Articles

Contributions of Substrate Binding to the Catalytic Activity of DsbC

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ABSTRACT: DsbA and DsbC are involved in protein disulfide bond formation in the periplasm of Gram-negative bacteria. The two proteins are thought to fulfill different functions in vivo, DsbA as a catalyst of disulfide bond formation and DsbC as a catalyst of disulfide bond rearrangement. To explore the basis of this catalytic complementarity, the reaction mechanism of DsbC has been examined using unstructured model peptides that contain only one or two cysteine residues as substrates. The reactions between the various forms of the peptide and DsbC occur at rates up to 10^6-fold faster than those that involve glutathione and DsbC, and they were constrained to occur at only one sulfur atom of disulfide bonds involving the peptide. Mixed disulfide complexes of DsbC and the peptide were 10^4-fold more stable than the corresponding mixed disulfides with glutathione. These observations suggest that noncovalent binding interactions occur between the peptide and DsbC, which contribute to the very rapid kinetics of substrate utilization. The interactions between DsbC and the peptide appear to be more substantial than those between DsbA and the same peptide. The differences in the reaction of the peptide at the active sites of DsbA and DsbC provide insight into why DsbC is the better catalyst of disulfide bond rearrangement and how the active site chemistry of these structurally related proteins has been adapted to fulfill complementary functions.

Disulfide bonds are a common feature of secretory proteins and are often required for the stability of the folded state, but their formation is a chemically slow process that can be an important rate-limiting step in protein folding (*1*). To overcome this problem, disulfide bond formation is assisted in vivo by the participation of a number of catalysts. In the secretory compartment of eukaryotic cells, the endoplasmic reticulum, the major catalyst is PDI[1] (*2*, *3*), which catalyzes protein disulfide bond formation, rearrangement, and reduction (*4*, *5*). In the bacterial periplasm, in contrast, several individual proteins have been associated with these processes (*6*−*12*). At least two proteins, DsbA and DsbC, are thought to be required to fulfill the functions of eukaryotic PDI (*13*, *14*). Both proteins catalyze disulfide bond formation, but only DsbC can catalyze disulfide bond rearrangement (*13*, *14*), albeit less effectively than PDI. This observation, together with genetic evidence and in vivo studies, has suggested that DsbA functions in the periplasm as the catalyst of disulfide bond formation and DsbC as the catalyst of disulfide bond rearrangement (*15*−*17*).

A common feature of PDI, DsbA, and DsbC is that they all contain Cys-X-Y-Cys active site sequences and are structurally related to thioredoxin (*18*−*23*). General features of these proteins are that the first cysteine residue in the active site is solvent exposed, has a low pK_a, and is highly reactive, whereas the second cysteine residue is buried (*14*, *24*−*30*). The catalytic mechanism of each of these proteins involves the cycling of the active site cysteine residues between the reduced and disulfide-bonded forms, via an intermediate with a covalent disulfide bond between the catalyst (E) and substrate protein (P) (*31*−*33*). In the process, a disulfide bond can be transferred to or from the catalyst, allowing it to serve as either a direct reductant or oxidant, depending upon the stability of the active site disulfide bond.

$$E_S^S + P_{SH}^{SH} \rightleftharpoons E_{SH}^{S-S}P_{SH} \rightleftharpoons E_{SH}^{SH} + P_S^S \quad (1)$$

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; $DsbC_{SH}^{SH}$ and $DsbC_S^S$, the dithiol and disulfide forms of protein DsbC; $DsbC_{101Ala}^{98SH}$, DsbC with Cys101 replaced by Ala; P_{SH}^{SH}, peptide substrate containing cysteine residues at positions 2 and 27 (when necessary, the two thiol groups are distinguished by their residue numbers); P_{2OH}^{27SH}, peptide substrate with a single cysteine residue at position 27 and a Ser residue at position 2 (the mixed disulfide forms of these peptides and of DsbC with glutathione are indicated by the sub- and superscripts -SSG); GSSG and GSH, the oxidized and reduced forms of glutathione, respectively; HPLC, high-pressure liquid chromatography; PDI, protein disulfide isomerase; TFA, trifluoroacetic acid.

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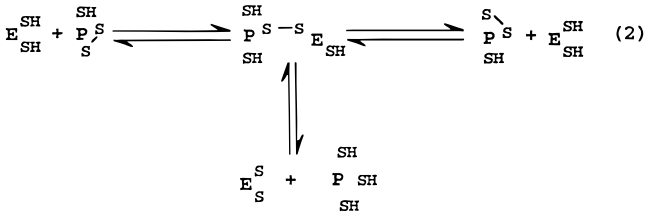
DsbA, DsbC, and PDI have unstable disulfide bonds at their active sites and consequently function as oxidants (*14*, *24*, *25*, *28*, *32*, *34*−*39*), whereas thioredoxin has a stable active site disulfide bond and functions as a reductant (*20*, *40*). Another possibility is that the intermediate complex reacts with an alternative cysteine residue of the substrate to produce disulfide bond rearrangement.



(2)

DsbA and DsbC are small proteins, each about 20 kDa in size and with a single active site, whereas PDI is a 55 kDa protein with two active sites located in two domains that are homologous to thioredoxin and with two additional major structural domains (*2*, *3*, *18*, *41*). In isolation, the two active site-containing domains of PDI are functionally very similar to DsbA, being excellent catalysts of disulfide bond formation, but much weaker catalysts of disulfide bond reduction and rearrangement compared to intact PDI (*37*). The similarities between DsbA and the individual thioredoxin-like domains of PDI plus their differences from intact PDI have raised questions about what features of the different catalysts are responsible for their individual spectra of functions. In particular, the features of these enzymes that are important for the catalysis of disulfide bond rearrangements remain unclear.

The small size and structural simplicity of the bacterial catalysts and of the individual domains of PDI are of considerable advantage in studying their reaction mechanisms, as the active site chemistries of these proteins have been established by direct methods (*14*, *25*, *28*, *42*). A second important advantage is the use of simplified substrates. Instead of using an intact protein with multiple cysteine residues and disulfide bonds, where many processes can take place simultaneously and in which conformational effects may further confuse interpretation, the processes can be readily studied using simple unfolded peptides that contain one or two cysteine residues (*5*). Using such peptides and active site mutants of DsbA, the catalytic cycle of DsbA has been analyzed in detail (*32*). Catalytic activity was found to result from both the ability of the highly reactive active site to participate in rapid thiol−disulfide exchange and from noncovalent binding of the substrate to the catalyst to increase rates further (*32*, *33*). In this report, we now extend these techniques to analyze the role of substrate binding in the catalytic mechanism of DsbC.

MATERIALS AND METHODS

Materials. The peptide substrate used in this study, P_{SH}^{SH}, is derived from residues 4−31 of BPTI and has the sequence FCLEPPYTGPSKARIIRYFYNAKAGLCQ, with the N- and C-terminal groups acetylated and amidated, respectively. A second peptide, P_{2OH}^{27SH}, in which Cys2 was replaced by a Ser residue was also used. The peptides were prepared and quantified as described previously (*5*). Mixed disulfide forms of the peptide were isolated by reverse phase HPLC at acid pH, as used in analysis of the various reaction products described below; they were recovered by lyophylization.

Normal DsbC and that with Cys101 replaced by Ala, Dsb C_{101Ala}^{98SH}, and the mixed disulfide form DsbC_{101Ala}^{98SSG} were purified as described previously (*14*). They were quantified using the molar absorbance coefficient of 16 170 at 280 nm for all forms. Dsb$C_{101Ala}^{98S-S27}P_{2OH}$ was prepared by reaction of DsbC_{101Ala}^{98SH} with a 1.5-fold molar excess of P_{2OH}^{27SSG} in 0.5 mL of 0.1 M Tris-HCl (pH 7.4) for 30 min, followed by gel filtration on a 13 cm × 1 cm Sephadex G-25 column equilibrated in 10 mM Tris-HCl (pH 7.4) to remove the unreacted peptide and GSH; it was quantified using a molar extinction coefficient of 20 010 at 280 nm.

Thiol−Disulfide Exchange Reactions. All reactions between peptide, thiol, and disulfide reagents and DsbC were carried out in 0.2 M KCl, 1 mM EDTA, buffered by 0.1 M Tris-HCl (pH 7.4). The total volumes of the reaction mixtures were in the range of 2−4 mL and were adjusted so that at least 5−10 pmol of reaction product could be generated for each HPLC analysis. The concentrations of the various reactants are indicated in the figure legends. A reaction mixture containing one of the reactants and the buffer components was pre-equilibrated at 25 °C for 10 min, and the reactions were then initiated by the addition of the second reactant, with simultaneous rapid mixing. After the desired time interval, reactions were quenched by addition of HCl to a final concentration of 0.1−0.3 M.

Analysis of Reaction Products. To resolve simultaneously the species P_{2OH}^{27SH}, P_{2OH}^{27SSG}, $P_{2OH}^{27S-S27}P_{2OH}$, DsbC_{101Ala}^{98SH}, DsbC_{101Ala}^{98SSG}, and Dsb$C_{101Ala}^{98S-S27}P_{2OH}$, quenched reaction mixtures were analyzed on a Vydac 25 cm × 0.46 cm 218TP54 column at 38 °C at a flow rate of 1 mL min^{-1}, using a 50 min gradient of 25 to 50% (v/v) acetonitrile in 0.1% (v/v) TFA (gradient A) (Figure 1a,b). An alternative gradient was used when only the DsbC-related species were to be resolved: 0−10 min, 38% (v/v) acetonitrile in 0.1% (v/v) TFA, followed by a 20 min gradient to 46% (v/v) acetonitrile (gradient B) (Figure 1c).

RESULTS

Reaction between the Model Peptide and Glutathione. The 28-residue model peptide used in this study, designated P_{2SH}^{27SH}, is based on residues 4−31 of BPTI, including Cys5 and Cys30, but with Cys14 replaced by Ser. Consequently, the peptide has two cysteine residues at positions 2 and 27. It is a simple model of an unfolded protein, as it adopts only local nonrandom conformations (*43*) that do not affect formation and breakage of its disulfide bond. This peptide, plus a variant with Cys2 replaced by Ser, P_{2OH}^{27SH}, were used in previous studies of the effect of PDI and DsbA on disulfide bond formation (*5*, *32*), using HPLC to resolve all the possible thiol- and disulfide-bonded forms of the peptide. Its chemical thiol−disulfide exchange reactions with GSSG and GSH at pH 7.4 were extensively characterized in these studies. Considering the two different mixed disulfide species together, the apparent rate and equilibrium constants are

The individual mixed disulfide species were distinguished, and that on Cys27 accumulated somewhat more than the other, probably due to weak electrostatic interactions of the

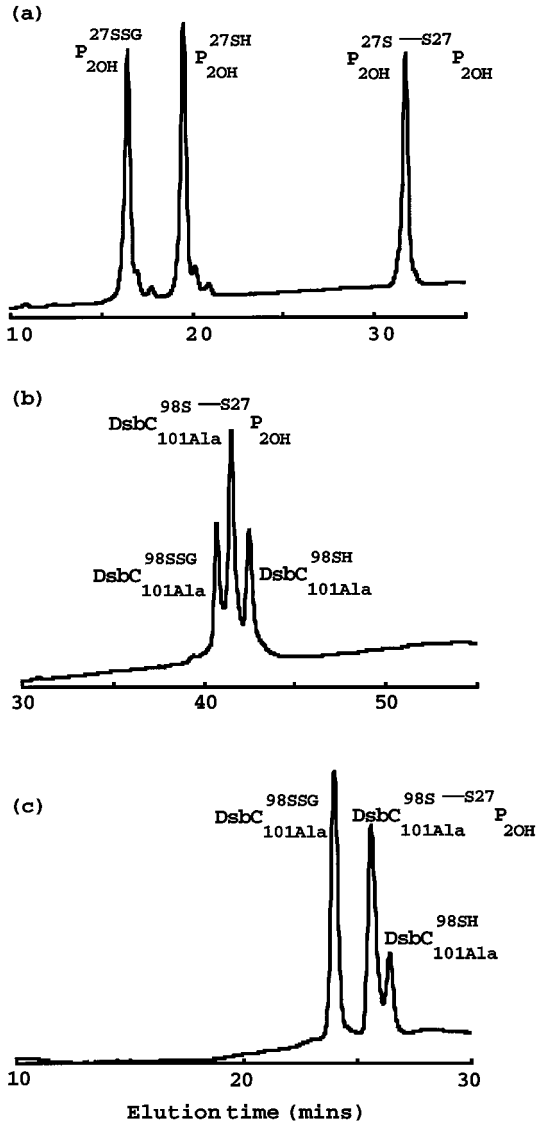
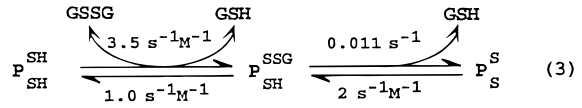


FIGURE 1: HPLC separation of the various forms of the peptide and their mixed disulfide complexes. (a) Separation of P_{2OH}^{27SH}, P_{2OH}^{27SSG}, and $P_{2OH}^{27S-S27}P_{2OH}$ using gradient A. (b) Separation of Dsb $C_{101Ala}^{98S-27S}P_{2OH}$, $DsbC_{101Ala}^{98SH}$, and $DsbC_{101Ala}^{98SSG}$ by HPLC using gradient A. (c) As (b), but separated using gradient B.

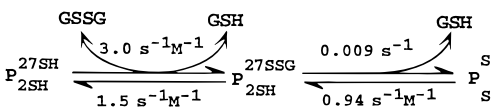


(3)

K_{3a}= 3.5 K_{3b} = 5.5 mM

$K_3 = K_{3a}K_{3b}$ = 1.9 x 10^{-2} M

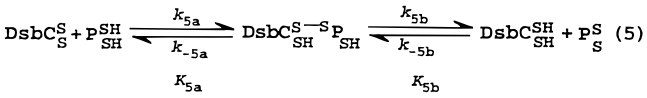
acidic glutathione with neighboring charged residues on the peptide. Combined with the results on P_{2OH}^{27SH}, the rate and equilibrium constants via this intermediate are



(4)

K_{4a}= 2.0 K_{4b} = 9.6 mM

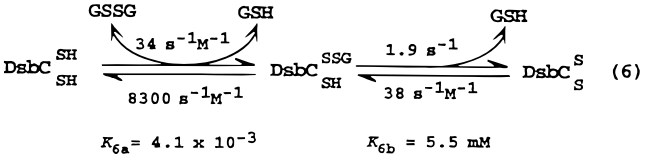
Direct Reactions between the Peptide and DsbC. In its normal role, DsbC is thought to function in its reduced form to catalyze disulfide bond rearrangement. Nevertheless, the relatively low stability of the active site disulfide bond of DsbC also allows it to function as a direct oxidant of protein thiol groups by transfer of its disulfide bond via an intermediate enzyme−peptide mixed disulfide species (*14*).



(5)

The mixed disulfide always occurs on the solvent-accessible Cys98 of DsbC (*14*). The overall rate constant for this process, 1.6 × 10^7 s^{-1} M^{-1} (Figure 2a), could be estimated at very low substrate concentrations (12.5 and 25 nM of each reactant) by following the rate of $DsbC_S^S$ reduction by P_{SH}^{SH}. The reaction rate was found to be proportional to the substrate concentration in this range. It will subsequently be shown that the rate of the first reaction in this scheme (k_{5a}) to form $DsbC_{101SH}^{98S-27S}P_{2SH}$ is probably limiting, which would explain why no mixed disulfide intermediate was observed.

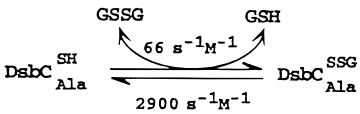
Mutation of the Buried Cysteine Residue of DsbC. The kinetics of formation and the stability of the intermediate enzyme−peptide mixed disulfide give important information about the reaction mechanism of DsbC and the role of substrate binding interactions in catalysis (*32*, *38*). Although it may be possible under some circumstances to observe the formation of the covalent complex using the normal enzyme, it is an inherently unstable species and might not be trapped quantitatively during the acid quenching procedure (*14*, *28*, *32*). To circumvent this problem, a mutant form of the enzyme can be used in which the buried Cys101 is replaced by an alanine residue. In this case, the normal intramolecular reaction to reform the protein disulfide bond (eq 5), which is rapid and competes with the acid trapping, cannot occur. The mutation of the buried cysteine residue, however, does cause some slight alterations in the stability of the mixed disulfide that can be formed between the accessible cysteine and glutathione (*14*).



(6)

K_{6a}= 4.1 x 10^{-3} K_{6b} = 5.5 mM

$K_6 = K_{6a}K_{6b}$ = 1.95 x 10^{-4} M



(7)

K_{7a}= 2.24 x 10^{-2}

Comparison of the two reactions to form the mixed disulfide indicates differences in rate constants of up to 3-fold and that the mutant form of the protein forms a glutathione mixed disulfide that is 5 times more stable than that measured with the normal protein. Similar observations have been made with the corresponding mutant forms of DsbA and active site containing domains of PDI (*26*, *28*), suggesting that either local perturbations in the structure of the mutants

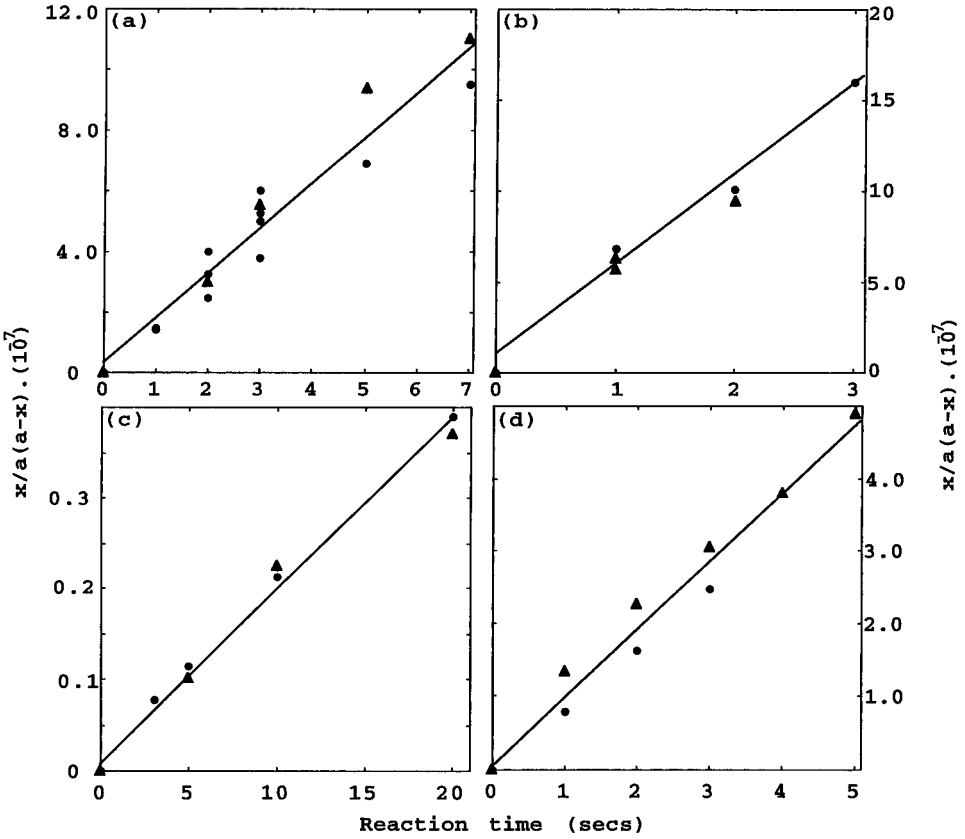


FIGURE 2: Kinetics of the reaction between (a) $DsbC^{S}_{S}$ and P^{SH}_{SH} to produce P^{S}_{S} and $DsbC^{SH}_{SH}$, (▲) 12.5 nM each reactant, (●) 25 nM each reactant; (b) $DsbC^{98SH}_{101Ala}$ and $P^{27S-S27}_{2OH}P_{2OH}$ to produce $DsbC^{98S-27S}_{101Ala}P_{2OH}$ and P^{27SH}_{2OH}, (●) 5 nM each reactant, (▲) 12.5 nM each reactant; (c) $DsbC^{98SSG}_{101Ala}$ and P^{27SH}_{2OH} to produce $DsbC^{98SH}_{101Ala}$ and P^{27SSG}_{2OH}, (▲) 12.5 nM each reactant, (●) 25 nM each reactant; (d) $DsbC^{98SH}_{101Ala}$ and P^{27SSG}_{2OH} to produce $DsbC^{98S-27S}_{101Ala}P_{2OH}$ and GSH, (▲) 12.5 nM each reactant, (●) 25 nM each reactant. Kinetics were analyzed using a special form of the second-order rate equation that applies when the starting concentrations of each reactant are equivalent, $x/a(a - x) = kt$ (*50*), where x is the concentration of product formed after time t from an initial concentration a of reactant and k is the rate constant. In a plot of $x/a(a - x)$ against reaction time, all the points for different values of a should fall on a straight line, the slope of which gives the value of the rate constant.

affect the reactivity of the accessible cysteine residue or acid trapping is not adequate with the normal protein. Nevertheless, the alterations in stability are relatively small, when it is considered that the apparent stability of the glutathione mixed disulfide form of the mutant is still 100-fold lower than a mixed disulfide with a normal cysteine residue (*44*). Reactions between DsbC and glutathione are not of physiological significance, but glutathione is commonly used as a reference compound in studies on disulfide bond stability and thiol and disulfide reactivity (*45*).

The activity of $DsbC^{98SH}_{101Ala}$ in disulfide bond rearrangement in a BPTI folding intermediate, (*30*−*51*, *14*−*38*) was similar to that of the normal protein (data not shown). Previously, it has been demonstrated that only the first cysteine residue of the active site is required for some catalytic activities of proteins such as DsbA and PDI (*36*, *46*, *47*). This finding is consistent with the proposed mechanism of disulfide bond rearrangement shown in eq 2, which only requires the reaction of the first active site cysteine residue of the catalyst with a disulfide bond in the substrate protein.

Formation of an Enzyme−*Peptide Mixed Disulfide.* Formation of the enzyme−peptide mixed disulfide is most readily achieved by reacting $DsbC^{98SH}_{101Ala}$ with the peptide−disulfide dimer.

$$DsbC^{SH}_{Ala} + P^{27S-S27}_{2OH}P_{2OH} \underset{(2.2 \times 10^5\ s^{-1}M^{-1})}{\overset{4.7 \times 10^7\ s^{-1}M^{-1}}{\rightleftharpoons}} DsbC^{S-S27}_{Ala}P_{2OH} + P^{27SH}_{2OH} \quad (8)$$

$$K_8 = 213 \pm 63$$

The rate constant for the forward reaction was extremely fast, but still just accessible to determination by manual mixing at very low substrate concentrations (Figure 2b). The reverse rate constant could not be directly determined, but was estimated from the forward rate constant and the value for the equilibrium constant,[2] which was determined directly with equilibrium mixtures. The peptide dimer reacts 7×10^5-fold more rapidly than does GSSG and forms a mixed disulfide complex that is 10^4-fold more stable. This difference is surprising and cannot be attributed to any differences in the thiol−disulfide chemistry of glutathione or the peptide substrate, which are chemically quite similar. The rates of reaction of the thiol groups of P^{27SH}_{2OH} and GSH with disulfide compounds are quite similar at pH 7.4 (1−3 s^{-1} M^{-1}), and the equilibrium constants for forming intermolecular disulfide bonds with the peptide or glutathione are close to the

[2] Rate and equilibrium constants that were not measured directly, but were calculated from other known rate and equilibrium constants, are depicted within brackets.

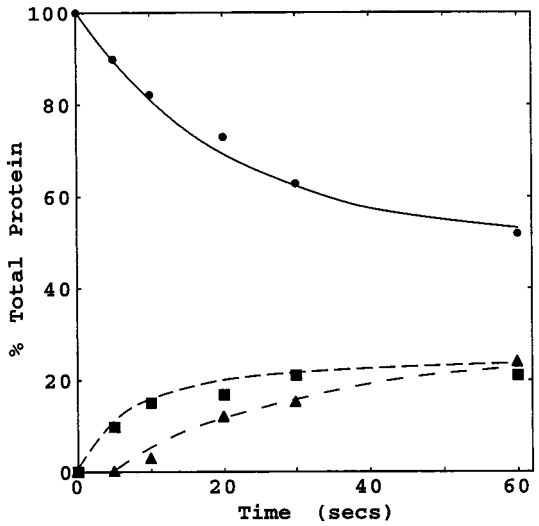
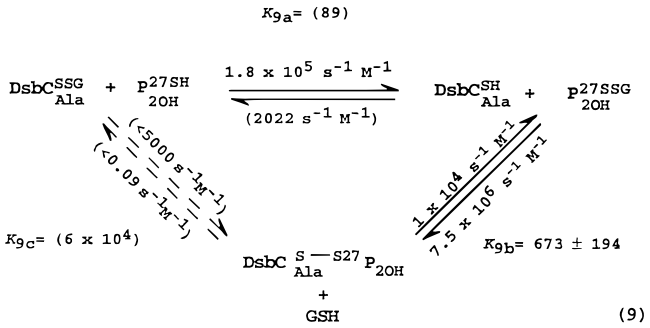


FIGURE 3: Kinetics of the reaction between $DsbC^{98SSG}_{101Ala}$ and P^{27SH}_{2OH}. The reaction between equal amounts (100 nM) of the two reactants was followed by acid trapping and HPLC analysis as in Figure 1. Symbols are (●) $DsbC^{98SSG}_{101Ala}$, (■) $DsbC^{98SH}_{101Ala}$, and (▲) $DsbC^{98S-27S}_{101Ala}P_{2OH}$. The curves shown are simply drawn through the points.

expected value of 2 in the absence of stabilizing factors, such as protein conformation (*5*, *32*, *44*).

The enzyme−peptide covalent complex could also be generated directly by reaction of $DsbC^{98SH}_{101Ala}$ with P^{27SSG}_{2OH}; there was no apparent formation of $DsbC^{98SSG}_{101Ala}$ or P^{27SH}_{2OH}. The alternative reaction between $DsbC^{98SSG}_{101Ala}$ and P^{27SH}_{2OH} did not form the complex directly, but instead generated $DsbC^{98SH}_{101Ala}$ and P^{27SSG}_{2OH}. After a lag phase (Figure 3), these initial products then reacted to form the enzyme−peptide complex. Taken together, these results are consistent with the following kinetic scheme:



(9)

The value of the equilibrium constant, K_{9a}, was calculated from the stabilities of the glutathione mixed disulfides of $DsbC^{98SH}_{101Ala}$ and P^{27SH}_{2OH} relative to GSSG (eqs 4 and 7).

$$K_{9a} = \frac{[DsbC^{SH}_{Ala}][P^{SSG}_{OH}]}{[DsbC^{SSG}_{Ala}][P^{SH}_{OH}]} = \frac{[DsbC^{SH}_{Ala}][GSSG][P^{SSG}_{OH}][GSH]}{[DsbC^{SSG}_{Ala}][GSH][P^{SH}_{OH}][GSSG]} = 2 \times 44.6 = (89) \quad (10)$$

The reaction indicated with the dashed arrows, and with equilibrium constant K_{9c}, was not detectable. The value of K_{9c} could be calculated from the values of the equilibrium constants K_{9a} and K_{9b}. The maximum values for the rate constants are based on this equilibrium constant and the assumption that, because it was not observed, the greatest rate constant is no greater than 3% of that for the alternative reaction, which does occur.

The rate of reaction of $DsbC^{98SSG}_{101Ala}$ with P^{27SH}_{2OH} to produce $DsbC^{98SH}_{101Ala}$ and P^{27SSG}_{2OH}(Figure 2c) and the rate of reaction of $DsbC^{98SH}_{101Ala}$ with P^{27SSG}_{2OH} to form $DsbC^{98S-S27}_{101Ala}P_{2OH}$ and GSH (Figure 2d) could be determined directly. Using the isolated enzyme−peptide mixed disulfide, its rate of reduction by GSH could also be determined directly. The only product of this reaction was $DsbC^{98SH}_{101Ala}$, with no detectable quantities of $DsbC^{98SSG}_{101Ala}$ being formed. As expected, the reaction came rapidly to equilibrium, and the rate constants in the forward and reverse directions were estimated by simulation of the kinetics to be 1×10^4 and 5×10^6 s^{-1} M^{-1}, respectively. These data are consistent with the directly determined rate constants and the directly determined equilibrium constant for this step (K_{9b}) of 673 ± 194.

This series of events in eq 9, in which the most stable products may not be formed directly, is identical to that observed with the corresponding series of reactions with DsbA in which the buried Cys has been mutated (*32*). It probably occurs because the reactive Cys98 of DsbC is a better leaving group than GSH in reactions that involve $DsbC^{98SSG}_{101Ala}$, and steric factors in the mixed disulfide protein complex prevent the reaction of Cys98 with an external thiol group.

Equilibrium and Rate Constants for the Reaction between the Peptide and DsbC. To understand fully the catalytic cycle of DsbC, the rate and equilibrium constants derived from measurements using P^{27SH}_{2OH} and $DsbC^{98SH}_{101Ala}$ need to be extrapolated to $DsbC^{SH}_{SH}$ and P^{SH}_{SH}. This is possible if the alterations of the cysteine residues to Ser or Ala have no or little effect. This is clearly the case for the peptide (eqs 3 and 4), and the changes in the C101A mutant of DsbC compared to the normal protein are small (eqs 6 and 7).

The equilibrium constant for the reaction between $DsbC^{S}_{S}$ and P^{SH}_{SH} can be calculated from the equilibrium between DsbC and the peptide mixed disulfide (eq 9), the stability of the DsbC active site disulfide bond (eq 6), and the equilibrium between the peptide and glutathione (eq 4):

$$K_{5a} = \frac{[DsbC^{S--S}_{SH}P_{SH}]}{[DsbC^{S}_{S}][P^{SH}_{SH}]} = \frac{[DsbC^{S--S}_{SH}P_{SH}][GSH]}{[DsbC^{SH}_{SH}][P^{27SSG}_{2SH}]} \frac{[DsbC^{SH}_{SH}][GSSG]}{[DsbC^{S}_{S}][GSH]^2} \frac{[P^{27SSG}_{2SH}][GSH]}{[P^{SH}_{SH}][GSSG]} \quad (11)$$

$$= 673 \times 5128 \times 2 = (6.9 \times 10^6 M^{-1})$$

Similarly,

$$K_{5b} = \frac{[DsbC^{SH}_{SH}][P^{S}_{S}]}{[DsbC^{S--S}_{SH}P_{SH}]} = \frac{[DsbC^{SH}_{SH}][P^{27SSG}_{2SH}]}{[DsbC^{S--S}_{SH}P_{SH}][GSH]} \frac{[P^{S}_{S}][GSH]}{[P^{27SSG}_{2SH}]} \quad (12)$$

$$= 9.6 \times 10^{-3} \times 1.5 \times 10^{-3} = (1.4 \times 10^{-5} M)$$

where the equilibrium constants are taken from eqs 4 and 9. Combining the two partial equilibrium constants K_{5a} and K_{5b}, the overall equilibrium constant for the transfer of the disulfide bond from $DsbC^{S}_{S}$ to P^{SH}_{SH} is 97.

The rate constants for the two reactions of eq 5 can be estimated with certain assumptions, that the rate of reaction of the Cys101 thiol group of DsbC with the mixed disulfide on Cys98 (k_{-5a}) is the same, (1.9 s^{-1}), whether the mixed disulfide is with glutathione (eq 6) or the peptide. From this value and the value of K_{5a} (eq 11), the value of k_{5a} can

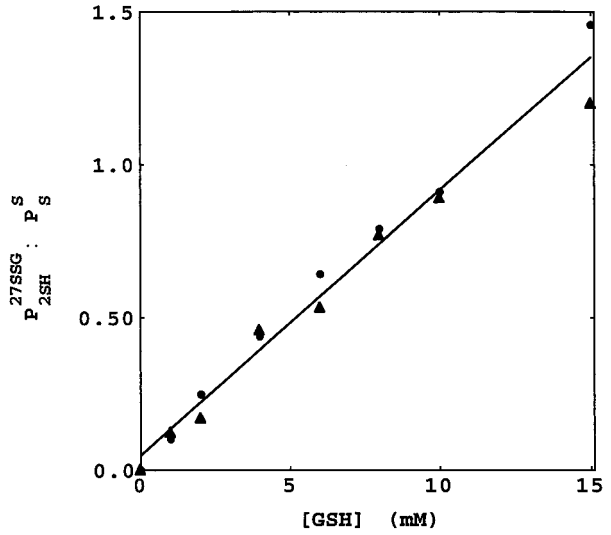


FIGURE 4: Competition between inter- and intramolecular disulfide bond formation from $DsbC^{98S-27S}_{101SH}P_{2SH}$ in the presence GSH. P^{SH}_{SH} (10 μM) was mixed with varying concentrations of GSH in 0.1 M Tris-HCl (pH 7.4), 0.2 M KCl, and 1 mM EDTA at 25 °C. Reactions were initiated by addition of $DsbC^{S}_{S}$ to a final concentration of 5 μM (●) or 10 μM (▲) and immediately trapped by addition of HCl to a concentration of 0.3 M. Time course studies showed that under these conditions the reaction had gone to completion within the mixing and trapping time. Samples were analyzed by reverse phase HPLC (gradient A) and the amounts of each peptide species quantified.

be calculated to be 1.4×10^7 s^{-1} M^{-1}. The similarity of this value to that for the reaction between $DsbC^{S}_{S}$ and P^{SH}_{SH} (1.6×10^7 s^{-1} M^{-1}) suggests that the first reaction, to form $DsbC^{S-S}_{SH}P_{SH}$, is rate determining and that the subsequent intramolecular reaction is much more rapid.

Rate constants for intramolecular formation of disulfide bonds can be determined by comparison with the rate of a competing intermolecular reaction (*45*). The value of k_{5b} can be estimated from the reaction between $DsbC^{S}_{S}$ and P^{SH}_{SH} in the presence of different amounts of GSH. In this case, there is competition between formation of the peptide disulfide bond from the intramolecular step and the breakage of the intermediate complex by GSH to form $DsbC^{SH}_{SH}$ and P^{27SSG}_{2SH} or P^{27SH}_{2SSG}. The rate of the latter reaction is known when P^{27SSG}_{2SH} is the product (1.0×10^4 s^{-1} M^{-1}, eq 9). The ratio of $[P^{27SSG}_{2SH}]$:$[P^{S}_{S}]$ formed during peptide oxidation was measured as a function of [GSH] (Figure 4). Equal amounts of these two products were formed at a [GSH] of 11.5 mM. The rate of the intermolecular reaction with GSH under these conditions is 115 s^{-1} (1×10^4 $s^{-1}M^{-1} \times 0.0115$ M), which should correspond to the rate of the competing intramolecular reaction, k_{5b}. The most likely source of error in this method of estimation would be the reduction of $DsbC^{S}_{S}$ by GSH before it has had a chance to react with the peptide. This reaction, however, is relatively slow when compared to the alternative reactions involving the peptide and would not be expected to be significant. In confirmation of this, the yield of disulfide-containing peptide products (P^{27SSG}_{2SH}, P^{27SH}_{2SSG}, and P^{S}_{S}) was near that expected and was similar in reactions that were carried out in the presence or absence of GSH. No significant quantities of the other possible species, the peptide dimer, were observed. Reactions that do not involve DsbC and only the different forms of the peptides would also be expected to be insignificant. Accumulation of P^{27SH}_{2SSG} was at a level of about 50% of P^{27SSG}_{2SH}. This is consistent with the known differences in reactivity of the two thiol groups in the peptide (*5*).

The value of k_{-5b} was calculated from K_{5b} and the value of k_{5a} to be 8×10^6 s^{-1} M^{-1}. The kinetic data are summarized below in eq 13a.

DISCUSSION

An important aspect of disulfide bond formation during protein folding is that of rearrangement of non-native disulfide bonds to form the correct isomers. In extreme cases, such as the BPTI folding pathway, disulfide bond rearrangements may be the most efficient route to the native state (*4*, *48*, *49*). Although each of the catalysts of thiol−disulfide exchange that has been characterized utilizes a common chemical reaction mechanism, by virtue of their relationship to thioredoxin, it is clear that additional features of the catalyst may be required for efficient disulfide bond rearrangement. Thus, DsbA and the active site-containing domains of PDI are efficient catalysts of disulfide bond formation, but are relatively weak catalysts of disulfide bond rearrangement compared to PDI itself, especially when the disulfide bonds occur within the context of a structured folding intermediate (*26*, *37*). In the case of PDI, evidence is emerging that the additional domains of this protein are also required for reactions involving disulfide bond rearrangement and that they are providing further sites of substrate interaction (*41*; N.J.D., unpublished data). Unfortunately, detailed characterization of the disulfide bond rearrangement mechanism by the structurally complex PDI molecule has proved very difficult. In the present work, we demonstrate that the simpler DsbC molecule, which is a relatively good catalyst of disulfide bond rearrangements, is a convenient model to study some aspects of this mechanism.

The approach adopted examines directly the properties of the intermediate disulfide-linked complex that is formed between the substrate and catalyst. Noncovalent interactions between substrate and catalyst are apparent from their effects on the stability of this intermediate complex. Although the affinity of the substrate for the catalyst is not measured, for example, as a K_m value, the present approach has the advantage that it analyzes the consequences of substrate binding on thiol−disulfide exchange at the active site and so is directly relevant to understanding the catalytic cycle.

Interactions between DsbC and the Peptide. There were a number of indications of noncovalent binding between the peptide and DsbC, which were manifested by their effects on the various thiol−disulfide exchange reactions. (1) The disulfide bond of $DsbC^{98SSG}_{101Ala}$ is very unstable, about 100-fold less stable than that of GSSG or of a protein or peptide disulfide bond in which there are no stabilizing interactions (eq 2; *44*). In contrast, the disulfide bond of the $DsbC^{98S-S27}_{101Ala}P_{2OH}$ complex was $\geq 10^4$-fold more stable than that of $DsbC^{98SSG}_{101Ala}$ (eqs 7−9), and has a stability similar to that of many protein structural disulfide bonds (*45*, *48*). Because the properties and reactivities of the cysteine residues of glutathione and P^{27SH}_{2OH} are practically equivalent, the most likely explanation for the stability of the disulfide bond of $DsbC^{98S-S27}_{101Ala}P_{2OH}$ is that there are substantial stabilizing interactions between DsbC and the covalently linked

peptide. (2) The various forms of the peptide reacted with either $DsbC_S^S$ or $DsbC_{SH}^{SH}$ 10^5-10^6-fold faster than with the corresponding forms of glutathione (eqs 6−9). A peptide dimer also reacted more rapidly than a peptide−glutathione mixed disulfide, suggesting that peptide size was also influential (eqs 8 and 9). (3) In the reaction of Cys98 of DsbC with P_{2OH}^{27SSG}, the formation of $DsbC_{101Ala}^{98S-S27}P_{2OH}$ was greatly favored over the alternative reaction in which Dsb C_{101Ala}^{98SSG} was the product (eq 9), although the peptide and glutathione cysteine sulfur atoms are chemically similar. This is probably a result of stereochemical restrictions imposed by the structure of the complex between the peptide and enzyme.

Similar observations have been made with DsbA (*32*), but the magnitude of the binding interaction with the peptide appears much stronger with DsbC than with DsbA. This was reflected by the fact that $DsbC_{101Ala}^{98S-S27}P_{2OH}$ is about 40−100-fold more stable than the corresponding DsbA−peptide mixed disulfide, even though the stabilities of the glutathione mixed disulfides of the two proteins are similar (eqs 13a and 13b below). Similarly, the rates of reaction of the various forms of the peptide with DsbC were generally much greater than with DsbA. Some other informative differences between DsbA and DsbC are also indicated. The reaction of P_{SH}^{SH} with $DsbA_{33SH}^{30SSG}$ is an exception to the general rule that forms of the peptide react more rapidly with DsbA than the corresponding forms of glutathione, suggesting that the presence of covalently bound glutathione at the active site of DsbA prevents interactions with the peptide (*32*). In the case of DsbC, however, reaction of P_{2OH}^{27SH} with $DsbC_{101SH}^{98SSG}$ was around 20-fold faster than the corresponding reaction with GSH (eqs 6 and 9). This might occur if the substrate binding area of DsbA was less extensive or more stereochemically restricted than that of DsbC, an explanation that would also be consistent with other indications that the peptide binds more tightly to DsbC than DsbA.

Substrate binding appears to play a more significant role in catalysis by DsbC than DsbA. The estimated rate enhancement due to peptide binding for DsbA is a factor of about 10^3, derived from comparison of the rate of reaction of $DsbA_S^S$ with P_{SH}^{SH} or GSH (*32*). For DsbC this rate enhancement is 100-fold greater, about 10^5-fold. We have shown, however, that such rate enhancements can occur with relatively weak binding of the peptide to the catalyst, which significantly increases the effective concentration of the reacting groups (*32*). In our previous analysis we have shown that effective concentrations of reacting groups in the DsbA−peptide complex are probably $\geq 10^{-2}$ M (*32*). Application of an identical form of analysis suggests that for the DsbC−peptide complex, the effective concentration of reacting groups is likely to be of the order of ≥ 1 M. Values of 10^2-10^5 M have been observed with thiol−disulfide exchange reactions in folded proteins (*48*).

It is clear that DsbA and DsbC each bind the 28-residue peptide substrate more tightly than glutathione. Glutathione is, however, a peptide so the possibility that it also binds to the catalyst, albeit weakly, cannot be eliminated. In the case of DsbA, it seems unlikely that such interactions can be significant, as the kinetic and equilibrium properties of thiol−disulfide exchange reactions with β-mercaptoethanol are similar to those with glutathione (*25*).

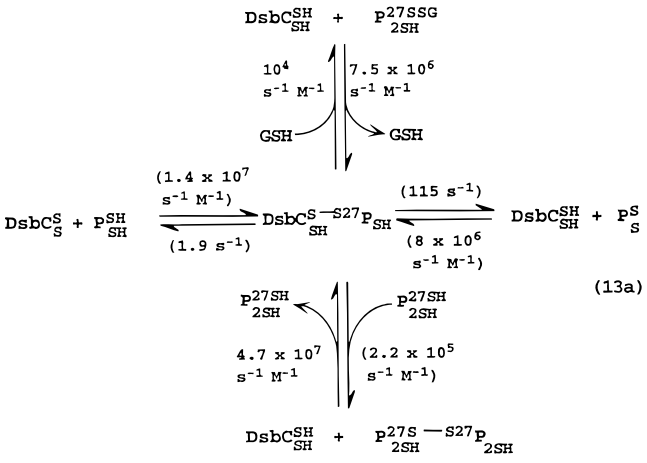
Effects of DsbC on Protein Conformational Transitions. Disulfide bond formation is occurring in proteins during protein folding, so it is relevant to ask if the catalysts participate directly in polypeptide conformational changes. This question can be answered by considering the intramolecular rate at which the peptide disulfide bond is made from $DsbC_{101SH}^{98S-27S}P_{2SH}$ (k_{5b}). This has a value of 115 s^{-1} which is1.3×10^4-fold faster than the intramolecular rate with the peptide−glutathione mixed disulfide (eq 4). To assess if this rate enhancement is due to conformational effects at the active site of DsbC increasing the intramolecular rate or simply due to an enhancement of thiol−disulfide exchange, this rate is compared to the intermolecular rate constant for reaction of the complex with GSH. The value of this ratio reflects the effective concentration of the cysteine residues in the peptide (i.e., their relative proximities). The value is 11 mM in the unbound peptide, which is in the range that might be expected for cysteine residues in an unfolded protein (*44*). This value is reduced to 1 mM when the peptide is complexed with DsbA, probably because of steric hindrance by the protein. In the complex with DsbC, the value is 11.5 mM, about the same as the unbound peptide. That formation of the peptide disulfide bond is not disfavored when bound to DsbC suggests that there are marked differences in the way in which the two Dsb proteins bind their substrates. One possibility is that the peptide binding site of DsbC is not a long cleft, which binds an extended peptide chain, as has been proposed for DsbA (*19*), but a site that favors binding of a more compact form of the peptide molecule.

Implications for the Reaction Mechanism of DsbC. Peptide binding enhances all the reactions between the various forms of peptide and DsbC. This can be clearly seen in the ability of $DsbC_S^S$ to transfer its disulfide bond to P_{SH}^{SH}, yet this is not thought to be the most important function of DsbC in vivo. Instead, DsbC is thought to function primarily in the reduced state to catalyze disulfide bond rearrangement, whereas DsbA is considered to be the primary oxidizing catalyst (*14*, *16*, *17*). It is therefore relevant to consider the reactions at the active sites of these two catalysts to understand how they have been adapted to fulfill their different functions.

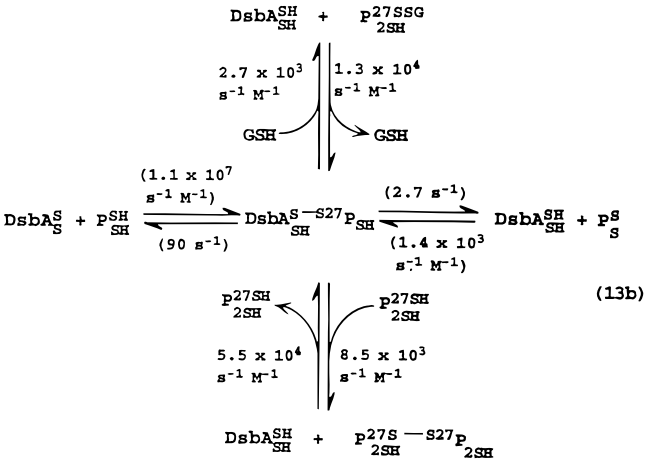
(i) The Stability of the Intermediate Complex. One feature of the reaction cycle of DsbC that might favor rearrangement is the relative stability of the enzyme−peptide mixed disulfide in comparison to the stability of a disulfide bond in an unfolded protein. The reaction of $DsbC_{101Ala}^{98S-S27}P_{2OH}$ with P_{2OH}^{27SH} to form the peptide dimer and reduced DsbC is very unfavorable because of the high stability of the complex. This is due, in part, to the relatively unstable disulfide bond that is formed between the two peptide monomers, which is not structurally stabilized; consequently, its breakage by the catalyst is favored. If the disulfide bond in the substrate molecule becomes more stable, as would be the case if it is structurally stabilized, the balance between forming the disulfide of the substrate−DsbC mixed disulfide and the substrate disulfide would change in favor of the latter. A stabilized substrate−DsbC mixed disulfide favors the breakage of unstable substrate disulfide bonds and provides the opportunity for a new more stable disulfide bond to be made by the reaction of an alternative cysteine residue. DsbA may

be less favorable as a catalyst of disulfide bond rearrangement, as the stability of its peptide−mixed disulfide complex is substantially lower than that of DsbC. Obviously, a careful balancing of the relative stabilities of all the disulfide bonds is required as it would be undesirable for a disulfide bond between DsbC and a substrate molecule to be so stable that the complex accumulates.

(ii) The Kinetics of Reaction at the Active Site. The data obtained are sufficient to account for the rates of all the possible reactions of DsbC with glutathione and Cys27 of the peptide



For comparison, the corresponding data with DsbA as the catalyst



Comparison of the kinetic schemes for DsbC and DsbA further suggests a kinetic explanation for the greater isomerase activity of DsbC. When catalyzed, this reaction probably occurs by the thiol group of the reduced catalyst reacting with the disulfide bond of the substrate protein that is to be rearranged, generating a transient mixed disulfide complex and liberating a protein thiol group. Disulfide rearrangement will result if a different protein thiol group reacts with the mixed disulfide to form a different protein disulfide bond (eq 2). In each of these steps, with the peptide, DsbC reacts more rapidly than does DsbA. The initial reaction of DsbC with the peptide disulfide bond occurs 6×10^3-fold more rapidly compared to DsbA. The reaction of a different protein thiol group with the mixed disulfide is probably modeled best by the reaction with another peptide molecule; this occurs 26-fold more rapidly with DsbC than DsbA. If the reaction with the thiol group of the second cysteine residue is considered (k_{5b}), DsbC reacts 43-fold more rapidly. These kinetic differences are likely to contribute to the greater isomerase activity of DsbC.

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