Unfolding and Refolding of a Type κ Immunoglobulin Light Chain and Its Variable and Constant Fragments

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ABSTRACT: By limited proteolysis of a type κ immunoglobulin light chain (Oku) with clostripain, both the V_L and C_L fragments were obtained with a high yield. The unfolding and refolding by guanidine hydrochloride of light chain Oku and its V_L and C_L fragments were studied at pH 7.5 and 25 °C with circular dichroism and tryptophyl fluorescence. The concentration of guanidine hydrochloride at the midpoint of the unfolding curve was 1.2 M for the V_L fragment and 0.9 M for the C_L fragment. As in the case of the C_L fragment of light chain Nag (type λ) [Goto, Y., & Hamaguchi, K. (1982) *J. Mol. Biol. 156*, 891–910], the unfolding and refolding kinetics of the C_L fragment could be explained in principle on the basis of the three-species mechanism $U_1 \rightleftharpoons U_2 \rightleftharpoons N$, where N is native protein and U_1 and U_2 are the slow-folding and fast-folding species, respectively, of unfolded protein. The unfolding and refolding kinetics of the V_L(Oku) fragment were described by a single exponential term. Double-jump experiments, however, showed that two forms of the unfolding molecule exist. The equilibrium and kinetics of unfolding of light chain Oku were explained by the sum of those of the V_L and C_L fragments. On the other hand, the refolding kinetics of light chain Oku were greatly different from the sum of those of the V_L and C_L fragments. The amplitude of the slow phase of the light chain was greater than that expected from the refolding kinetics of the isolated domains, and an additional slow phase that did not exist in the isolated domains appeared. These results indicate that while the unfolding and refolding of the light chain were approximately explained in terms of the independent folding of the two domains, some interactions exist between the domains during the kinetically determined refolding process.

The immunoglobulin light chain consists of two structural units, each with a molecular weight of about 12 000, the amino-terminal variable domain (V_L domain) and the carboxy-terminal constant domain (C_L domain). The V_L and C_L domains have similar tertiary structures, each consisting of two β-sheets with one intrachain disulfide bond buried in the interior hydrophobic region between the sheets (Beale & Feinstein, 1976; Amzel & Poljak, 1979). While the folding kinetics of small globular proteins have been studied extensively, the folding kinetics of larger proteins with multidomain structures are complex (Baldwin, 1975; Kim & Baldwin, 1982; Goldberg, 1985; Jaenicke & Rudolph, 1986). In some multidomain proteins, folding of a domain is influenced by the presence of the other domains (Teale & Benjamin, 1976, 1977; Dautry-Varsat & Garel, 1978; Desmadril & Yon, 1984), while in other proteins the refolding rates are similar for the isolated fragments and for the protein (Dautry & Garel, 1981; Zetina & Goldberg, 1982). The immunoglobulin light chain that consists of two domains may be a simple model for understanding the folding mechanism of such large proteins. From this viewpoint, we previously studied the refolding kinetics of type λ light chains and isolated V_L and C_L fragments from the unfolded state in 4 M Gdn-HCl[1] (Goto et al., 1979). The results showed that while the spectral properties of the light chain can be explained in terms of the independent folding of the two domains, the refolding process cannot be expressed as the simple sum of the refolding reactions of the isolated V_L and C_L fragments. In that study, however, we were unable to use the constituent V_L and C_L fragments obtained from a light chain.

In this paper, we report the results on the unfolding and refolding of a type κ light chain and its V_L and C_L fragments induced by Gdn-HCl. In these experiments, we first attempted to isolate both the V_L and C_L fragments from a light chain. On the basis of the amino acid sequences of the type κ light chains, we noticed that the arginyl residue is one of the least frequent residues and that one arginyl residue is located at position 108 in the switch region for all the type κ light chains. We found that by limited proteolysis of a type κ light chain with clostripain (EC 3.4.22.8), an arginine-specific endoproteinase, both the V_L and C_L fragments could be successfully isolated at a high yield. Using these fragments and their parent type κ light chain, we carried out equilibrium and kinetic measurements of unfolding and refolding induced by Gdn-HCl. We found that the CD and fluorescence spectra of the light chain are expressed as the simple sum of the spectra of the V_L and C_L fragments and that while the unfolding equilibrium and unfolding kinetics of the light chain are explained in terms of the independent unfolding of the V_L and C_L fragments, the refolding kinetics of the light chain cannot be simply explained in terms of the independent refolding of the two domains. Folding reactions of the type κ light chain and V_L and C_L fragments are also compared with those of the respective type λ proteins.

Materials and Methods

Materials. Bence–Jones protein Oku (type κ) was prepared from the urine of a multiple-myeloma patient by precipitation with ammonium sulfate and ion-exchange chromatography (DE-52) as described previously (Goto et al., 1979). The urine of multiple-myeloma patient Tod contained a V_L fragment in addition to the parent Bence–Jones protein (type κ), and the fragment was purified from the urine as described previously (Azuma et al., 1978).

[1] Abbreviations: CD, circular dichroism; Gdn-HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

[2] The numbering system used in this paper is based on protein Ag (Putnam, 1969) for the type κ light chain and protein New (Chen & Poljak, 1974) for the type λ light chain.

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Table I: N-Terminal and C-Terminal Sequences of Fragments of Light Chain Oku (Type κ) Obtained by Limited Proteolysis with Clostripain

DEAE fraction	identification	N-terminal sequence[a]	C-terminal sequence[b]	molecular weight[c]	yield from 1 g of light chain (mg)
II-1	V_L	H-Glu[1]-Ile[2]-Val[3]-Met[4]-Ser[5]-Gln[6]-Ser[7]-Pro[8]-Leu[9]-	-Arg[108]-OH	12 700	200
II-2	V_L	H-Glu[1]-Ile[2]-	-Arg[108]-OH	12 700	100
III-1	C_L	H-Thr[109]-Val[110]-	-Arg[211]-OH	11 500	20
III-2	C_L	H-Thr[109]-Val[110]-Ala[111]-Ala[112]-	-Arg[211]-Gly[212]-Glu[213]-Cys[214]-OH	11 200	150

[a] The N-terminal sequences were determined by Edman degradation (Blombäck et al., 1966). [b] The C-terminals of II-1, II-2, and III-1 were determined by hydrazinolysis (Niu & Fraenkel-Conrat, 1955). The C-terminal sequence of III-2 was determined by digestion with carboxypeptidase Y. The C-terminal Cys residue of III-2 was detected as (carboxamidomethyl)cysteine. [c] Molecular weights were determined from amino acid compositions.

Clostripain was obtained from Cooper Biomedical and carboxypeptidase Y from Oriental Yeast. Gdn-HCl (specially purified grade) was obtained from Nakarai Chemicals. Other reagents were of reagent grade and were used without further purification.

Preparation of V_L and C_L Fragments of Light Chain Oku. The V_L and C_L fragments of light chain Oku were prepared by limited proteolysis with clostripain. Bence–Jones protein Oku (1%), in which the interchain disulfide bond had been reduced and alkylated with iodoacetamide, was digested in 0.1 M Tris-HCl buffer at pH 7.5 containing 0.15 M NaCl and 5 mM dithiothreitol at 37 °C for 40 min with clostripain at a substrate-to-enzyme ratio of 500:1 (w/w). The digestion was stopped by addition of iodoacetamide to give a final concentration of 35 mM. The digestion product was subjected to gel filtration on a Sephadex G-75 column (1.5 × 115 cm) equilibrated with 10 mM Tris-HCl buffer at pH 8.6. Three peaks were separated. The first peak corresponded to the undigested protein. The second and third peaks corresponded to proteins with molecular weights of about 12 000 and 11 000, respectively, as estimated by SDS–polyacryamide gel electrophoresis. The fractions of the second and third peaks were pooled and subjected separately to ion-exchange chromatography on a DEAE-cellulose column (1 × 22 cm) equilibrated with 10 mM Tris-HCl buffer at pH 8.6. The proteins of the second peak were separated into an unadsorbed protein (II-1) and an adsorbed protein. The adsorbed protein was eluted as a peak (II-2) with a linear gradient of NaCl (0–0.1 M). The proteins of the third peak were adsorbed to the DEAE-cellulose column and separated into two peaks (III-1 and III-2) with a linear gradient of NaCl (0–0.1 M). The fractions corresponding to these peaks were dialyzed against water and lyophilized.

Table I shows the N-terminal and C-terminal sequences of the respective fragments. The N-terminal sequence of fragment II-1 corresponds to the sequence from 1 to 9 of the type κ (subgroup III) light chain (Nisonoff et al., 1975). On the basis of the sequence of the type κ light chain, the Arg-108–Thr-109 bond was shown to be cleaved by clostripain. Fragments II-1 and II-2 were identified to be the V_L fragment, and fragments III-1 and III-2 were identified to be the C_L fragment. The C-terminal of fragment III-1 was produced by hydrolysis with clostripain of the peptide bond Arg-211–Gly-212. On electrophoresis in the absence of SDS at pH 9.4, the mobilities of the four fragments were III-2, III-1, II-2, and II-1, in the order of fastest to slowest. The amino acid compositions of the two V_L fragments were the same (not shown), and the reason why the two forms were separated by ion-exchange chromatography is unknown at present. One possible reason is that deamination occurs in fragment II-2. In most of our present experiments, we used fragments II-1 and III-2.

Unfolding Equilibrium. All the measurements in the present studies were carried out in 50 mM Tris-HCl buffer at pH 7.5 containing 0.15 M NaCl at 25 °C. Unfolding transitions by Gdn-HCl of light chain Oku and its V_L and C_L fragments were measured by CD at 218 nm and tryptophyl fluorescence at 350 nm. The protein solutions (about 0.1 mg/mL) at different concentrations of Gdn-HCl were allowed to stand overnight at room temperature before measurement. The protein concentration was 0.2 mg/mL for the CD measurement and below 0.08 mg/mL for the fluorescence measurement. The fluorescence at 350 nm was linearly dependent on protein concentration at all the protein concentrations used (the optical density was 0.1 or less at 280 nm).

CD measurements were carried out with a Jasco spectropolarimeter, Model J-500A, equipped with a DP-501 data processor. The details of the CD measurements have been described previously (Goto & Hamaguchi, 1986). Fluorescence was measured with a Hitachi fluorescence spectrophotometer, Model MPF-4, equipped with a spectral corrector. Tryptophyl fluorescence was measured with 295-nm light for the excitation.

Unfolding and Refolding Kinetics. Unfolding and refolding kinetics were measured on a Union Giken stopped-flow spectrophotometer, Model RA-401, by use of fluorescence detection. The details of the apparatus have been described previously (Goto & Hamaguchi, 1982). The unfolding was initiated by mixing a protein solution in 0 M Gdn-HCl at pH 7.5 with a Gdn-HCl solution at a given concentration, also at pH 7.5, in a 1:1 ratio. For the measurements of refolding reactions, the proteins were initially exposed to 0.4 M Gdn-HCl at pH 1.9, under which conditions the proteins were unfolded as judged from the maximum wavelength of the tryptophyl fluorescence. The refolding was initiated by mixing the protein solution with 45 mM Tris-HCl buffer containing Gdn-HCl at a given concentration at pH 8.6 in a 1:1 ratio to give a final pH of 7.5.

Slow unfolding and refolding reactions were also measured with the Hitachi fluorescence spectrophotometer. A protein solution (0.05 mL) at pH 7.5 was manually mixed with 2.5 mL of a buffer at pH 7.5 containing Gdn-HCl at a given concentration. Initial concentrations of Gdn-HCl for the unfolding and refolding measurements were 0 and 4 M, respectively, at pH 7.5.

All the kinetic data were analyzed as described previously (Goto & Hamaguchi, 1982).

Amino Acid Analysis. The amino acid compositions of light chain Oku and its V_L and C_L fragments were determined with an Irica amino acid analyzer, Model A-5500 (Kyoto, Japan). The samples were hydrolyzed in evacuated, sealed tubes with 6 N HCl at 110 °C for 24 h. The amino-terminal sequences of the fragments were determined by manual Edman degradation (Blombäck et al., 1966), and the phenylthiohydantoin derivatives of amino acids were identified by high-performance liquid chromatography (Zimmerman et al., 1977) or thin-layer chromatography. The carboxyl terminals of the fragments

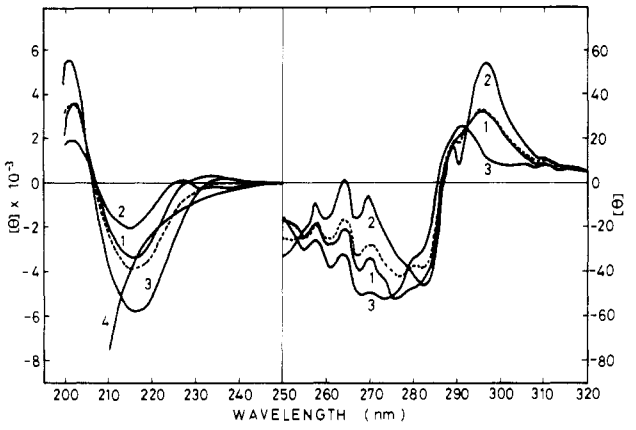

FIGURE 1: CD spectra of light chain Oku (1) and its V_L fragment (2) and C_L fragment (3) in 50 mM Tris-HCl buffer at pH 7.5 containing 0.15 M NaCl at 25 °C and that of the denatured light chain Oku (4) in 4 M Gdn-HCl at pH 7.5 and 25 °C. The dotted line indicates the CD spectrum of light chain Oku calculated from the spectra of the V_L and C_L fragments and assuming the independent folding of the two domains. The concentrations of the light chain and V_L and C_L fragments were 0.2 mg/mL.

were determined by hydrazinolysis (Niu & Fraenkel-Conrat, 1955) or by proteolysis with carboxypeptidase Y. The amino acids liberated on digestion with carboxypeptidase Y were quantified by the amino acid analyzer.

Protein Concentration. The concentrations of the V_L fragment (II-1), C_L fragment (III-2), and light chain Oku were determined spectrophotometrically with absorption coefficients at 280 nm for a 1% solution in a 1.0-cm cell of 12.7, 10.8, and 12.6, respectively. These values were determined by amino acid analysis of a protein solution with known absorbance.

pH Measurement. pH was measured with a Radiometer PHM26c meter at 25 °C.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis on 15% gels in the presence of SDS was carried out according to the method of Weber and Osborn (1969). Polyacrylamide gel electrophoresis on 7.5% gels in the absence of SDS was carried out according to the method of Davis (1964).

RESULTS

Conformations of Light Chain Oku and Its V_L and C_L Fragments. Figure 1 shows the CD spectra of light chain Oku and its V_L and C_L fragments. The CD spectra of the two forms (II-1 and II-2) of the V_L fragments were the same, as were the CD spectra of the two forms (III-1 and III-2) of the C_L fragments. The CD spectrum of the light chain had a negative maximum at 218 nm ($[\theta] = -3500$ deg cm^2 dmol^{-1}). The CD spectrum of the C_L fragment had a negative maximum at 218 nm with $[\theta] = -6000$ deg cm^2 dmol^{-1}, which is larger than the negative maximum for the V_L fragment ($[\theta] = -2000$ deg cm^2 dmol^{-1} at 215 nm). The CD spectra of the these proteins in 4 M Gdn-HCl were almost the same. The CD spectra of light chain Oku and its V_L and C_L fragments in the far-ultraviolet and aromatic absorption regions are very similar to the respective spectra reported by Ikeda et al. (1968) and by Ghose and Jirgensons (1971). Assuming that the V_L and C_L domains in the light chain fold independently, the CD spectrum of light chain Oku was calculated from the observed spectra of the V_L and C_L fragments. As shown in Figure 1, the calculated spectrum of light chain Oku was almost the same as the observed spectrum.

Figure 2 shows the fluorescence spectra of light chain Oku and its V_L and C_L fragments in Tris-HCl buffer at pH 7.5 relative to the fluorescence spectra of the respective proteins

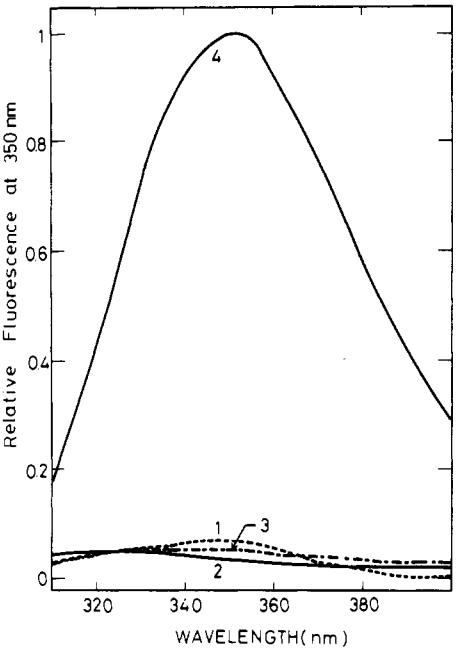

FIGURE 2: Fluorescence spectra of light chain Oku (1) and its V_L fragment (2) and C_L fragment (3) in 50 mM Tris-HCl buffer at pH 7.5 containing 0.15 M NaCl at 25 °C and the denatured proteins (4) in 4 M Gdn-HCl at pH 7.5 and 25 °C. The ordinate represents the fluorescence relative to that in the presence of 4 M Gdn-HCl. Excitation was at 295 nm. The concentrations of the light chain and V_L and C_L fragments were 0.04 mg/mL.

in 4 M Gdn-HCl. The fluorescence intensities of these proteins in the absence of denaturant were greatly quenched and were less than 5% of the fluorescence in 4 M Gdn-HCl. Similar observations have been made for type κ Bence–Jones proteins (Pollet et al., 1972). The C_L domain of the type κ light chain contains only one tryptophyl residue at position 148. From the ultraviolet absorption in 6 M Gdn-HCl and the number of tyrosyl residues determined by amino acid analysis, the number of tryptophyl residues in the V_L fragment of light chain Oku was determined to be one by the method of Edelhoch (1967). From the amino acid sequences of the type κ light chains (Nisonoff et al., 1975), this tryptophyl residue should be located at position 43. Each of these tryptophyl residues is buried in the interior of the domain and is located close to the intrachain disulfide bond (Amzel & Poljak, 1979). The tryptophyl fluorescences of these proteins are greatly quenched by the disulfide bond (Cowgill, 1967).

Unfolding Equilibria. Figure 3 shows the unfolding curves obtained with Gdn-HCl of light chain Oku and its V_L and C_L fragments measured by fluorescence at 350 nm. The transition curves were normalized by assuming that the fluorescences for the native and unfolded proteins which were observed before and after the transition zone, respectively, can be extrapolated linearly into the transition zone. The transition curves of the V_L and C_L fragments measured by CD agreed well with the curves measured by fluorescence. The unfolding processes for the three proteins were practically reversible. The Gdn-HCl concentrations at the midpoints of the unfolding curves for the light chain and the V_L and C_L fragments were 1.0, 1.3, and 0.9 M, respectively.

When the unfolding reactions of the V_L and C_L domains in the light chain proceed independently, the fraction (f_D) of the unfolded light chain can be expressed by

$$f_D = pf_D^V + qf_D^C \quad (1)$$

where p and q are the changes in fluorescence due to unfolding of the V_L and C_L domains, respectively, relative to the total

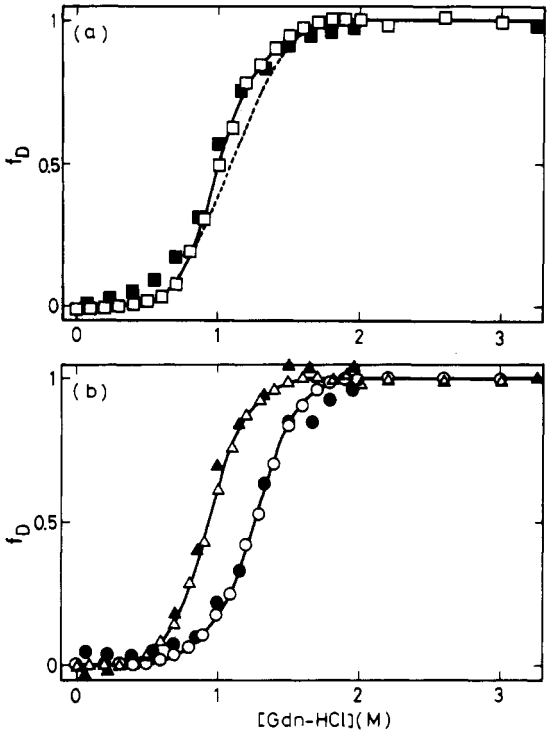

FIGURE 3: Unfolding transitions by Gdn-HCl of light chain Oku (squares in panel a) and its V_L fragment (circles in panel b) and C_L fragment (triangles in panel b) at pH 7.5 and 25 °C. The ordinate represents the fraction of unfolded protein (f_D). Unfolding transitions were measured in terms of the change in fluorescence at 350 nm. Excitation was at 295 nm. The values obtained by unfolding from 0 M Gdn-HCl to the indicated concentrations of Gdn-HCl are shown by clear symbols, and the values obtained by refolding from 4 M Gdn-HCl to indicated concentrations of Gdn-HCl are shown by solid symbols. The solid lines indicate the theoretical curves calculated from the values of $\Delta G_D^{H_2O}$ and Δn (see Table II). The dotted line in (a) indicates the transition curve of the light chain from the transition curves for the V_L and C_L fragments (see eq 1).

changes in fluorescence of the light chain and $p + q = 1$. f_D^V and f_D^C are the fractions of the unfolded V_L and C_L domains, respectively. As shown in Figure 2, the changes in fluorescence on unfolding of the V_L and C_L fragments were the same, and we assumed $p = q = 0.5$. In this case

$$f_D = 0.5(f_D^V + f_D^C) \quad (2)$$

As shown in Figure 3, the transition curve calculated from eq 2 was nearly the same as the observed transition curve for Oku protein. The transition curve obtained for a mixture of the V_L and C_L fragment (molar ratio 1:1) was found to be the same as the calculated curve. V_L fragments are known to be in an equilibrium between the dimer and monomer (Cathou & Dorrington, 1975; Azuma et al., 1978; Maeda et al., 1976, 1978). In order to clarify whether the monomer–dimer equilibrium affects the unfolding equilibrium, we measured the unfolding equilibria of the V_L fragment at two protein concentrations, 0.2 and 0.04 mg/mL. The transition curves were found to be the same.

Unfolding Kinetics. Unfolding kinetics of light chain Oku and its V_L and C_L fragments were measured in terms of tryptophyl fluorescence. Figures 4–6 show the dependence on Gdn-HCl concentration of the kinetic parameters of C_L, V_L, and light chain Oku, respectively.

The unfolding kinetics of the C_L fragment inside the transition zone were described by two exponential decay terms:

$$F(t) - F(\infty) = F_1 \exp(-\lambda_1 t) + F_2 \exp(-\lambda_2 t) \quad (3)$$

where λ_1 and λ_2 are the apparent rate constants of the slow and fast phases, respectively, and F_1 and F_2 are the amplitudes of the respective phases. The amplitudes of the slow and fast phases relative to the total fluorescence change are described by α_1 and α_2, respectively, where $\alpha_1 + \alpha_2 = 1$. The relative amplitude varied greatly with Gdn-HCl concentration, and the total change in the fluorescence above 2.0 M Gdn-HCl was expressed by a single exponential term (Figure 4). These results are similar to those of the unfolding kinetics of the type λ C_L fragment (Goto & Hamaguchi, 1982).

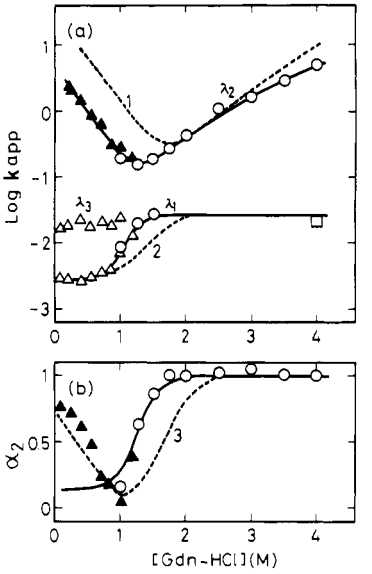

FIGURE 4: Dependence on Gdn-HCl concentration of (a) the apparent rate constants (λ_1, λ_2, and λ_3) and (b) the relative amplitude (α_2) of the fast phase for the unfolding and refolding kinetics of the C_L(Oku) fragment at pH 7.5 and 25 °C. (a) (○) From unfolding kinetics obtained by stopped-flow fluorescence measurements; (▲) from refolding kinetics obtained by stopped-flow fluorescence measurements; (△) from refolding kinetics measured by fluorescence at 350 nm with manual mixing; (□) the apparent rate constant of the isomerization process of U_2 to U_1 in mechanism 1 after unfolding measured by double-jump experiments. (b) Continuous line indicates the values of α_2 calculated from the equation $\alpha_2 = (1 + f_N K_{21})^{-1}$ (Hagerman & Baldwin, 1976), with $K_{21} = 6$ and the fraction of folded protein (f_N) obtained from equilibrium measurements. The values of α_2 for the unfolding (○) and refolding (▲) kinetics measured by fluorescence at 350 nm with manual mixing were obtained by assuming that the kinetics consist of the same phases as those observed by stopped-flow measurements and that the starting points for unfolding or refolding lie on a straight line extending the linear portion which corresponds to the unfolded or native state in the transition curve determined by fluorescence measurements at 350 nm. The dotted lines 1–3 are values of λ_2, λ_1, and α_2, respectively, for the C_L(Nag, λ) fragment cited from the previous paper (Goto & Hamaguchi, 1982).

The unfolding kinetics of the V_L fragment of Oku protein were described by a single exponential term with an apparent rate constant λ (Figure 5). The value of λ was smaller than the value of λ_2 for the C_L fragment when compared at the same concentration of Gdn-HCl, but with increasing Gdn-HCl concentration, the value of λ for the V_L fragment increased more sharply than the value of λ_2 for the C_L fragment.

For comparison, the unfolding kinetics of the V_L fragment of light chain Tod (type λ) were studied (Figure 5). Above 2.5 M Gdn-HCl, the kinetics were described by a single exponential term, and the values of the apparent rate constant above 3.5 M Gdn-HCl were similar to the values for the V_L(Oku) fragment.

The unfolding kinetics of light chain Oku above 1.5 M Gdn-HCl were described by two exponential terms (Figure 6). The amplitudes of the fast (α_2) and slow (α_1) phases of light chain Oku were almost the same (0.5 each) and were constant above 1.5 M Gdn-HCl. In Figure 6, the rate con-

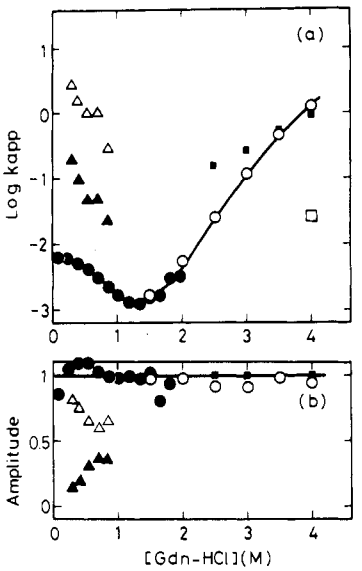

FIGURE 5: Dependence on Gdn-HCl concentration of (a) the apparent rate constant for the V_L(Oku) fragment (circles) and the apparent rate constant for the V_L(Tod) fragment (triangles and solid squares) and (b) the relative amplitude at pH 7.5 and 25 °C. (○) From unfolding kinetics of V_L(Oku) obtained by stopped-flow fluorescence measurements (at concentrations above 2.5 M Gdn-HCl) and from unfolding kinetics measured by fluorescence at 350 nm with manual mixing (1.5 and 2.0 M Gdn-HCl); (●) from refolding kinetics of V_L(Oku) measured by fluorescence at 350 nm with manual mixing; (■) from unfolding kinetics of V_L(Tod) obtained by stopped-flow fluorescence measurements; (△, ▲), from refolding kinetics of V_L(Tod) obtained by stopped-flow fluorescence measurements; (□) apparent rate constant obtained by double-jump experiments.

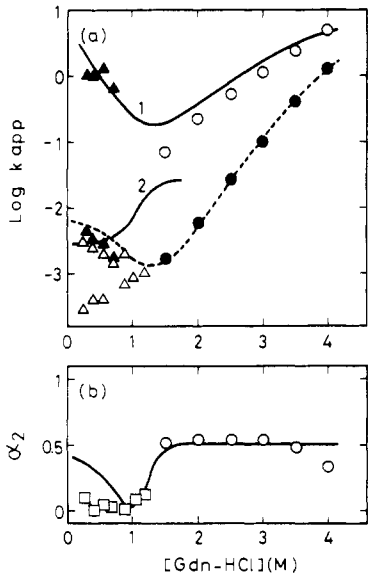

FIGURE 6: Dependence on Gdn-HCl concentration of (a) the apparent rate constants of unfolding and refolding and (b) the relative amplitude (α_2) of the fast phase for light chain Oku at pH 7.5 and 25 °C. (a) (○, ●) From unfolding kinetics obtained by stopped-flow fluorescence measurements; (△) from refolding kinetics measured by fluorescence at 350 nm with manual mixing; (▲) from refolding kinetics measured by stopped-flow fluorescence measurements. (b) (○) Amplitude of the fast phase of unfolding obtained by stopped-flow measurements; (□) amplitude of the fast phase of refolding. The solid lines 1 and 2 in (a) indicate the values of λ_2 and λ_1, respectively, of the C_L(Oku) fragment (see Figure 4). The dotted line indicates the apparent rate constant of the V_L(Oku) fragment (see Figure 5). The solid line in (b) indicates the curve for the amplitude of the fast phase of light chain Oku calculated by assuming that the V_L and C_L domains unfold and refold independently.

stants, λ_1 and λ_2, for the C_L fragment (Figure 4) and λ for the V_L fragment (Figure 5) are also shown. As can be seen, the apparent rate constants for the slow and fast phases of Oku protein agreed well with the rate constant for the V_L fragment and the rate constant for the C_L fragment, respectively. This shows that the unfolding kinetics of the light chain are explained with the unfolding kinetics of the isolated domains. The unfolding rate constant of the V_L fragment was not dependent on protein concentration in the concentration range of 0.04–0.2 mg/mL.

Refolding Kinetics. Figures 4–6 show the dependence on Gdn-HCl concentration of the kinetic parameters for refolding of C_L, V_L, and light chain Oku, respectively. The kinetic parameters of the slow refolding process initiated by raising the pH from the unfolded state in 0.4 M Gdn-HCl and at pH 1.9 were the same as those of refolding by dilution from the unfolded state in 4.0 M Gdn-HCl.

Refolding of the C_L fragment below 1 M Gdn-HCl was described by three exponential terms: a fast phase with an apparent rate constant λ_2 and relative amplitude α_2, a slow phase with an apparent rate constant λ_1 and relative amplitude α_1, and an additional slow phase with an apparent rate constant λ_3 and relative amplitude α_3 (Figure 4). At around 1.2 M Gdn-HCl, the values of λ_1, λ_2, α_1, and α_2 for the refolding kinetics agreed well with the respective values for the unfolding kinetics. The apparent rate constant (λ_2) and amplitude (α_2) of the fast phase of refolding increased sharply with decreasing concentration of Gdn-HCl. The apparent rate constant (λ_1) of the slow phase was nearly constant below 1 M Gdn-HCl, and its amplitude (α_1) decreased with decreasing concentration of Gdn-HCl. The apparent rate constant of the additional slow phase was about 7 times larger than that of the slow phase, and its amplitude was small (0.05–0.15) and not reproducible. The dependence on Gdn-HCl concentration of the kinetic parameters of refolding of the C_L fragment of Oku protein was similar to that for the C_L fragment of Nag protein (type λ) (Goto & Hamaguchi, 1982), but the apparent rate constant (λ_2) of the fast phase for the type κ C_L fragment was 7–10 times smaller than that for type λ C_L fragment.

Refolding kinetics of the V_L fragment of Oku protein were described by a single exponential term (Figure 5). The apparent rate constant of refolding agreed well with that of unfolding at around 1.8 M Gdn-HCl. The apparent rate constant of refolding increased with decreasing concentration of Gdn-HCl. The refolding rate constant was not dependent on protein concentration in the concentration range of 0.014–0.177 mg/mL.

The kinetics of refolding of the V_L fragment of Tod protein (type λ) were described by two exponential terms (Figure 5). The rate constants were both greater than the rate constant of refolding of the V_L(Oku) fragment.

The kinetics of refolding of light chain Oku were described by three exponential terms (Figure 6): a fast phase with a relative amplitude of about 5%, a slow phase with a relative amplitude of about 80%, and an additional slow phase with a relative amplitude of about 15%. The apparent refolding rate constant of the fast phase of the light chain was similar to that of the fast phase of the C_L fragment, and the apparent rate constant of the slow phase of the light chain was similar to that of the V_L(Oku) fragment or that of the slow phase of the C_L fragment. The apparent rate constant of the additional slow phase was 5–10 times smaller than that of the slow phase. The amplitude of the fast phase was much smaller than that of the fast phase expected from the independent refolding of the V_L and C_L domains (the solid line in Figure 6b).

The immunoglobulin light chains form a dimer by interactions between the V_L domains. In order to clarify the effect of the monomer–dimer equilibrium on the refolding kinetics, we measured the refolding of light chain Oku at two different protein concentrations (0.22 and 0.01 mg/mL). However, we found no significant difference in the refolding kinetics.

Double-Jump Experiment. We performed double-jump experiments (Brandts et al., 1975; Nall et al., 1978) in order to determine whether two forms of the unfolded protein exist in the unfolded state. The C_L fragment was first unfolded by 4 M Gdn-HCl, and then, refolding was initiated at 0.6 M Gdn-HCl after various intervals under the unfolding conditions. If there is a slow equilibrium in the unfolded state ($U_2 \rightleftharpoons U_1$), the refolding kinetics should depend on the time elapsed in the unfolded condition. We measured the amplitudes of the slow phases (α_1) under the refolding conditions after various times of exposure of the V_L and C_L fragments to 4 M Gdn-HCl. The dependence was expressed by one exponential decay process, and the apparent rate constant was 0.021 s^{-1} for the C_L fragment and 0.025 s^{-1} for the V_L fragment.

Discussion

Conformations and Stabilities of Light Chain Oku and Its V_L and C_L Fragments. As shown in Figure 1, the CD spectrum of light chain Oku is similar to the spectrum calculated with the spectra of the constituent V_L and C_L fragments. The unfolding curve by Gdn-HCl of light chain Oku is almost the same as the curve calculated by use of the unfolding curves for the V_L and C_L fragments and by assuming the independent unfolding of the two domains (Figure 3). These results indicate that the folding of the V_L and C_L domains in the light chain molecule is almost independent. On the basis of the results of spectral and unfolding experiments, the independent folding of the domains has been shown for other light chains (Ghose & Jirgensons, 1971; Björk et al., 1971; Karlsson et al., 1972; Rowe & Tanford, 1973; Goto et al., 1979) and for the Fc fragment of an immunoglobulin G (Sumi & Hamaguchi, 1982).

The unfolding curves produced with Gdn-HCl of the V_L and C_L fragments were analyzed by assuming the two-state approximation, and the free-energy changes of unfolding in the absence of Gdn-HCl, $\Delta G_D^{H_2O}$, were estimated. Table II shows the values for the C_L and V_L fragments of light chain Oku and other immunoglobulin domains. The $C_L(\kappa,Oku)$ fragment is more unstable than the $C_L(\lambda,Nag)$ and $V_L(\kappa,Oku)$ fragments by 0.6 and 1 kcal/mol, respectively. Although there are some differences in the free-energy change of unfolding, each domain of the immunoglobulin molecule has a similar stability of about 5–6 kcal/mol. The V_L fragments are in an equilibrium between the dimer and monomer. The faster elution position of the V_L fragment compared with the C_L fragment on gel filtration (see Materials and Methods) is due to the dimer formation of the V_L fragment, which may contribute to the higher stability of the V_L fragment in comparison with the C_L fragment.

Folding Kinetics of the C_L Fragment. Previously we reported that the kinetics of the unfolding and refolding of a type λ C_L fragment can be described almost entirely by two exponential terms, although there is a third phase with a small amplitude below the transition zone (Goto & Hamaguchi, 1982). The unfolding and refolding kinetics of the type λ C_L fragment at Gdn-HCl concentrations above 1.0 M could be explained on the basis of a three-species mechanism (mechanism 1), which was first proposed for the unfolding and refolding kinetics of ribonuclease A (Garel & Baldwin, 1975; Brandts et al., 1975):

Table II: Transition Midpoints and Free-Energy Changes of Unfolding by Gdn-HCl of Immunoglobulin Domains at pH 7.5 and 25 °C[a]

protein	C_m (M)	$\Delta G_D^{H_2O}$ (kcal/mol)	Δn	ref
C_L(Oku, κ)	0.9	5.2 ± 0.2	32.6 ± 0.9	this study
V_L(Oku, κ)	1.3	6.3 ± 0.2	32.3 ± 1.2	this study
C_L(Nag, λ)	1.2	5.7	29	Goto & Hamaguchi, 1979
Fc(t)	2.0	5.7	22.5	Sumi & Hamaguchi, 1982
pFc′	2.0	5.7	20.4	Sumi & Hamaguchi, 1982
light chain (κ)	1.15	5.5		Rowe & Tanford, 1973

[a] C_m is the concentration of Gdn-HCl at the midpoint of the unfolding transition. The transition curves were analyzed by assuming the two-state approximation N (native) $\rightleftharpoons$ D (unfolded). The equilibrium constant of unfolding (K_D) was determined by $K_D = f_D/(1 - f_D)$ at each concentration of Gdn-HCl. $\Delta G_D^{H_2O}$ is the free-energy change of unfolding in the absence of Gdn-HCl and was obtained by the equation proposed by Tanford (1970): $\Delta G_D = \Delta G_D^{H_2O} - \Delta nRT \ln (1 + ka_*)$, where Δn is the difference in the number of binding sites between unfolded and folded states, k is the average binding constant of the sites, and a_* is the mean activity of Gdn-HCl. We used 0.6 M^{-1} as the value of k [Pace & Vanderburg, 1979; see Goto and Hamaguchi (1979)].

mechanism 1

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

where N is native protein, U_1 and U_2 are the slow-folding and fast-folding species, respectively, of unfolded protein, and k_{12}, k_{21}, k_{23}, and k_{32} are the microscopic rate constants for the respective processes. U_1 and U_2 are indistinguishable from one another on the basis of CD and fluorescence properties. The equilibrium between U_1 and U_2 was independent of the concentration of Gdn-HCl, and the value of $K_{21}(=k_{21}/k_{12})$ was 10. When the refolding kinetics follow mechanism 1, the relative amplitude of the fast phase (α_2) should be constant below the transition zone. The value of α_2 increased sharply, however, with decreasing concentration of Gdn-HCl. This was well explained on the basis of mechanism 2 in which an alternative pathway of refolding of U_1 is involved:

mechanism 2

$$\begin{array}{ccc} U_1 & \overset{\text{slow}}{\rightleftharpoons} & U_2 \\ {\scriptstyle \text{fast}}\uparrow\downarrow & & \downarrow\uparrow{\scriptstyle \text{fast}} \\ I & \underset{\text{slow}}{\longrightarrow} & N \end{array}$$

where I represents an intermediate. We found previously that the intermediate accumulates substantially under refolding conditions and the intermediate has a compact structure with a native-like structure (Goto & Hamaguchi, 1982).

As shown in Figure 4, the dependence on Gdn-HCl concentration of the kinetic parameters (λ_1, λ_2, and α_2) for the type κ C_L fragment is similar to that for the type λ C_L fragment. The double-jump experiments showed that fast-folding and slow-folding species exist in the unfolded state of the C_L fragment of light chain Oku. These findings indicate that the unfolding and refolding of the type κ C_L fragment can also be explained by mechanism 1 or 2.

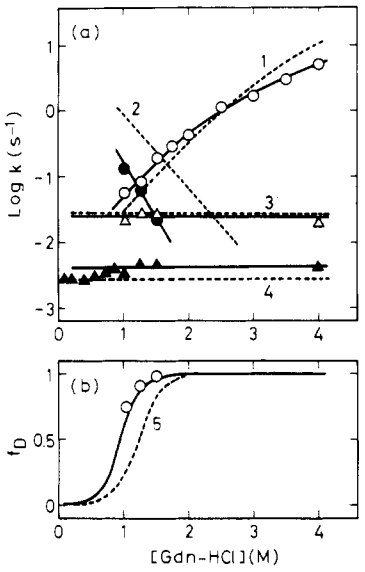

FIGURE 7: Dependence on Gdn-HCl concentration of the microscopic rate constants estimated on the basis of mechanism 1 for folding of the C_L(Oku) fragment (a) and the transition curve for the unfolding calculated with the microscopic rate constants (b): pH 7.5, 25 °C. (○) k_{32}; (●) k_{23}; (△) k_{21}; (▲) k_{12}. The continuous line in (b) indicates the transition curve for unfolding obtained from equilibrium measurements. The dotted curves 1–5 represent the values of k_{32}, k_{23}, k_{21}, and k_{12} and the transition curve for the C_L(Nag) fragment cited from the previous paper (Goto & Hamaguchi, 1982).

The microscopic rate constants in mechanism 1 were determined as follows. The value of $k_{12} + k_{21}$ in 4 M Gdn-HCl was determined to be 0.021 s^{-1} from the double-jump experiments. The value of K_{21} ($=k_{21}/k_{12}$) was estimated to be 6 by assuming that the apparent rate constant (λ_1) of the slow phase of refolding below the transition zone corresponds to k_{12} and that the value of K_{21} is constant over the concentration range of Gdn-HCl studied. The values of k_{12}, k_{21}, k_{23}, and k_{32} were then estimated from the experimentally determined values of λ_1, λ_2, and α_2 (Figure 4) (Hagerman & Baldwin, 1976). Figure 7 shows the microscopic rate constants thus obtained as a function of the concentration of Gdn-HCl. As can be seen, the rate constants for the interconversion between U_1 and U_2 below 1.5 M Gdn-HCl are essentially constant and agree with the value obtained by the double-jump experiment with 4 M Gdn-HCl. The unfolding rate constant (k_{32}) increases, and the refolding rate constant (k_{23}) decreases sharply with increasing concentration of Gdn-HCl.

Figure 7 also shows the microscopic rate constants for the folding reaction of the type λ C_L fragment reported previously (Goto & Hamaguchi, 1982). The dependence on the Gdn-HCl concentration of the unfolding rate constant (k_{32}) for the type κ C_L fragment is the same as that for the type λ C_L fragment. On the other hand, the refolding rate constants (k_{23}) for the type λ C_L fragment are 7–10 times greater than those for the type κ C_L fragment. Thus, the high stability of the type λ C_L fragment compared with the type κ C_L fragment must be due largely to the greater refolding rate constant of the type λ C_L fragment. Thus, the high stability of the type λ C_L fragment compared with the type κ C_L fragment must be due largely to the greater refolding rate constant of the type λ C_L fragment.

The interconversion between the two forms (U_1 and U_2 in mechanism 1) in the unfolded state has been interpreted in terms of the cis–trans isomerization of the prolyl residues (Brandts et al., 1975). The type λ C_L domain has nine prolyl residues, one of which (Pro-143) is in the cis configuration in the native conformation (Marquart et al., 1980). From comparison of the refolding kinetics of several immunoglobulin domains, it was found that slow refolding with a time constant of about 300 s was observed only for the domains with cis proline at the position corresponding to Pro-143 of the type λ C_L domain, and we suggested that the slow phase of refolding of the C_L fragment is due to the cis–trans isomerization of Pro-143 (Goto & Hamaguchi, 1982). The type κ C_L domain has five proline residues, one of which (Pro-141) is located at the position corresponding to Pro-143 in the type λ C_L domain. As shown in Figure 7, the microscopic rate constants of the interconversion between U_1 and U_2 for the type κ C_L fragment are similar to those for the type λ C_L domain. Although the cis–trans isomerism of Pro-141 of the type κ C_L domain is unknown, the present findings strongly suggest that Pro-141 is in the cis configuration in the native state and that it isomerizes to trans in the unfolded state to produce the slow refolding species.

Folding Kinetics of the V_L Fragment. Contrary to the C_L fragment, the unfolding and refolding kinetics of the V_L fragment of Oku protein were described by a single exponential term at all concentrations of Gdn-HCl studied (Figure 5). The apparent rate constant was minimal at 1.3 M Gdn-HCl, which corresponds to the midpoint of the unfolding transition. These findings would be expected if the unfolding and refolding kinetics followed the two-state transition. The double-jump experiments (not shown) demonstrated, however, that two species of the unfolded molecule exist in the unfolded state of the type κ V_L fragment. This indicates that the folding kinetics of the V_L fragment cannot be explained simply by the two-species mechanism and that a more complex mechanism is needed.

Previously, we reported that the refolding of the V_L fragments of type λ Tod and Fu proteins is fast (Goto et al., 1979). As shown in Figure 5, the kinetics of refolding of the V_L(Tod) fragment consist of two phases, and both rate constants are greater than the rate constant of refolding of the V_L(Oku) fragment. It is not clear whether the difference in the refolding kinetics between V_L(Tod) and V_L(Oku) is due to the difference between type λ and type κ or simply to the difference between the species.

Folding Kinetics of Light Chain Oku. The unfolding kinetics of light chain Oku were explained in terms of the independent unfolding of the V_L and C_L domains. The unfolding kinetics of light chain Oku above 2 M Gdn-HCl consist of two phases, and the rate constants for the fast and slow phases correspond to the unfolding rate constants for the C_L and V_L fragments, respectively. Sumi and Hamaguchi (1982) revealed the independent unfolding kinetics of the C_H2 and C_H3 domains in the Fc fragment of an immunoglobulin G. At high concentrations of Gdn-HCl, the interactions between the V_L and C_L domains, which might be important under physiological conditions, must be destroyed first, and the two domains unfold, as do the isolated fragments.

On the other hand, the refolding kinetics of light chain Oku were not explained simply in terms of the refolding kinetics of the constituent V_L and C_L fragments. As described above, although the two apparent rate constants of refolding of the light chain were similar to the rate constants for the C_L and V_L fragments, the relative amplitudes were quite different from those for the C_L and V_L fragments, and the amplitude for the fast phase of the light chain was much smaller than that expected from the refolding kinetics of the V_L and C_L fragments. Furthermore, an additional phase with a slower rate constant appeared in the refolding of the light chain. Therefore, the apparent refolding reaction of the light chain

was much slower than that of either the V_L or C_L fragment. In the in vitro refolding of the light chain from the unfolded state, both the V_L and C_L domains start to refold at the same time. The two domains are linked by the switch peptide, and they interact with each other as an intermediate formed during the refolding process. Such interdomain interactions during in vitro refolding may also have an adverse influence on the effective refolding of other large proteins with multidomain structures. The work of Bergman and Kuehl (1979a,b) has shown that the intrachain disulfide bond of the V_L domain of MPC 11 light chain is formed before the completion of the primary structure of the light chain. This strongly suggests that in the folding of immunoglobulin chains in vivo the domain synthesized first must fold before the next domain is completely synthesized.

The domain has been assumed to divide the folding unit and to accelerate the folding. However, folding of large proteins with multidomain structures has been observed to be sometimes irreversible (Goldberg, 1985). Such irreversible unfolding has been interpreted in terms of intermolecular, rather than intramolecular, interactions between the regions of an intermediate formed during refolding. The intermolecular interactions inhibit the folding or proceed to the formation of irreversible aggregates (Zettlmeissl et al., 1979; Jaenicke & Rudolph, 1986). The present results indicate that not only the intermolecular interactions but also the interdomain interactions within the molecule affect the folding rate of each domain and hence the whole molecule. The effect of the interdomain interactions on the folding has also been suggested by Zettlmeissl et al. (1979).

Registry No. Guanidine monohydrochloride, 50-01-1.

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