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Extrinsic 33-Kilodalton Protein of Spinach Oxygen-Evolving Complexes: Kinetic Studies of Folding and Disulfide Reduction†

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ABSTRACT: The 33-kDa protein is one of the three extrinsic proteins in the oxygen-evolving photosystem II complexes. The protein has one intrachain disulfide bond. On reduction of this disulfide bond, the protein was unfolded and lost its activity. On the basis of the unfolding equilibrium curve obtained by using guanidine hydrochloride, the free energy change of unfolding in the absence of guanidine hydrochloride was estimated to be 4.4 kcal/mol using the Tanford method [Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95] and 2.8 kcal/mol using the linear extrapolation method. The unfolding of the 33-kDa protein caused by reduction was explained in terms of the entropy change associated with reduction of the intrachain disulfide bond. The kinetics of the reduction of the disulfide bond using dithiothreitol were studied at various concentrations of guanidine hydrochloride at pH 7.5 and 25 °C. The disulfide bond was reduced even in the absence of guanidine hydrochloride. The unfolding and refolding kinetics of the 33-kDa protein using guanidine hydrochloride were also studied under the same conditions, and the results were compared with those for the reduction kinetics. It was shown that the reduction of the disulfide bond proceeds through a species in which the disulfide bond is exposed by local fluctuations.

The 33-kDa protein is an extrinsic component of the oxygen-evolving photosystem II (PS II)[1] complexes located on the thylakoid membrane of chloroplasts (Inoue et al., 1983, 1984; Miyao & Murata, 1984; Abramowicz & Dismukes, 1984). The 33-kDa protein consists of 247 amino acid residues and has only 1 intrachain disulfide bond between Cys-28 and Cys-51 and 1 Trp at position 241 (Oh-oka et al., 1986). Recently, Tanaka and Wada (1988) reported that the intrachain disulfide bond is essential for maintaining the functional conformation of the molecule. In this study, we investigated the role of the disulfide bond in the stability of the 33-kDa protein. We also examined the kinetics of the reduction of the disulfide bond with DTT at various concentrations of Gdn-HCl, and the results were compared with those of the unfolding and refolding kinetics obtained with Gdn-HCl. We found that the disulfide bond is located in the interior of the molecule and that the reduction proceeds through a species in which the disulfide bond is exposed by local fluctuations.

Materials and Methods

Materials. The 33-kDa protein was extracted from NaCl-treated PS II complex by 1.0 M $CaCl_2$ treatment and purified by column chromatography on DEAE-Sepharose CL-6B as described by Kuwabara and Murata (1982). The purified protein was dialyzed against 10 mM NH_4HCO_3 and lyophilized. All the procedures were done at 0–7 °C. The reduced 33-kDa protein was prepared by reduction of the intrachain disulfide bond with a 1000 molar excess of 2-mercaptoethanol in the presence of 6 M Gdn-HCl for 2 h at 25 °C and then separated from the residual reagents on a column of Sephadex G-25 equilibrated with 10 mM acetate buffer at pH 4.0 containing 30 mM NaCl and 5 μM EDTA.

DTT and Gdn-HCl (specially purified grade) were obtained from Nakarai Chemicals, and DEAE-Sepharose CL-6B was obtained from Pharmacia Fine Chemicals. Other reagents were obtained from Wako Pure Chemicals and Nakarai Chemicals and were used without further purification.

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[1] Abbreviations: CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Gdn-HCl, guanidine hydrochloride; reduced 33-kDa protein, 33-kDa protein in which the intrachain disulfide bond is reduced; PS, photosystem.

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Fluorescence Measurement. Fluorescence was measured with a Hitachi fluorescence spectrophotometer, Model MPF-4, equipped with a spectral corrector. The temperature was kept at 25 °C with a thermostatically controlled cell holder.

Reduction Kinetics of the Disulfide Bond of the 33-kDa Protein. The buffer used was 50 mM Tris-HCl at pH 7.5. DTT was used to reduce the disulfide bond. Reduction was always carried out under a nitrogen atmosphere after the buffer had been degassed. On reduction of the disulfide bond, the fluorescence spectrum of the 33-kDa protein was changed greatly (see Figure 1). Therefore, we used the change in the tryptophyl fluorescence at 314 nm, at which the fluorescence change on reduction was greatest, to follow the reduction reaction. The temperature was kept at 25 °C with a thermostatically controlled cell holder.

Unfolding Equilibrium. The unfolding equilibrium of the 33-kDa protein using Gdn-HCl was measured at 25 °C at pH 7.5 (50 mM Tris-HCl buffer) by the tryptophyl fluorescence at 350 nm.

Unfolding and Refolding Kinetics. Refolding reactions and slow unfolding reactions were measured with the Hitachi fluorescence spectrophotometer. Mixing was done manually. The initial concentration of Gdn-HCl used for the refolding measurements was 4 M at pH 7.5. Fast unfolding reactions were measured on a Union Giken stopped-flow spectrophotometer, Model RA-401, equipped with an RA-450 data processor, using fluorescence detection. The details of the apparatus have been described previously by Goto and Hamaguchi (1982). The excitation wavelength was set at 280 nm, and a Toshiba UV-33 glass filter was used to cut off the fluorescence at wavelengths shorter than 330 nm. The unfolding was initiated by mixing the protein solution in 0 M Gdn-HCl at pH 7.5 with a Gdn-HCl solution of a given concentration, also at pH 7.5, in a 1:1 ratio. The protein concentration was 2.0 μM, and the temperature was kept at 25 °C with a thermostatically controlled cell holder.

All kinetic data can be described by an equation of the form

$$F(t) - F(\infty) = \sum \alpha_i \exp(-k_{app,i}t) \quad (1)$$

where $F(t)$ is the fluorescence at time t and $F(\infty)$ is the fluorescence after equilibration. $k_{app,i}$ is the apparent rate constant of phase i, and α_i is the amplitude of the phase.

Protein Concentration. The concentrations of the intact and reduced 33-kDa protein were determined spectrophotometrically with absorption coefficients at 276 nm for a 1 mM solution in a 1.0-cm cell of 20 and 18 (Tanaka & Wada, 1988).

Results

Fluorescence Spectrum. As shown in Figure 1a, the CD spectrum of the reduced 33-kDa protein is very similar to that of the unfolded protein in 4 M Gdn-HCl, and the reduced protein neither binds with the $CaCl_2$-treated PS II complexes nor has oxygen-evolving activity (Tanaka & Wada, 1988).

Figure 1b shows the fluorescence spectra of the intact and reduced 33-kDa protein. The spectrum of the intact protein had a maximum at 314 nm. On reduction of the disulfide bond, the spectrum became very similar to that of the unfolded protein in 4 M Gdn-HCl and had a maximum at 350 nm. The fluorescence intensity was decreased slightly. These findings indicate that Trp-241 of the intact 33-kDa protein is located in the hydrophobic interior of the molecule and is not quenched by other residues and that on reduction of the disulfide bond the conformation of the intact protein is changed greatly and the tryptophyl residue is exposed to the aqueous environment.

Unfolding Equilibrium. Figure 2 shows the unfolding equilibrium curve of the 33-kDa protein as measured by the

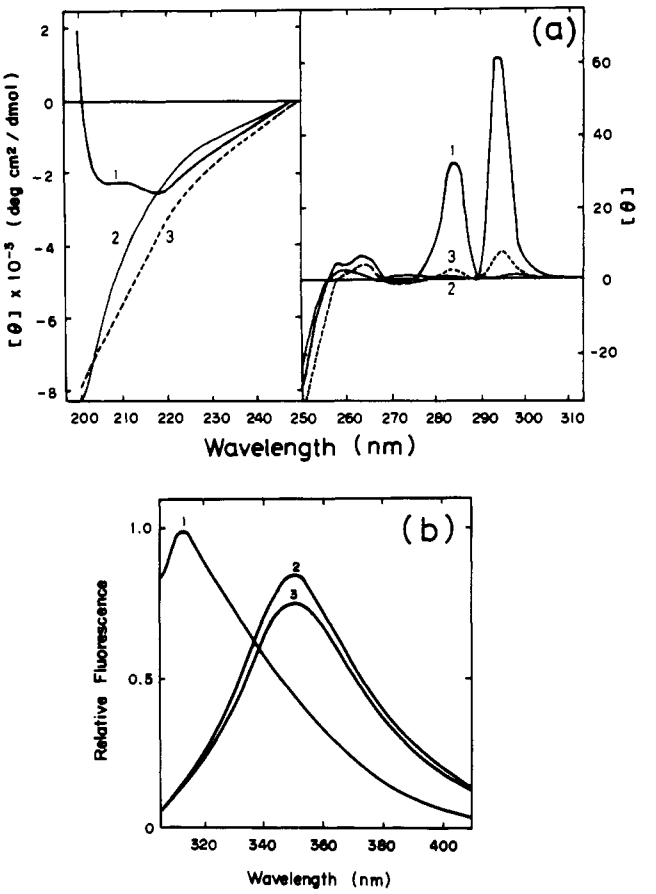


FIGURE 1: CD spectra (a) and fluorescence spectra (b) of the intact 33-kDa protein (1), the denatured 33-kDa protein (2) in 4 M Gdn-HCl, and the reduced 33-kDa protein (3) at pH 7.5 and 25 °C. (a) The protein concentrations were 20 μM for measurements in the region of 250–310 nm and 6 μM for measurements in the region of 200–250 nm, respectively. (b) The protein concentrations were 2 μM. Excitation was at 295 nm. A correction was made to correct fluorescence intensities for the solvent Raman scattering.

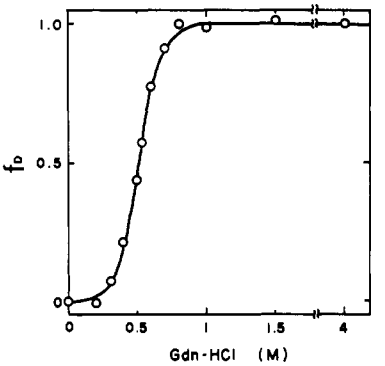


FIGURE 2: Unfolding transition curve of the 33-kDa protein at pH 7.5 and 25 °C. The ordinate represents the fraction of the unfolded protein (f_D). The unfolding transition was measured in terms of the change in fluorescence at 350 nm. Excitation was at 295 nm. The solid line indicates the theoretical curves calculated by using eq 3 and the values of $\Delta G_D^{H_2O}$ = 4.4 kcal mol^{-1} and Δn = 42.

fluorescence at 350 nm. The unfolding began from a Gdn-HCl concentration of about 0.2 M and ended at 0.8 M with a midpoint concentration of 0.5 M at pH 7.5 and 25 °C. The unfolding curve obtained by CD at 212 nm was the same as that shown in Figure 2. The 33-kDa protein refolded reversibly. The unfolding curve was normalized by assuming that the fluorescence of the native and unfolded proteins, which were observed before and after the transition zone, respectively, can be extrapolated linearly into the transition zone.

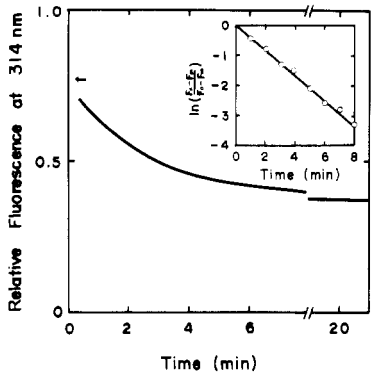


FIGURE 3: Unfolding kinetics of the 33-kDa protein in 0.5 M Gdn-HCl at pH 7.5 and 25 °C obtained by measuring the fluorescence at 314 nm. Protein concentration was 2 μM. The arrow indicates the fluorescence intensity for the 33-kDa protein in 0 M Gdn-HCl.

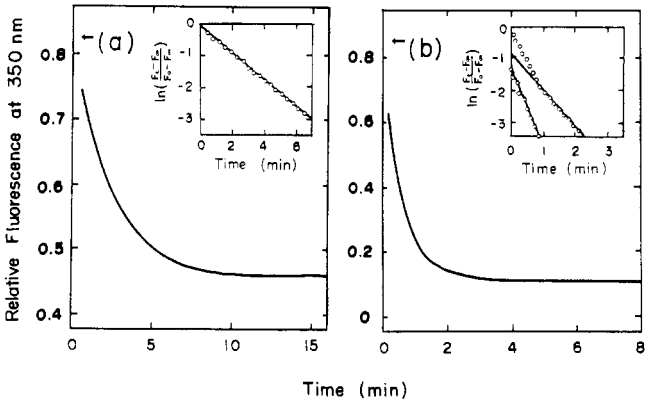


FIGURE 4: Refolding kinetics of the 33-kDa protein obtained by the fluorescence at 350 nm, pH 7.5 and 25 °C. The protein concentration was 2 μM. The initial concentration of Gdn-HCl was 4 M, and final concentrations were 0.5 M (a) and 0.1 M (b). The insets (a) and (b) show that the refolding kinetics were described by single- and double-exponential decay terms, respectively.

Unfolding and Refolding Kinetics. The kinetics of unfolding by Gdn-HCl of the 33-kDa protein were measured by the change in fluorescence. Figure 3 shows the unfolding kinetics in 0.5 M Gdn-HCl obtained by manual mixing. Stopped-flow mixing was also used to measure the very fast unfolding kinetics that were completed within a few seconds. The unfolding kinetics in a Gdn-HCl concentration range from 0.2 to 4 M were all described by a single-exponential decay term.

The kinetics of refolding of the 33-kDa protein were measured by manual mixing. Figure 4a shows the refolding kinetics in 0.5 M Gdn-HCl diluted from 4 M as an example of the refolding pattern inside the transition zone. As shown in the inset of this figure, the refolding kinetics inside the transition zone were described by a single extrapolation decay term. The initial point obtained by extrapolation to zero time fell on the fluorescence of the 33-kDa protein in 4 M Gdn-HCl, and we may conclude that any fast phase that escaped observation was not present in the refolding kinetics in the transition zone.

Figure 5 shows the apparent rate constants for the unfolding and refolding at various concentrations of Gdn-HCl. At any concentration of Gdn-HCl inside the transition zone, the apparent rate constant obtained from the unfolding experiments agreed well with that obtained from the refolding experiments. There was a minimum at 0.5 M Gdn-HCl, which is the same as the midpoint concentration of the unfolding transition curve.

For the refolding kinetics in the region before the transition (0.05–0.15 M Gdn-HCl) from 4 M Gdn-HCl, an additional exponential term was necessary to describe the total fluorescence change (Figure 4b). This additional term has a large amplitude, about half the total fluorescence change and a greater apparent rate constant than the other.

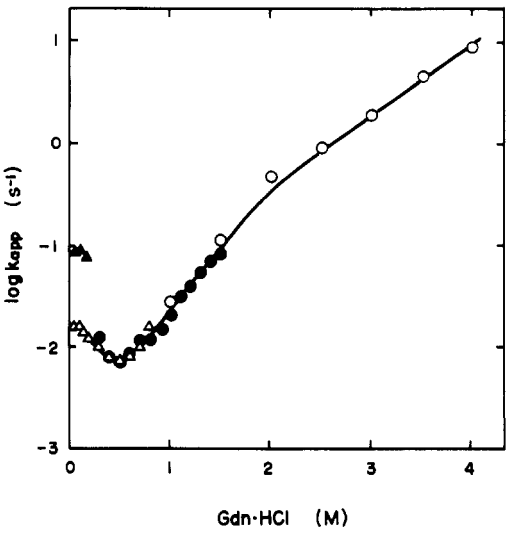


FIGURE 5: Apparent rate constants for unfolding and refolding kinetics of the 33-kDa protein at various concentrations of Gdn-HCl at pH 7.5 and 25 °C. (○) From unfolding kinetics determined by stopped-flow measurements; (●) from unfolding kinetics determined by manual mixing measurements; (△) from refolding kinetics determined by manual mixing measurements. The closed triangles show the apparent rate constants for the additional phase of the refolding kinetics below the transition zone.

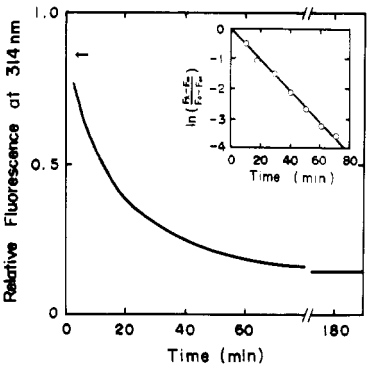


FIGURE 6: Kinetics of reduction with DTT of the S–S bond of the 33-kDa protein in the absence of Gdn-HCl at pH 7.5 and 25 °C. The reaction was followed by measuring the fluorescence at 314 nm. Excitation was at 295 nm. The arrow indicates the fluorescence intensity of the 33-kDa protein in the absence of DTT. The final concentrations of the 33-kDa protein and DTT were 2 μM and 100 mM, respectively. The inset shows the first-order plot of the kinetics.

In order to examine whether two forms of the unfolded 33-kDa protein exist in the unfolded state, we performed double-jump experiments (Brandts et al., 1975; Nall et al., 1978). The 33-kDa protein was first unfolded in 4 M Gdn-HCl, and then refolding was initiated at 0.1 M Gdn-HCl after various intervals under the unfolding conditions. Unfolding of the 33-kDa protein in 4 M Gdn-HCl was complete within 1 s. If there is a slow equilibrium in the unfolded state, the refolding kinetics should depend on the time elapsed in the unfolded condition. We found that the amplitude, relative to the total fluorescence change, of the slow phase is completely independent of the time of exposure to 4 M Gdn-HCl (data not shown). It was therefore concluded that there is no slow equilibrium of the 33-kDa protein in the unfolded state.

Kinetics of Reduction of the Disulfide Bond. The kinetics of the reduction with DTT of the disulfide bond of the 33-kDa protein in the absence and presence (0.1 and 0.2 M) of

Gdn-HCl were measured by using the change in fluorescence at 314 nm. Figure 6 shows the decrease in the fluorescence at 314 nm with time in the absence of Gdn-HCl after the addition of DTT to give a final concentration of 100 mM. The change follows first-order kinetics, as shown in the inset. The apparent rate constant was found to be 0.9×10^{-3} s^{-1}.

Discussion

Stability of the 33-kDa Protein. As shown in Figure 1a, the CD spectrum of the reduced 33-kDa protein is very similar to that of the unfolded protein. The fluorescence spectrum of the reduced 33-kDa protein (Figure 1b) also shows that the protein is unfolded on reduction of the disulfide bond.

Assuming a two-state transition, we analyzed the unfolding curve shown in Figure 2 and obtained the free energy of unfolding at a given concentration of Gdn-HCl (ΔG_D) from the equation:

$$\Delta G_D = -RT \ln [f_D/(1 - f_D)] \quad (2)$$

where f_D is the fraction of the unfolded molecule. We then estimated the free energy change of unfolding in the absence of Gdn-HCl, $\Delta G_D^{H_2O}$, using the equation proposed by Tanford (1970):

$$\Delta G_D = \Delta G_D^{H_2O} - \Delta nRT \ln (1 + ka_{\pm}) \quad (3)$$

where Δn is the difference in the number of binding sites between the unfolded and folded states, k is the average binding constant of the sites, and $a_{\pm}$ is the activity of Gdn-HCl. We used 0.6 M^{-1} as the value of k (Pace, 1986). The values of $\Delta G_D^{H_2O}$ and Δn were found to be 4.4 kcal/mol and 42, respectively. The value of $\Delta G_D^{H_2O}$ was also estimated by linear extrapolation of the plot of $\Delta G_D^{H_2O}$ against the concentration of Gdn-HCl. The values of $\Delta G_D^{H_2O}$ and the slope of the plot were found to be 2.8 kcal/mol and 4.5 kcal/(mol·M), respectively. It is generally observed that the value of $\Delta G_D^{H_2O}$ estimated by the linear extrapolation is smaller than the value estimated by the ligand binding model (Pace, 1986; Goto et al., 1988). At present, however, it is not clear which method is appropriate to estimate the value of $\Delta G_D^{H_2O}$. The value of $\Delta G_D^{H_2O}$ (4.4 or 2.8 kcal/mol) for the 33-kDa protein is one of the smallest values for proteins so far estimated.

The change in the conformational entropy on reduction of the disulfide bond in the unfolded state can be estimated by using Flory's equation (1956):

$$\Delta S = 0.75\nu R(\ln N_s/\nu + 3) \quad (4)$$

where ν is the number of connected chains (twice the number of disulfide bonds) and N_s is the number of amino acid residues in the loop formed by the disulfide bond. In the case of the 33-kDa protein, $\nu = 2$ and $N_s = 22$, and the entropy change associated with the reduction of the disulfide bond is calculated to be 16.1 cal/(deg·mol), which contributes 4.7 kcal/mol to the free energy change. This value is larger than or comparable to the value of $\Delta G_D^{H_2O}$ estimated above. This may explain why the 33-kDa protein is unfolded when the disulfide bond is reduced.

Kinetics of Unfolding and Refolding. The unfolding and refolding kinetics of the 33-kDa protein were described by a single-exponential term inside and above the transition zone. The apparent rate constant was minimal at 0.5 M Gdn-HCl, which corresponds to the midpoint of the unfolding transition (Figure 5). Only in the region before the transition, the refolding kinetics cannot be described by a single-exponential term. Even in this region, however, the double-jump experiment showed that the amplitudes of the two phases were independent of the time of exposure to 4 M Gdn-HCl. These findings indicate that in principle the unfolding and refolding kinetics of the 33-kDa protein follow the two-state transition (mechanism 1):

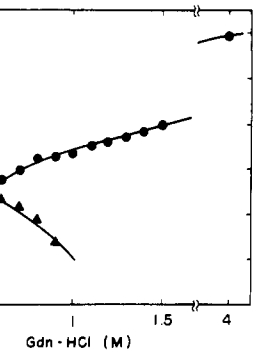


FIGURE 7: Rate constants k_1^g (●) and k_2^g (▲) in mechanism 1 for the unfolding and refolding kinetics and rate constants k_1 (○) and k_2 (△) in mechanism 3 for the reduction kinetics at various concentrations of Gdn-HCl, pH 7.5 and 25 °C.

$$N \underset{k_2^g}{\overset{k_1^g}{\rightleftharpoons}} U$$

where N is the native protein and U is the unfolded one. k_1^g and k_2^g are the unfolding and refolding rate constants, respectively. The values of k_1^g and k_2^g were estimated as follows. The value of the apparent rate constant (k_{app}) was assumed to be equal to k_1^g above the transition zone. Inside the transition zone, the apparent rate constant and equilibrium constant ($K = [U]/[N]$) are expressed by

$$k_{app} = k_1^g + k_2^g \quad (5)$$

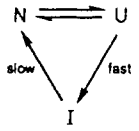
$$K = k_1^g/k_2^g \quad (6)$$

From the values of K estimated from the transition curve (Figure 2) and the values of k_{app} (Figure 5), the values of k_1^g and k_2^g were determined. In Figure 7, the values of k_1^g and k_2^g are plotted against the concentration of Gdn-HCl above 0.2 M.

As described above, while the refolding kinetics of the 33-kDa protein inside the transition zone were described in terms of mechanism 1 and the apparent rate constants obtained by the unfolding experiments were in agreement with those obtained by the refolding experiments, the refolding kinetics below the transition zone were not explained completely on the basis of mechanism 1. Recent studies on the kinetics of unfolding and refolding of ribonuclease A (Brandts et al., 1975; Garel & Baldwin, 1975; Garel et al., 1976; Hagerman & Baldwin, 1976) and the constant fragment of the immunoglobulin light chain (Goto & Hamaguchi, 1979, 1982a; Tsunenaga et al., 1987) show that in principle the kinetics follow the three-species mechanism $U_1 \rightleftharpoons U_2 \rightleftharpoons N$, where U_1 and U_2 are two forms of the unfolded molecule and N is the native molecule. While U_2 refolds to N very rapidly, U_1 refolds slowly through conversion to U_2. Brandts et al. (1975) proposed the idea that the slow-refolding species are produced by introduction of wrong isomers of the peptide bond of X-Pro, X being any preceding amino acid residue, after unfolding. This proposition has been gradually accepted on the basis of many observations (Baldwin & Creighton, 1980). Judging from the results of the double-jump experiment, however, the additional phase of refolding kinetics of the 33-kDa protein below the transition zone cannot be ascribed to the proline isomerizing equilibrium in the unfolded state. It is interesting to note that the unfolding and refolding kinetics of the 33-kDa

protein, which contains 14 proline residues, do not follow the 3-species mechanism.

The refolding kinetics of the 33-kDa protein in strongly native conditions (0.05–0.15 M Gdn-HCl) may be explained on the basis of mechanism 2:



where I represents an intermediate, which is any species different from, but very similar to, the native form and is not distinguishable spectrophotometrically. This intermediate is formed only in the refolding process under strongly native conditions. Such an intermediate has also been found in the refolding processes of ribonuclease A (Cook et al., 1979; Schmid, 1981; Schmid & Blaschek, 1981) and the constant fragment of the immunoglobulin light chain (Goto & Hamaguchi, 1979, 1982b; Tsunenaga et al., 1987).

Kinetics of the Reduction of the Disulfide Bond. As shown in Figure 6, the disulfide bond of the 33-kDa protein is reduced even in the absence of denaturant. When the disulfide bond is located in the interior of the molecule, the disulfide bond must be exposed and accessible to reducing agent in order to be reduced. The reduction kinetics may be expressed by mechanism 3:

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X \xrightarrow{k_3[DTT]} R$$

where N is the native protein, R is the protein in which the disulfide bond is reduced, and X is any conformation of the protein in which the disulfide bond is exposed. k_1 and k_2 are the rate constants for the reaction N $\rightleftharpoons$ X, and k_3 is the second-order rate constant for the reduction of the disulfide bond.

When k_1 and k_2 in mechanism 3 are the same as k_1^g and k_2^g, respectively, in mechanism 1, the reduction of the disulfide bond will occur through a species in which the disulfide bond is exposed by global unfolding. When k_1 and k_2 are different from k_1^g and k_2^g, respectively, the reduction of the disulfide bond will occur through a species in which the disulfide bond is exposed by fluctuations different from global unfolding. In order to clarify the mechanism by which the disulfide bond of the 33-kDa protein is reduced, we performed the present experiments of the reduction kinetics. In the case of the constant fragment of the immunoglobulin light chain, the reduction of the disulfide bond was found to follow the former mechanism (Kikuchi et al., 1985).

The apparent rate constant (k_{app}) is expressed by (Hvidt & Nielsen, 1966; Miller & Narutis, 1984)

$$k_{app} = \frac{k_1 k_3[DTT]}{k_1 + k_2 + k_3[DTT]} \quad (7)$$

This equation is basically the same as that used for the hydrogen–deuterium exchange kinetics. In hydrogen exchange kinetics, the intrinsic rate constant with which the exposed hydrogen is replaced with deuterium, corresponding to the X → R step in this case, is constant at a given pH. Then the kinetics mechanism is invariable. In the reduction kinetics, however, this can be changed because k_3[DTT] is able to be varied appropriately by changing the concentration of DTT.

To simplify eq 7, the significant question is the relative magnitude of k_1, k_2, and k_3[DTT] (Vas & Boross, 1974). In the present experiments, the following ramifications were appropriate to analyze.

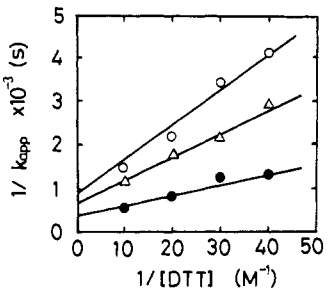


FIGURE 8: Plots of $1/k_{app}$ against $1/[DTT]$ according to eq 9 for the reduction kinetics of the disulfide bond of the 33-kDa protein in 0 (○), 0.1 (△), and 0.2 M Gdn-HCl (●) at pH 7.5 and 25 °C.

When $k_1 \ll k_2 + k_3[DTT]$, the apparent first-order rate constant (k_{app}) is approximated by

$$k_{app} = k_1 k_3[DTT]/(k_2 + k_3[DTT]) \quad (8)$$

or

$$1/k_{app} = 1/k_1 + (k_2/k_1 k_3)(1/[DTT]) \quad (9)$$

When $k_3[DTT] \ll k_1 + k_2$, the apparent first-order rate constant is approximated by

$$k_{app} = k_1 k_3[DTT]/(k_1 + k_2) \quad (10)$$

or

$$1/k_{app} = 1/k_3[DTT] + k_2/k_1 k_3[DTT] \quad (11)$$

The plot of k_{app} against [DTT] gives a straight line for the latter case but not for the former. Plotting $1/k_{app}$ against $1/[DTT]$ gives a straight line in both cases. However, the plot gives $1/k_1$ on the ordinate when $1/[DTT]$ is equal to zero in the former case, whereas the plot extrapolates back through the origin in the latter case.

Figure 8 shows the plots of $1/k_{app}$ against $1/[DTT]$ for the reactions in 0, 0.1, and 0.2 M Gdn-HCl. Each plot gave a straight line and did not extrapolate back through the origin. Therefore, the reduction reaction of the disulfide bond of the 33-kDa protein with DTT can be analyzed by assuming $k_1 \ll k_2 + k_3[DTT]$ in mechanism 3. The value of k_1 can be obtained from the intercept of the straight line with the ordinate when $1/[DTT]$ is equal to zero. The slope of the line is equal to $k_2/k_1 k_3$.

In order to determine the value of k_2, the value of k_3 should be obtained. It was shown that the intrinsic rate constant (k_3) of the disulfide exchange reaction varies considerably in unfolded proteins (Snyder et al., 1981) and in native proteins (Shaked et al., 1980) owing to electrostatic and steric effects around the disulfide bond. If we assume that the reduction reaction of the 33-kDa protein occurs through a species in which the disulfide bond is exposed to global unfolding, the value of k_3 determined previously for the reduction of the disulfide bond in the unfolded constant fragment (Kikuchi et al., 1986) could be applied to this case. For the 33-kDa protein, a cystine bridge is formed between Cys-28 and Cys-51. The sequences around Cys-28 and Cys-51 are -Gln-Cys-Pro- and -Phe-Cys-Leu-, respectively. For the constant fragment, a cystine bridge is formed between Cys-136 and Cys-195. The sequences around Cys-136 and Cys-195 are -Val-Cys-Leu- and -Ser-Cys-Gln-, respectively (Chen & Poljak, 1974). There are no ionizable amino acid residues adjacent to the cystine residues, and two (Gln and Leu) of the four residues are identical for both proteins. Thus, the difference in the mag-

nitude of the electrostatic and steric effects on the rate constant (k_3) of the disulfide reduction would be small, if any. We assumed that the electrostatic and steric effects on the disulfide reduction rate for the 33-kDa protein are similar to those for the constant fragment in the unfolded state and used the following equation obtained for the constant fragment (Kikuchi et al., 1986) to estimate the value of k_3:

$$\log k_3 = 0.96 - 0.13[\text{Gdn-HCl}] \quad (12)$$

where [Gdn-HCl] is the concentration of Gdn-HCl.

The value of k_2 was estimated by using the value of k_3 obtained from eq 12 and k_1 at a given concentration of Gdn-HCl. The values of k_1 and k_2 thus estimated at various concentrations of Gdn-HCl are given and compared with the values of k_1^s and k_2^s in Figure 7.

As shown in Figure 6, the apparent rate constant for the reduction of the disulfide bond in the absence of Gdn-HCl was found to be 0.9×10^{-3} s^{-1}. The rate constant for the reduction of the exposed disulfide bond by 100 mM DTT, the concentration used in the experiment, in the absence of Gdn-HCl was calculated to be 0.9 s^{-1} using eq 12. Thus, the reduction reaction of the disulfide bond of the 33-kDa protein is 1000 times slower than the reduction rate of the exposed disulfide bond. This suggests that the disulfide bond of the 33-kDa protein is located somewhere in the interior of the molecule and not exposed and that the reduction is rate-limited by the exposure of the disulfide bond as a result of fluctuation.

Figure 7 shows that the values of k_1 and k_2 for the reaction $N \rightleftharpoons X$ (mechanism 3) are much larger than k_1^s and k_2^s, respectively, for the reaction $N \rightleftharpoons U$ (mechanism 1). This indicates that the reduction of the disulfide bond of the 33-kDa protein does not proceed through a species with a conformation very similar to that of the fully unfolded one but through a species in which the disulfide bond is exposed by local fluctuations. In the case of the constant fragment of the immunoglobulin light chain, the disulfide bond, which is completely buried in the interior of the molecule, is reduced through a species with a completely unfolded conformation (Kikuchi et al., 1986). In the case of the 33-kDa protein, the disulfide bond is also located in the interior of the molecule but may not be completely buried or is located in a part where local fluctuation occurs frequently.

Recently, Kawata et al. (1988) examined the hydrogen–deuterium exchange kinetics of the tryptophan indole NH proton buried in the interior of the constant fragment of the immunoglobulin light chain and demonstrated that the protein undergoes not only global unfolding but also local fluctuations in water. Also in the case of the 33-kDa protein, exchangeable protons around the disulfide bond would be exchanged with deuterium by the local fluctuation. Thus, generally, such local fluctuations might be related to the hydrogen exchange mechanism in proteins.

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