between the DsbA(C33A)_{SH} and DsbB_{S,S,S} is described by Equation 1. At time t, the mass of the reaction intermediate of the DsbA-DsbB complex is given by Equations 2 and 3, where [DsbA]_0 is the initial concentration of reduced DsbA_{SH,SH}, [DsbB]_0 is the initial concentration of oxidized DsbB_{S,S,S}, [DsbA-S] is the concentration of the DsbA-DsbB intermediate, M_w is the molecular weight of DsbA_{SH,SH}, and [DsbB]_0 >> [DsbB]_0.

\[
\text{DsbA}_{SH,SH} + \text{DsbB}_{S,S,S} \rightleftharpoons \text{DsbA-DsbB} \rightarrow \text{DsbA-S} + \text{DsbB}_{S,S,S} \\
\quad (\text{Eq. 1})
\]

\[
[DsbA-DsbB]_t = (e^{−kt} - e^{−kt}) \\
\quad (\text{Eq. 2})
\]

\[
\Delta m_t = M_w \cdot (e^{−kt} - e^{−kt}) \\
\quad (\text{Eq. 3})
\]

\[
X = \frac{P - \sqrt{P^2 - 4Q}}{2} \\
\quad (\text{Eq. 4})
\]

\[
Y = \frac{f + \sqrt{f^2 - 4Q}}{2} \\
\quad (\text{Eq. 5})
\]

\[
Z = \frac{k_1[DsbA]_0[DsbB]_0}{Y - X} \\
\quad (\text{Eq. 6})
\]

\[
P = k_1[DsbA]_0 + k_−_1 + k_cat \\
\quad (\text{Eq. 7})
\]

\[
Q = k_1k_cat[DsbA]_0 \\
\quad (\text{Eq. 8})
\]

We followed the formation and degradation of the DsbA-DsbB intermediate on the QCM as frequency (mass) changes over time (curve a in Fig. 3A). Then we excluded nonspecific binding (curve b in Fig. 3A) from the ladle-shaped curve before curve-fitting. The formation and reverse rate constants (k_1 and k_−1, respectively) of the intermediate and the release rate constant (k_cat) were obtained from curve fittings on the basis of Equation 3. More detailed information about transient kinetic analysis on a QCM has been described in previous studies (24, 25).

The DsbA(C33A)_{SH} variant could form the DsbA-DsbB intermediate with DsbB_{S,S,S} but could not proceed to the oxidation step. The formation of an intermediate between the DsbA(C33A)_{SH} variant and DsbB_{S,S,S} is described by Equation 9. At time t the mass of the intermediate is given by Equations 10–12, as previously described (20).

\[
\text{DsbA}_{SH,SH} + \text{DsbB}_{S,S,S} \rightleftharpoons \text{DsbA-DsbB} \\
\quad (\text{Eq. 9})
\]

\[
[DsbA-DsbB]_t = [DsbA-DsbB]_{max} \cdot [1 - \exp(-t/\tau)] \\
\quad (\text{Eq. 10})
\]

\[
\Delta m_t = \Delta m_{max} \cdot [1 - \exp(-t/\tau)] \\
\quad (\text{Eq. 11})
\]

\[
\tau = k_1[DsbA]_0 + k_−_1 \\
\quad (\text{Eq. 12})
\]

By following the formation of the DsbA(C33A)_{SH}-DsbB intermediate over time on the QCM, the relaxation time (τ) was obtained from curve fittings of the simple QCM frequency
decrease (mass increase) on the basis of Equation 11. When binding experiments were carried out at various concentrations of the DsbA(C33A)SH variant to obtain each relaxation time, the formation and reverse rate constants ($k_f$ and $k_r$, respectively) of the DsbA(C33A)SH variant for DsbBS-S,S-S were obtained from the slope and intercept of the plot for Equation 12, respectively. The parameter corresponding to the dissociation constant ($K_d$) was calculated from the ratio of $k_r$ to $k_f$. Kinetic analysis of DsbA(C33A)SH with DsbB(C41A,C44A)S-S variants was also carried out in the same manner.

**RESULTS**

Construction of DsbB-embedded Supported Lipid Bilayers on QCM—In vitro experiments on membrane proteins, the activities of hydrophobic membrane proteins are usually measured in a surfactant-solubilized form (6, 11, 13, 33). In the presence of a surfactant, however, enzymatic activity may be reduced, due to the creation of an unnatural environment for the membrane protein. Although using a proteoliposome is another method for solubilizing membrane proteins in solution, it is
unsuitable for quantitative measurements due to the small and unclear amounts of active membrane proteins in bulk solutions. To observe and quantify thiol-disulfide exchange reactions between soluble DsbA-SS-H and membrane-embedded DsbB-SS-S-S, we examined the construction of DsbB-SS-S-S-embedded supported lipid bilayers on a QCM plate (Fig. 2). We first prepared biotin-BCCP-DsbB using an E. coli protein expression system as a fusion protein consisting of the BCCP tag, which included a region biotinylated by an endogenous biotin ligase in E. coli. Biotin-BCCP-DsbB was immobilized on a NeutrAvidin-modified QCM plate through biotin-avidin linking in the presence of DDM. This immobilization method has the advantage of regulating the amount of protein bound onto the QCM and the unidirectional orientation of membrane proteins (Fig. 2B). Next, lipid bilayers were formed around the biotin-BCCP-DsbB immobilized on the QCM plate by the addition of lipid-surfactant-mixed micelles followed by slow removal of the surfactants. We observed frequency decreases (mass increases) due to the binding of the mixed micelle around the DsbB (data not shown). After saturation of the frequency decrease, the buffer was replaced by a buffer without DDM several times to achieve a lower DDM concentration than the critical micelle concentration of DDM. To confirm the formation of lipid bilayers on the QCM plate, we also prepared a lipid bilayer on the QCM plate in the same manner by using a mixture of E. coli lipid extracts, DDM, and biotinylated lipid (5 mol%), and we subsequently observed the binding of avidin onto the biotinylated lipid bilayer (data not shown). The amount of bound avidin was consistent with the amount of avidin adsorbed onto a planar substrate, such as the gold electrode of a QCM, indicating that planar lipid bilayers around DsbB were formed on the QCM plate.

**Observation of Reactions between DsbA and DsbB**—Fig. 3A indicates typical frequency changes as a function of time for 27-MHz QCM on which DsbB-SS-S-S-embedded lipid bilayers was immobilized, in response to the addition of native DsbA-SS-H, DsbA(C30A)-SS-H, and DsbA(C33A)-SS-H variants. After the addition of DsbA-SS-H, the frequency decreased (i.e. the mass increased) rapidly and then increased gradually to return to the original frequency (curve a in Fig. 3A). This behavior corresponds to the formation of the DsbA-DsbB intermediate followed by the release of oxidized DsbA-S-S from reduced DsbB-SS-S-SS-H in the membrane (Fig. 2C). When the DsbA(C30A)-SS-H variant was added onto the DsbB-SS-S-S-SS-H immobilized QCM plate, only a very slight change was observed (curve b in Fig. 3A), indicating that Cys-30 of DsbA was essential for the formation of the DsbA-DsbB intermediate. This is consistent with x-ray crystallography reports demonstrating that a disulfide bond is formed between Cys-30 of DsbA-SS-H and Cys-104 of DsbB-SS-S-S (Fig. 2C) (3). We also confirmed that oxidized DsbA-S-S could not interact with DsbB-SS-S-S or reduced DsbB-SS-S-S-H on the QCM (curves a and b in Fig. 3B). When the DsbA(C33A)-SS-H variant was added, it could bind to, but not be released from DsbB-SS-S-S-SS-H (curve c in Fig. 3A). This indicated that Cys-30 of DsbA-SS-H formed an interprotein disulfide bond with Cys-104 of DsbB-SS-S-S-S and that Cys-33 of DsbA-SS-H was essential for the oxidation of DsbA-SS-H to allow it to be released from reduced DsbB-SS-S-S-H (Fig. 2C). Thus, we could detect the intermediate of the covalently bonded DsbA-DsbB complex on a DsbB-immobilized QCM.

We also confirmed that supported bilayer structures are important for formation of DsbA-DsbB intermediates. When DsbB-SS-S-S was covered with DDM micelles, the amounts and formation rates of DsbA-DsbB intermediates produced by the addition of DsbA-SS-H (curve a in Fig. 3C) and DsbA(C33A)-SS-H (curve c) were very small and slow compared with those shown in Fig. 3A. This clearly indicated that DDM inhibited interactions between DsbA-SS-H and DsbB-SS-S-S and/or that lipid bilayers were required to activate DsbB-SS-S-S.

**Importance of the Two Cysteine Residues (Cys-41 and Cys-44) of DsbB-SS-S-S for the Reaction with DsbA-SS-H**—Previously, two potential models were proposed to represent the reaction pathway of the oxidation mechanism of DsbA-SS-H by DsbB-SS-S-S as follows. 1) Oxidized DsbA-S-S is released from the DsbA-DsbB intermediate before intraprotein isomerization in DsbB (attack of the reduced Cys-130 on Cys-41 of DsbB, pathway I in Fig. 1), or 2) oxidized DsbA-S-S is released after the formation of the interloop Cys-41–Cys-130 disulfide bond of DsbB (pathway II). In the case of pathway I, only two cysteine residues (Cys-104 and Cys-130) of DsbB are involved in the formation of the DsbA-DsbB intermediate. For pathway II, four cysteine residues (Cys-41, Cys-44, Cys-104, and Cys-130) of DsbB are required for the formation of the DsbA-DsbB intermediate. To investigate the importance of these cysteine residues, we used the DsbB(C41A,C44A)-SS-H variant, which has only two cysteine residues (Cys-104 and Cys-130). As shown in Fig. 4A, both DsbA-SS-H and the DsbA(C33A)-SS-H variant formed only small amounts of the DsbA-DsbB intermediate when reacted with the DsbB(C41A,C44A)-SS-H variant under the same conditions as used in Fig. 3A. Meanwhile, when using a high concentration of the DsbA(C33A)-SS-H variant, we found that the amount of the interprotein disulfide-bonded intermediate formed by DsbA(C33A)-SS-H with DsbB(C41A,C44A)-SS-H was equal to that with DsbB-SS-S-S (Fig. 4B). These data indicated that the DsbB(C41A,C44A)-SS-H variant was not inactivated by Ala mutations and that the stability of the DsbA-DsbB(C41A,C44A)-SS-H intermediate was clearly lower than that of the DsbA-DsbB intermediate, which included all four DsbB cysteine residues.

**Effects of Ubiquinone in DsbB-SS-S-S on the Reaction with DsbA-SS-H**—The intermediates of the covalently bonded DsbA-DsbB complex detected by QCM included electron-transfer complexes between DsbA-SS-H and DsbB-SS-S-S. We predicted that ubiquinone, an acceptor of two electrons (and two protons) from reduced DsbB-SS-S-S-H in the DsbA-DsbB system, may play a key role in the formation of the stable intermediate as a one-electron-transfer complex. Ala mutations in the unstable DsbA-DsbB(C41A,C44A)-SS-H intermediate seemed to block electron transfer from DsbA-SS-H to ubiquinone via DsbB-SS-S-S. To investigate whether ubiquinone contributes to the stability of the DsbA-DsbB intermediate, we observed the formation of the DsbA-DsbB intermediate in the presence of excessive ubiquinone-10 (UQ) that was added in the supported lipid bilayers (Fig. 5A) and in the absence of endogenous ubiquinone (Fig. 5B) using DsbB-SS-S-S with reduced ubiquinol (UQ2−). DsbB-SS-S-S(UQ2−) was prepared as described under “Experimental Procedures.” Surprisingly, the
formation curve of the DsbA-DsbB intermediate from the reaction of DsbA(C33A)SH and DsbB-SS,SS was almost the same, independent of the redox state of ubiquinone (UQ or UQ\(_2^+/\)) (Fig. 5, A and B). Similar results were obtained using native DsbA-SS,SS. The plateau phase (3–10 s) found in curve a in Fig. 5A in the presence of excess UQ can be explained by the turnover of DsbA-SS,SS to DsbB-SS,SS, recycled by UQ in supported lipid bilayers. These results implied that the formation of the DsbA-DsbB intermediate and the oxidation of DsbA should occur nearly independently of ubiquinone and that ubiquinone functions in the re-oxidation of DsbB-SS,SS rather than DsbA.

**SDS-PAGE Analysis of Oxidation Products from Reactions between DsbA and DsbB in the Bulk Solution**—Although we could follow the oxidation reaction of DsbA-SS,SH with DsbB-SS,SS embedded in supported bilayers on the QCM as changes in frequency, we needed to confirm the actual products formed in this reaction. Therefore, we next performed nonreducing SDS-PAGE analysis for the reaction between DsbA-SS,SH and DsbB-SS,SS in the DDM micellar solution. After reactions of DsbA-SS,SH with DsbB-SS,SS or DsbB(C41A,C44A)-SS, we confirmed that oxidized DsbA-SS appeared as the reaction product (reaction time 3 min) at 21 kDa together with unreacted DsbA-SS as a reference. Oxidized DsbA-SS was identified on 12% nonreducing gels by SDS-PAGE after staining with Coomassie Brilliant blue.

**FIGURE 5. Effects of ubiquinone on time courses of frequency changes in the QCM experiments.** Shown are reactions of DsbB-SS,SS embedded in supported lipid bilayers including additional excessive UQ (A) and reactions of DsbB-SS,SS that were coexistent with ubiquinol (UQ\(_2^−/\)) (B). All experiments were carried out under the same conditions in Fig. 3A.

**FIGURE 6. SDS-PAGE of DsbA-SS,SH and DsbB-SS,SS or DsbB(C41A,C44A)-SS reaction products in the bulk solution with DDM micelles.** Reactions were carried out in 100 mM citrate, pH 6.0, with 0.1% DDM for 3 min and terminated by the addition of trichloroacetic acid. Oxidized DsbA-SS (21 kDa) and AMS-modified DsbA (22 kDa) were identified on 12% nonreducing gels by SDS-PAGE after staining with Coomassie Brilliant blue.

| Reaction Product | Oxidized DsbA-SS | AMS-modified DsbA |
|------------------|-----------------|------------------|
| DsbA-SS,SH       | +               | +                |
| DsbB-SS,SS       | −               | −                |
| DsbB(C41A,C44A)-SS | −             | −                |
| AMS              | +               | +                |
| AMS-modified DsbA | Oxidized DsbA (Reacted product) | Oxidized DsbA-SS |

**FIGURE 4. Effects of the two cysteine residues (Cys-41 and Cys-44) of DsbB-SS,SS for the reaction with DsbA-SS,SH.** A, typical time courses of frequency changes in the QCM on which DsbB(C41A,C44A)-SS, immobilized in supported bilayers was immobilized in response to the addition of DsbA-SS,SH (a) and DsbA(C33A)-SH (b). The experimental condition was the same as that of reactions between DsbA-SS,SH and DsbB-SS,SS in Fig. 3A, B, saturation curves of mass increases due to the intermediate formation between DsbA(C33A)-SH and DsbB-SS,SS (a) or DsbB(C41A,C44A)-SS, immobilized on a QCM. Maximum binding amounts (−ΔF\(_\text{max}\)) were calculated to be 321 ± 12 Hz for DsbB-SS,SS and 392 ± 78 Hz for DsbB(C41A,C44A)-SS with experimental errors, respectively.
Kinetic Analysis of Reactions between DsbA and DsbB—To assess the dynamic behavior of the formation of the DsbA-DsbB intermediate, we performed kinetic analysis of the time dependences of the \( \mathcal{F} \) curves. The ladle-shaped curve of the reaction between DsbA$_{SH,SH}$ and DsbB$_{S,S,S,S}$ (curve a in Fig. 3A) is described by Equations 1–3 under “Experimental Procedures” (24, 25). Kinetic parameters, i.e. the formation and reverse rate constants \((k_f \text{ and } k_r)\) respectively and the oxidation rate constant \((k_{cat})\), were obtained from curve-fittings as optimized coefficients in these equations. Experimental curves, generated using different concentrations of DsbA$_{SH,SH}$ (Fig. 7A) were fitted well with theoretical curves described by Equations 2 and 3. The \( k_f \) and \( k_{cat} \) values were obtained from one experimental curve, and the average of values at three different concentrations of DsbA$_{SH,SH}$ in Fig. 7A were calculated. The obtained binding rate constants \((k_f = 5.9 \times 10^6 \text{ M}^{-1} \text{s}^{-1})\) and catalytic rate constants \((k_{cat} = 0.12 \text{ s}^{-1})\) are summarized in Table 1. The \( k_r \) value could not be accurately measured because it was negligibly smaller than the \( k_{cat} \) value.
Oxidation Reaction of DsbA and DsbB on QCM

TABLE 1

Kinetic parameters of the thiol-disulfide exchange reaction between DsbA and DsbB embedded in supported bilayers on the 27-MHz QCM

| DsbA          | DsbB          | $k_f$   | $k_r$   | $k_d = k_f/k_r$ | $k_{cat}$ |
|---------------|---------------|---------|---------|----------------|-----------|
| DsbA(C33A)$_{SH}$ | DsbB$_{S-S}$ | 5.9 ± 1.8 | 10$^6$ M$^{-1}$ s$^{-1}$ | 0.0020 ± 0.004 | 0.08 ± 0.002 |
| DsbA(C33A)$_{SH}$ | DsbB$_{S-S}$ | 2.3 ± 0.2 | 10$^6$ M$^{-1}$ s$^{-1}$ | 0.12 ± 0.02 | 2300 ± 447 |
| DsbA($C_{33}A$)$_{SH}$ | DsbB(C41A,C44A)$_{SS}$ | 0.5 ± 0.05 | 10$^6$ M$^{-1}$ s$^{-1}$ | 0.0012 ± 0.004 | 0.004 ± 0.002 |
| DsbA$_{SH,SH}$ | DsbB$_{S-S}$($\Delta$UQ) | 7.8 ± 2.6 | 10$^6$ M$^{-1}$ s$^{-1}$ | 3 ± 10 | 13 ± 11 |
| DsbA(C33A)$_{SH}$ | DsbB$_{S,S,S}$ | 3.6 ± 0.2 | 10$^6$ M$^{-1}$ s$^{-1}$ | 0.0024 ± 0.002 | 0.15 ± 0.06 |

The experimental conditions were 50 mM citrate, pH 6.0, 100 mM NaCl, 25 °C. Each kinetic parameter was obtained from Equations 2 and 3 and from 4–6 and is reported with experimental errors. The formation and reverse rate constants ($k_f$ and $k_r$, respectively) of the intermediate and the DsbA oxidation rate constant ($k_{cat}$) were obtained using our QCM method with different experimental conditions, the formation rate of the covalent DsbA-DsbB complex in this study was similar to the rate constant ($k_{cat}$) obtained in the previous study (reaction conditions: 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 0.04% DDM at 10 °C) (13). Despite different experimental conditions, the $k_f$ value (5.9 × 10$^5$ M$^{-1}$ s$^{-1}$) obtained showing purple color, see Stable intermediate in Fig. 9B from the formation of the covalent DsbA-DsbB complex in this study was similar to the rate constant (~5 × 10$^5$ M$^{-1}$ s$^{-1}$) of formation of the DsbB-ubiquinone complex (purple complex) in the process of electron transfer from DsbA to ubiquinone reported in the previous study (reaction conditions: 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 0.04% DDM at 10 °C) (13). This suggests that the rate-limiting step is likely the covalent binding of DsbA to DsbB. On the other hand, the rate of DsbA oxidation (~2 s$^{-1}$ at pH 8.0), which was found to be the same as the rate of quinone reduction in the previous work, is larger than the $k_{cat}$ value (0.12 s$^{-1}$ at pH 6.0) obtained in the current work. This may be due to the higher pH used in the previous work, as the presence of the thiolate anion at Cys-33(DsbA) increases with increasing pH. We confirmed the similar $k_f$ value and increased $k_{cat}$ value at pH 8.0 in our other studies (34). Unlike absorbance
measurements, where the solution condition requires a pH greater than 8.0 to obtain the purple complex of DsbB-ubiquinone.

**The Mechanism of DsbA/DsbB Reaction Based on Kinetic Parameters**—The destiny of the DsbA-DsbB intermediate can be determined by both the $k_r$ and $k_{cat}$ values (Fig. 8B). On comparing the first-order rate constants, $k_r$ and $k_{cat}$, between DsbA$_{SH,SH}$ and DsbB$_{S,S,S}$, we found that the $k_{cat}$ value (0.12 s$^{-1}$) was 60 times larger than the estimated $k_r$ value (0.0020 s$^{-1}$). This suggested that the reaction of DsbA$_{SH,SH}$ with DsbB$_{S,S,S}$ was a consecutive reaction. Moreover, the $k_r$ value in the reaction between DsbA$_{SH,SH}$ and the DsbB(C41A,C44A)$_{S,S}$ variant (0.12 s$^{-1}$) was also 60 times larger than the $k_r$ value in the reaction between DsbA$_{SH,SH}$ and DsbB$_{S,S,S}$ (0.0020 s$^{-1}$; Fig. 9A). These results clearly suggested that the stability of the DsbA-DsbB(C41A,C44A)$_{S,S}$ intermediate was largely reduced due to the lack of these two cysteine residues.

In the thiol-disulfide exchange reaction between DsbA$_{SH,SH}$ and DsbB$_{S,S,S}$, the interprotein disulfide bond of Cys-30 with Cys-104 is first formed by nucleophilic attack of Cys-30 of DsbA$_{SH,SH}$ on Cys-104 of DsbB$_{S,S,S}$. Then, reduced Cys-130 attacks Cys-104 or Cys-41 of the Cys-41–Cys-44 disulfide bond of DsbB. The attack on Cys-104 results in the reverse reaction, whereas the attack on Cys-41 results in the formation of the interloop Cys-41–Cys-130 disulfide bond in pathway II.

The formation of the interloop Cys-41–Cys-130 prevents the reverse reaction caused by the attack of Cys-130 on Cys-104 as a stable intermediate. We found that the formation of the stable intermediate reduced the rate of the reverse reaction from $k_r = 0.12$ s$^{-1}$ to $k_r = 0.0020$ s$^{-1}$. This allowed the forward reaction to proceed (Fig. 9B). Thus, our data demonstrated that the thiol-disulfide exchange reaction between DsbA$_{SH,SH}$ and DsbB$_{S,S,S}$ could occur efficiently through the stable intermediate, as indicated by pathway II.

**Ubiquinone-independent Oxidation of DsbA on DsbB**—We expected that ubiquinone in DsbB could contribute to the reduction of the reverse rate constant ($k_r$) in the stable intermediate due to delocalization of the electrons transferred from DsbA because ubiquinone can interact with the Cys-44 thiolate anion produced by the cleavage of the Cys-41–Cys-44 disulfide bond of DsbB (14). However, we obtained kinetic parameters
Oxidation Reaction of DsbA and DsbB on QCM

(k_p, k_r, and k_cat) that were virtually unchanged in the presence or absence of ubiquinone. These data indicated that a stable intermediate could form between DsbA_{SH,SH} and DsbB_{S,S-S,S} without ubiquinone and that the reaction step involving two electron transfers from DsbA_{SH,SH} to DsbB_{S,S-S,S} could proceed even in the absence of ubiquinone. In fact, it has been reported that quinone-free DsbB_{S,S-S,S} can produce oxidative DsbA_{SH,SH} (31). In the stable intermediate in pathway II, the interloop Cys-41–Cys-130, which prevents the reverse reaction, may be retained due to the reduced nucleophilicity of the thiolate anion at pH 6.6, the reported pK_a of the thiol group of Cys-44(DsbB) (14) instead of electron transfer to ubiquinone. In other words, the stable separation of a transferred electron from DsbA_{SH,SH} to Cys-44(DsbB) instead of ubiquinone should contribute to the stability of the DsbA-DsbB intermediate, resulting in the efficient oxidation of DsbA_{SH,SH}. Thus, DsbB has two functions in the DsbA-DsbB system; that is, DsbA_{SH,SH} oxidation and ubiquinone reduction. The optimum pH for the DsbA_{SH,SH} oxidation reaction by DsbB_{S,S-S,S} is reported to be 5.5–6.0 (12). We expect that the oxidation of DsbA_{SH,SH} at the proper pH (i.e., pH 6.0) can proceed independently of the reduction of ubiquinone. We present this reaction model, referred to as modified pathway II, in Fig. 9C.

Pathway of the Oxidation Reaction of DsbA and DsbB—Next, we examined the possibility that the oxidation reaction proceeds through pathway I (Fig. 1). A small amount of oxidized DsbA_{S,S} was found in the reaction between DsbA_{SH,SH} and the DsbB(C41A,C44A)_{S,S} variant in SDS-PAGE experiments (Fig. 6), indicating that the oxidation reaction occurred without passage through the stable intermediate. This result also supported the hypothesis that DsbA oxidation can occur independent of ubiquinone (31). Given the kinetic parameters obtained in our current study, the forward reaction should become kinetically competitive with the reverse reaction (k_r = 0.12 s^{-1} and k_cat = 0.12 s^{-1}) in the reaction mechanism. The ratio of the amount of product generated through pathways II and I can be estimated from a comparison of the rates of product formation (=k_cat[DsbA-DsbB]). Because the existence of the stable intermediate reduced the k_r value by 60-fold, we estimated that the ratio of the pathway I intermediate to the pathway II stable intermediate would be 1:60 (Fig. 1). This suggested that pathway II occurs ~98% of the time, whereas pathway I occurs only ~2% of the time. Therefore, we concluded that the thiol-disulfide exchange reaction between DsbA_{SH,SH} and DsbB_{S,S-S,S} proceeds mainly through pathway II, which involves formation of the stable intermediate, and that pathway I may sometimes occur due to the more rapid attack of Cys-33 on Cys-30 than that of Cys-130 on Cys-104 in the intermediate step.

Driving Force of the Disulfide Bond Exchange Reaction between DsbA and DsbB—The thiol-disulfide bond exchange reaction between DsbA_{SH,SH} and DsbB_{S,S-S,S} involves the transfer of two electrons (and two protons) from DsbA_{SH,SH} to DsbB_{S,S-S,S}. The driving force of the reaction can be explained by differences between the redox potential values of DsbA_{SH,SH} (~120 mV) and ubiquinone (+110 mV) (6, 31, 33). Because the redox potentials of DsbB_{S,S-S,S} (Cys-41–Cys-44: −207 mV, Cys-104–Cys-130 and Cys-41–Cys-130: −224 mV) are reductive toward DsbA_{SH,SH}, the oxidative power of ubiquinone was previously thought to be essential for the oxidation of DsbA. In our results, however, the effects of ubiquinone on the kinetic parameters of the formation of the DsbA-DsbB intermediate (one-electron transfer complex) and the oxidation of DsbA (two-electron transfer reaction) were not apparent. In addition, the DsbB(C41A,C44A)_{S,S} variant lacking two cysteine residues to transfer the electron to ubiquinone could form the DsbA-DsbB intermediate (one-electron transfer complex) and could oxidize DsbA_{SH,SH} although the reaction efficiency was low. These results imply that the oxidative power of ubiquinone is not a prerequisite for DsbA oxidation. This surprising consequence is consistent with a previous report demonstrating that quinone-free DsbB_{S,S-S,S} and the DsbB(C41S,C44S)_{S,S} variant could oxidize DsbA_{SH,SH} (31). To interpret the mechanism involved in ubiquinone-independent oxidation of DsbA, we present two possibilities: 1) the redox potentials of the disulfide bonds of DsbB_{S,S-S,S} in the DsbA-DsbB complex are oxidative in comparison with DsbA_{SH,SH}, and 2) the electron transfer reaction from DsbA_{SH,SH} to DsbB_{S,S-S,S} proceeds through a kinetically controlled pathway. We confirmed that reverse electron-transfer from reduced DsbB_{SH,SH} to oxidized DsbA_{S,S} could not occur on the QCM despite a favorable reaction in terms of the redox potential (curve b in Fig. 3B). Thus, our results supported that the thiol-disulfide bond exchange reaction between DsbA_{SH,SH} and DsbB_{S,S-S,S} should proceed in a kinetically favorable direction.

Innovation—We demonstrated that the DsbA-DsbB intermediate could be directly detected using a QCM device on which DsbB_{S,S-S,S} was embedded in supported lipid bilayers according to the ladle-type frequency curve of the thiol-disulfide exchange reaction between DsbA_{SH,SH} and DsbB_{S,S-S,S}. The kinetic parameters (k_p, k_r, and k_cat) obtained from in situ monitoring of the DsbA-DsbB complex intermediate allowed us to examine the reaction mechanism kinetically. Biomolecular reactions in vivo may be controlled not only thermodynamically but also kinetically. Thus, elucidation of the transient kinetics of this reaction intermediate broadens our understanding of the kinetics of biomolecular reaction mechanisms.

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