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Reduction of the Buried Intrachain Disulfide Bond of the Constant Fragment of the Immunoglobulin Light Chain: Global Unfolding under Physiological Conditions

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ABSTRACT: The constant (C_L) fragment of the immunoglobulin light chain contains only one intrachain disulfide bond buried in the interior of the molecule. The kinetics of reduction with dithiothreitol of the disulfide bond were studied at various concentrations of guanidine hydrochloride at pH 8.0 and 25 °C. It was found that the disulfide bond is reduced even in the absence of guanidine hydrochloride. The results of the reduction kinetics were compared with those of the unfolding and refolding kinetics of the C_L fragment previously reported [Goto, Y., & Hamaguchi, K. (1982) *J. Mol. Biol. 156*, 891–910]. It was shown that the reduction of the disulfide bond proceeds through a species with a conformation very similar to that of the fully unfolded one and that the C_L fragment undergoes global unfolding transition even in water.

Fluctuations of the protein molecule are believed to play an important role in biological functions and have been studied extensively by various methods such as X-ray crystallography, nuclear magnetic resonance, fluorescence quenching, fluorescence anisotropy, and hydrogen isotope exchange (Gurd & Rothgeb, 1979; Karplus & McCammon, 1981; Ringe & Petsko, 1985). The immunoglobulin molecule consists of two regions, Fab and Fc, which are flexibly linked through a hinge

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region (Amzel & Poljak, 1979). Fluorescence emission anisotropy measurements have shown that the immunoglobulin G molecule exhibits segmental flexibility in the nanosecond time range (Yguerabide et al., 1970). Recently the structural role of the hinge in molecular flexibility has been studied by the nuclear magnetic resonance method (Endo & Arata, 1985). However, the internal fluctuations occurring in each domain have not been studied in detail. The molecular fluctuations in the immunoglobulin domains are important for understanding their functions in view of the recent studies on atomic mobility in antigen–antibody reactions (Tainer et al., 1985).

Each of the immunoglobulin domains contains only one intrachain disulfide bond buried in the interior of the domain (Amzel & Poljak, 1979). The accessible surface area of the disulfide bond in several V_L and V_H domains has been calculated to be zero, and so it must also be zero in the C_L[1] domain (B. Sutton, private communication). Usually the reduction of the disulfide bond is carried out in the presence of concentrated denaturing agent. However, if the domain fluctuates so as to expose the disulfide bond to solvent, the disulfide bond would be expected to be reduced even in the absence of denaturant. Previously, Goto and Hamaguchi (1979) studied the unfolding equilibrium by Gdn-HCl of the C_L fragment obtained by papain digestion of a type λ light chain. They also analyzed the kinetics of the unfolding and refolding of the C_L fragment by Gdn-HCl on the basis of the three-species mechanism, and the rate constants for the respective processes were determined (Goto & Hamaguchi, 1982a,b). Comparison of the kinetics of the reduction of the disulfide bond with the kinetics of the unfolding and refolding would provide an important insight into the molecular fluctuations (global or local unfolding) under physiological conditions.

In this study, we have examined the kinetics of reduction with DTT of the intrachain disulfide bond of the C_L fragment at various concentrations of Gdn-HCl. We found that the disulfide bond buried in the interior of the C_L fragment molecule is reduced with DTT even in the absence of denaturant and that the unfolding and refolding rates evaluated from the kinetics of the reduction are consistent with the rates determined previously from the unfolding and refolding kinetics. These findings indicate that the reduction of the disulfide bond of the C_L fragment occurs through full unfolding and that the C_L fragment molecule undergoes global unfolding transition even in water.

Materials and Methods

Materials. The C_L fragment of Bence-Jones protein Nag (type λ) was obtained by digestion with papain as previously described (Goto & Hamaguchi, 1979). Gdn-HCl (specially purified grade), DTT, iodoacetamide, and other reagents were obtained from Nakarai Chemicals Co. and Wako Pure Chemical Co. and used without further purification.

Unfolding Equilibrium. Unfolding equilibria of the intact C_L and reduced C_L fragments by Gdn-HCl were measured at 25 °C and pH 7.5 (50 mM Tris-HCl buffer) by fluorescence at 350 nm. The fluorescence was measured with a Hitachi fluorescence spectrophotometer, Model MPF-4, equipped with a spectral corrector. The fluorescence at 350 nm was measured by using 295-nm light for the excitation. The protein concentration was 0.04 mg/mL.

Reduction of the Disulfide Bond of the C_L Fragment. The buffer used was 0.1 M Tris-HCl at pH 8.0 containing 0.15 M KCl and 0.1 mM EDTA. DTT was used to reduce the disulfide bond. Reduction was always carried out under a nitrogen atmosphere after buffers had been degassed. We used two methods to follow the reduction reaction. In the Gdn-HCl concentration range from 0.5 to 1 M, where the reaction proceeds relatively fast, the reduction was followed by fluorescence measurements. As reported previously (Goto & Hamaguchi, 1979), the tryptophyl fluorescence of the C_L frgament is quenched to a great extent owing to one of the two tryptophyl residues being located near the intrachain disulfide bond. When the disulfide bond is reduced, the fluorescence increases, because the quenching effect of the disulfide bond is removed (see Figure 1). Therefore, reduction of the disulfide bond with DTT can be followed by measuring the change in the tryptophyl fluorescence. In this case, we used the change in the fluorescence intensity at 350 nm, and the excitation wavelength was 295 nm. The protein concentration was 4.8 μM, and the final concentration of DTT was 20–100 mM. The temperature was kept at 25 °C with a thermostatically controlled cell holder.

In the absence of Gdn-HCl or the presence of 0.2 M Gdn-HCl, where the reduction reactions were extremely slow, the fluorescence measurements could not be used to follow the reaction because of instrumental fluctuations. In the presence of more than 2 M Gdn-HCl, where the C_L fragment is completely unfolded, there is no change in the tryptophyl fluorescence on reduction of the disulfide bond (see Figure 1). Therefore, the reduction reactions in the absence and presence of 0.2 M Gdn-HCl and more than 2 M Gdn-HCl were followed by polyacrylamide gel electrophoresis. The final concentration of the protein was about 40 μM. The reduction was started by adding DTT to give a final concentration of 20–100 mM to this solution. Samples of 0.2 mL of the reaction mixture were withdrawn at appropriate intervals, and the reaction was stopped by the addition of an equivalent volume of 200–400 mM iodoacetamide in 0.1 M acetate buffer at pH 5.3. In the presence of Gdn-HCl, the alkylated sample was separated from the residual reagents on a column of Sephadex G-25 or by dialysis and then lyophilized. The lyophilized sample was dissolved in buffer containing 30 mM H_3PO_4 and 60 mM Tris at pH 6.9, and the products were analyzed by gel electrophoresis. As reported previously (Goto & Hamaguchi, 1979), the reduced and alkylated C_L fragment is unfolded to a great extent, and its hydrodynamic volume is larger than that of the intact C_L fragment. Thus the two species, the intact C_L and the reduced and alkylated C_L, can be separated on the basis of the sieving effect of polyacylamide gel (Goto & Hamaguchi, 1981). The electrophoresis was carried out at pH 9.4 in 12.5% (w/v) polyacrylamide gel according to the method of Davis (1964). Samples (50 μL) were layered on the stacking gel, and electrophoresis was carried out for 3–4 h at 2 mA/tube. Gels were stained with 2.5% (w/v) Coomassie brilliant blue R-250 in 45% (v/v) methanol and 7.5% (v/v) acetic acid and destained in 5% (v/v) methanol and 7.5% (v/v) acetic acid. Densitometric scanning of the gels was done at 565 nm with a Toyo DMU-33C digital densitometer.

The concentration of DTT present after the reaction was determined by titration with DTNB at pH 8.0. The molar extinction coefficient of reduced DTNB was assumed to be

[1] Abbreviations: C_L fragment, the constant fragment of the immunoglobulin light chain; reduced C_L fragment, C_L fragment in which the intrachain disulfide bond is reduced; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Gdn-HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane.

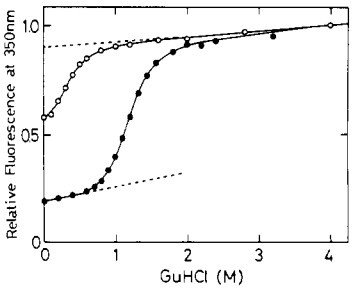


FIGURE 1: Unfolding equilibria of the intact C_L fragment (●) and reduced C_L fragment (○) by Gdn-HCl as measured by fluorescence intensity at 350 nm, pH 7.5, and 25 °C.

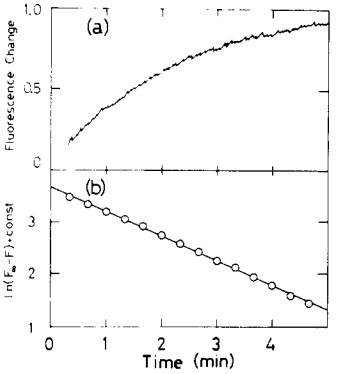


FIGURE 2: Kinetics of reduction with DTT of the disulfide bond of the C_L fragment in 1 M Gdn-HCl. Change with time in the fluorescence intensity at 350 nm (a) and the first-order plot (b) at pH 8.0, 25 °C. The final concentrations of the C_L fragment and DTT were 4.8 μM and 41 mM, respectively.

13 600 M^{-1} cm^{-1} at 412 nm (Ellman, 1958; Gething & Davidson, 1972).

Protein Concentration. The concentration of the C_L fragment was determined spectrophotometrically by using a 280-nm absorption coefficient of $A^{1\%}_{1cm}$ = 14.6 (Karlsson et al., 1972). A value of 11 500 was used as the molecular weight of the C_L fragment.

pH Measurement. pH was measued with a Radiometer pHM26c at 25 °C.

RESULTS

Previously, Goto and Hamaguchi (1979) reported the reversible equilibria of the unfolding of the intact C_L and reduced C_L fragments by Gdn-HCl as measured by the circular dichroism at 218 nm. Figure 1 shows the reversible unfolding curves of the intact C_L and reduced C_L frgments as measured by fluorescence at 350 nm. In the absence of Gdn-HCl, the intensity of the tryptophyl fluorescence of the reduced C_L fragment is much higher than that of the intact C_L fragment. This is due to the quenching effect of the intrachain disulfide bond on the tryptophyl fluroescence being removed on reduction of the disulfide bond. The unfolding of the intact C_L fragment began from about 0.7 M Gdn-HCl and ended at about 2 M Gdn-HCl with a midpoint of 1.2 M Gdn-HCl at pH 7.5 and 25 °C. The unfolding of the reduced C_L fragment began from a very low concentration of Gdn-HCl and ended at about 1 M Gdn-HCl with a midpoint of 0.4 M Gdn-HCl. These curves were the same as the respective curves obtained by the circular dichroism at 218 nm.

The kinetics of the reduction with DTT of the disulfide bond of the C_L fragment in the presence of Gdn-HCl from 0.5 to 1.0 M were measured by using the fluorescence change at 350 nm. Figure 2a shows the increase in the tryptophyl fluorescence at 350 nm with time in 1 M Gdn-HCl after the addition of DTT to give a final concentration of 41 mM. The change

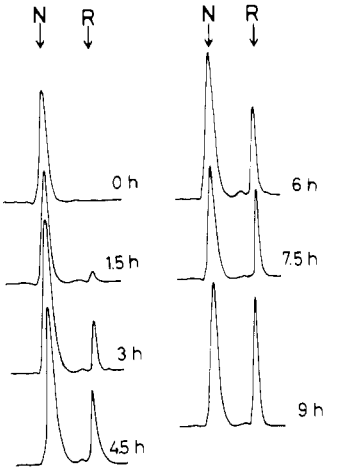


FIGURE 3: Densitometric tracings of the species separated by gel electrophoresis in 12.5% polyacrylamide at pH 9.4 during the reduction with DTT of the disulfide bond of the C_L fragment in 0.1 M Tris-HCl buffer at pH 8.0, 25 °C. N and R indicate the intact C_L and reduced C_L fragments, respectively. The final concentration of DTT was 88 mM.

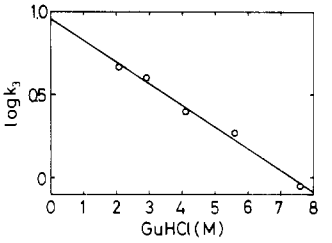


FIGURE 4: Second-order rate constants for the reduction of the disulfide bond of the unfolded C_L fragment in the concentration range of 2.1–7.6 M Gdn-HCl, pH 8.0, 25 °C. The straight line is expressed by the equation log k_3 = 0.96 − 0.13[Gdn-HCl].

followed first-order kinetics as shown in Figure 2b. The apparent rate constant increased in a nonlinear manner with increased concentration of DTT in the range from 20 to 100 mM.

Figure 3 shows the densitometric tracings of the polyacrylamide gels of the reduced C_L fragment trapped as the alkylated protein in the course of the reduction of the C_L fragment with 88 mM DTT in the absence of Gdn-HCl. It is clearly apparent that the disulfide bond of the C_L fragment is reduced even in the absence of Gdn-HCl. The change with time of the appearance of the reduced C_L fragment followed first-order kinetics, and the apparent first-order rate constant increased in a nonlinear manner with an increase in the concentration of DTT.

As described below, in order to analyze the kinetics of the reduction of the disulfide bond, it is necessary to know the rate of reduction with DTT of the exposed disulfide bond. The kinetics of the reduction reaction were measued in the concentration range of 2–8 M Gdn-HCl, where the C_L fragment is completely unfolded (Figure 1). Figure 4 shows the dependence of the second-order rate constant (k_3) on the Gdn-HCl concentration. The dependence was linear and is expressed by

$$\log k_3 = 0.96 - 0.13[\text{Gdn-HCl}] \quad (1)$$

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

DISCUSSION

The intrachain disulfide bond of the C_L fragment is buried in the interior of the protein molecule (Amzel & Poljak, 1979), and its accessible surface area is zero (B. Sutton, private

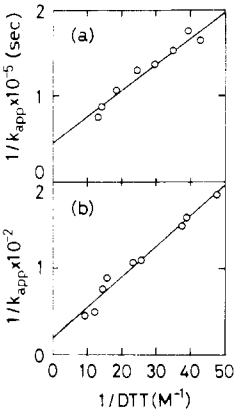


FIGURE 5: Plots of $1/k_{app}$ against 1/[DTT] according to eq 2 for the reduction in 1.0 (a) and 0 M Gdn-HCl (b) at pH 8.0 and 25 °C.

communication). Nevertheless, the disulfide bond is reduced with DTT even in the absence of any denaturing agents, though a high concentration of DTT is needed. In order to be reduced with DTT, the disulfide bond of the C_L fragment must be accessible to DTT. Thus, the reduction of the disulfide bond with DTT may be explained on the basis of the mechanism shown in Scheme I.

Scheme I

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

In Scheme I, N, the native C_L fragment containing the disulfide bond buried in the interior of the molecule and thus not accessible to solvent, is in equilibrium with X. X is any conformation of the C_L fragment molecule in which the disulfide bond is accessible to DTT. R is the C_L fragment in which the disulfide bond is reduced. k_1, k_2, and k_3 are the rate constants for the respective processes. Under the experimental conditions used here, the reduction of the disulfide bond was irrerversible.

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

$$k_{app} = k_1k_3[DTT]/(k_2 + k_3[DTT]) \quad (2)$$

or

$$1/k_{app} = 1/k_1 + (k_2/k_1k_3)(1/[DTT])$$

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

$$k_{app} = k_1k_3[DTT]/(k_1 + k_2) \quad (3)$$

or

$$1/k_{app} = 1/(k_3[DTT]) + k_2/(k_1k_3[DTT])$$

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 give $1/k_1$ 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.

Parts a and b of Figure 5 show the plots of $1/k_{app}$ against 1/[DTT] for the reactions in 1.0 and 0 M Gdn-HCl, respectively. As can be seen, each plot gave a straight line and did not extrapolate back through the origin in either case. Therefore the reduction reaction of the disulfide bond with DTT can be analyzed assuming $k_1 \ll k_2 + k_3[DTT]$ in Scheme I. 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_1k_3. Since the value of k_3 at a given concentration of Gdn-HCl is estimated

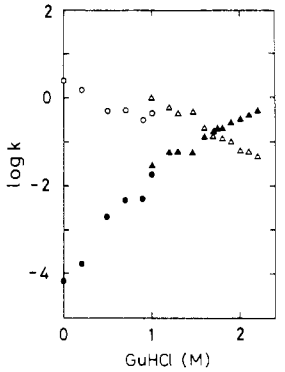


FIGURE 6: Rate constants k_1 (●) and k_2 (○) in Scheme I as a funciton of concentration of Gdn-HCl. The solid and open triangles represent the rate constants k_{32} and k_{23}, respectively, in Scheme II. See text for details.

by using eq 1, the value of k_2 can be determined. The values of k_1 and k_2 thus obtained at various concentrations of Gdn-HCl are shown in Figure 6.

Goto and Hamaguchi (1982a) determined the rate constants for the unfolding and refolding of the C_L fragment by Gdn-HCl using the three-species mechanism shown in Scheme II.

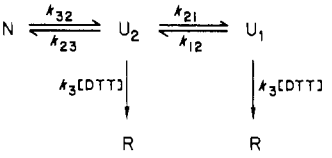
Scheme II

$$N \underset{k_{23}}{\overset{k_{32}}{\rightleftharpoons}} U_2 \underset{k_{12}}{\overset{k_{21}}{\rightleftharpoons}} U_1$$

In Scheme II, N is native protein, and U_1 and U_2 are the slow-folding and fast-folding species, respectively, of the unfolded protein. k_{12}, k_{21}, k_{23}, and k_{32} are the rate constants for the respective processes. The values of k_{23} and k_{32} determined by Goto and Hamaguchi (1982a) are shown in Figure 6.

When the unfolding and refolding of the C_L fragment follow the three-species mechanism and if the conformation of species X is the same as that of U in Scheme II, the reduction reaction may be expressed by the mechanism shown in Scheme III.

Scheme III



If $k_3[DTT]$ is much larger than the rate constant for the reaction from U_2 to U_1 (k_{21}), Scheme III is reduced to Scheme I. When the value of $k_3[DTT]$ is not large enough compared with the value of k_{21}, the values of k_1 and k_2 obtained with eq 2 are slightly smaller than the values of k_{32} and k_{23}, respectively. In the concentration range of DTT used in the present experiments, however, $k_3[DTT]$ is 5–30 times as large as k_{21}, and the reaciton given by Scheme III may be approximated by Scheme I. As shown in Figure 6, the values of k_1 and k_2 below 1 M Gdn-HCl lie on the respective curves obtained by extension of the curves for k_{32} and k_{23}. This shows that even in the absence of Gdn-HCl the C_L fragment containing a disulfide bond that can react with DTT (species X in Scheme I) has a conformation very similar to that of the fully unfolded molecule and that k_1 and k_2 correspond to the rates of unfolding and refolding, respectively, of the C_L fragment.

The free energy change for the reaction N → X in the absence of Gdn-HCl in Scheme I is calculated to be 6.2 kcal/mol by using the values of k_1 and k_2. This value is close to the free energy change (7.1 kcal/mol) for the reaction N → U_2 in Scheme II estimated by extrapolating the unfolding equilibrium curve to 0 M Gdn-HCl (Goto & Hamaguchi,

1979, 1982). This shows that the free energy change obtained by extrapolation of the unfolding equilibrium curve to 0 M denaturant represents well the free energy change for the unfolding reaction that actually occurs in the absence of denaturant. The same may hold well for the extrapolated free energy changes obtained for other proteins (Pace, 1975).

Hydrogen isotope exchange has been used extensively as one of the methods to study the fluctuations of the protein molecule. In order for hydrogen atoms located in the interior of the protein molecule to be exchanged with deuterium, the former must be exposed to solvent. Although it is established that hydrogen isotope exchange proceeds through global unfolding in the unfolding transition zone, it is not clear from hydrogen exchange experiments whether global unfolding of the protein molecule occurs as well under native conditions (Woodward & Tüchsen, 1982). However, the results obtained in the present study strongly support the proposal that the C_L fragment molecule undergoes global unfolding even under native conditions.

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Physical–Chemical Model for the Entry of Water-Insoluble Compounds into Cells. Studies of Fatty Acid Uptake by the Liver†

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ABSTRACT: The spontaneous transfer of water-insoluble substances from plasma to the interior of cells would involve a series of steps in which the substance of interest dissociates from albumin in plasma, enters the outer half of the plasma membrane of a cell, crosses the bilayer, and then dissociates from the inner half of the plasma membrane to enter cell cytosol and diffuses to sites of its metabolism. We have examined the behavior of long-chain fatty acids in the uptake process, assuming that none of these steps is facilitated by the cell during the entry of fatty acids into the liver. Comparison of the spontaneous rates for each individual step with rates of uptake of fatty acid by perfused liver leads to the conclusion that the uptake of fatty acids is not limited by kinetic factors but is determined instead by the equilibrium distribution (K_{eq}) of fatty acids between albumin in plasma and the phospholipids of the plasma membrane. This idea was examined further by determining whether there was a relationship between the value for K_{eq} and rates of uptake of a fatty acid and the pattern of kinetics for uptake. The data indicate that there is a linear relationship between K_{eq} and the rate of uptake, that uptake rates can be predicted with a high degree of accuracy from thermodynamic data, and that the pattern of kinetics of uptake is compatible with the idea that the uptake rate is determined by the relative affinity of a fatty acid for albumin and membranes.

Mechanisms for the uptake of water-insoluble substances by tissues have been considered in terms of processes for uptake of water-soluble substances. Thus, most if not all, treatments of the subject are conceptualized as requiring specific mechanisms for the transport into tissues of water-insoluble compounds (Mahadevan & Sauer, 1971, 1974; Samuel et al., 1976; Weisiger et al., 1981; Abumrad et al. 1981, 1984; Stremmel et al., 1985). The problem for the cell in internalizing polar compounds is to short-circuit the high energy barrier to the passage of these substances across the apolar plasma membranes of the cell. By contrast, many biologically important compounds with limited solubility in water are highly soluble in membrane lipids and in fact penetrate lipid bilayers in a

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