Folding Pathways of Immunoglobulin Domains. The Folding Kinetics of the $C_\gamma 3$ Domain of Human IgG1†

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ABSTRACT: The in vitro folding kinetics of a fragment corresponding to an intact dimer of the $C_\gamma 3$ domain of human IgG1 (pFc′) were monitored via the large changes in tryptophan fluorescence which accompany these processes. In going from the guanidine hydrochloride (Gdn·HCl) induced unfolded state (4.0 M Gdn·HCl) to the native state (0.5 M Gdn·HCl), three well-separated first-order processes were observed having time constants of 5, 50, and 350 s and roughly equal amplitudes. These values were concentration independent, a fact consistent with there being no fluorescence change accompanying dimerization. These time constants are one to two orders of magnitude slower than those observed for proteins of similar size such as ribonuclease or cytochrome *c*, most probably reflecting the complex processes involved in forming the correct β-sheet arrangement of immunoglobulin domains. The corresponding unfolding transition is biphasic having time constant values of 50 and 500 s, the latter comprising 80% of the fluorescence change. These data indicate the presence of at least one species with intermediate fluorescence along the unfolding pathway. Gdn·HCl concentration jumps were also performed over various intervals within the transition zone. The results are not consistent with a fully reversible mechanism. In the absence of the intrachain disulfide bond, pFc′ exists in an unfolded state even at 0.5 M Gdn·HCl. In a concomitant refolding and reoxidation experiment (at 0.5 M Gdn·HCl and using an optimal disulfide interchange catalytic system), the time constant for disulfide formation was in the range of 80–200 s and the fluorescence change revealed a lag phase analyzable in terms of rate-limiting reoxidation and refolding times consistent with those observed for the initially disulfide bonded species. Under similar conditions but at 4 M Gdn·HCl, reoxidation was more than two orders of magnitude slower, suggesting that reoxidation is directed by a refolding nucleation event.

The kinetics and mechanism of protein folding have been the subject of considerable experimental and theoretical investigation for a number of years (see reviews by Tanford, 1970; Anfinsen & Scheraga, 1975; Baldwin, 1975). Despite this, the molecular details of this crucial biological process are not yet well understood. In general, equilibrium studies of protein folding have failed to detect the existence of stable intermediates; however, more recent kinetic studies provide ample evidence for the participation of one or more kinetic intermediates in the folding process (see Baldwin, 1975; Tsong, 1976; Hagerman & Baldwin, 1976, and references therein). The intrachain disulfide bond is a common, although not universal, structural feature of proteins. While the conformational integrity of some proteins is absolutely dependent on the presence of intact intrachain disulfides (White, 1961; Harrington & Sela, 1959), other proteins retain a large portion of their native structure (as judged by chiroptical criteria) when these bonds are cleaved (Yutani et al., 1968; Saxena & Wetlaufer, 1970).

Immunoglobulin chains are folded into a linear series of compact domains, each domain corresponding to one of the homology regions apparent in the primary structure (Edelman & Gall, 1969; Cathou & Dorrington, 1975). The absolute conservation and periodic arrangement of the two half-cystines constituting the single intrachain disulfide bond in each homology region of the immunoglobulin molecule suggested an important role for this structural element in maintaining the native conformation of these molecules. Previous work on isolated immunoglobulin domains and on β_2-microglobulin, which resembles an individual immunoglobulin domain (Peterson et al., 1972), has demonstrated that, in the absence of an intact intrachain disulfide, these proteins exist in a largely unfolded state (Isenman, 1976; Isenman et al., 1975) as judged by chiroptical criteria. In examining the X-ray structures of immunoglobulin domains, a crucial role for the intrachain disulfide in maintaining the overall conformation is not readily apparent. Thus, it has been suggested that the importance of this bridge in immunoglobulins relates to its role in providing for a kinetically accessible pathway for domain folding (Isenman et al., 1975). It has further been proposed that an early nucleation event in the folding process would bring the two one-dimensionally distant SH groups into close apposition, thereby facilitating their oxidation. The formation of the disulfide bond would stabilize the putative nucleus and folding of the chain could then continue, presumably passing through several intermediate states on the way to the native structure. The formation of nuclei (Wetlaufer, 1973) within each of these homology regions was indeed proposed as a mechanism for limiting the overall time required to attain the native structure of a polypeptide having the size and complexity of an immunoglobulin chain (Isenman et al., 1975).

In the present paper, we examined the kinetic features of the in vitro folding processes of a single immunoglobulin domain. This initial study was performed on the peptic Fc′ fragment (pFc′)[1] which corresponds to an intact noncovalent dimer of the $C_\gamma 3$ domain of human IgG1. One must in general exert caution in extrapolating the conclusions reached about the folding processes in a cleaved fragment to those processes which might occur in the intact chain as, in some cases, the

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[1] Abreviations used: pFc′, peptic fragment corresponding to the $C_\gamma 3$ domain of immunoglobulin G; ACD, acid citrate dextrose; TBS, Tris-buffered saline; Gdn·HCl (Gu·HCl in figures), guanidine hydrochloride; DTT, dithiothreitol; NATA, *N*-acetyl-L-trytophanamide; GSSG, oxidized glutathione; GSH, reduced glutathione; τ, the time constant (reciprocal of the apparent rate constant) of a chemical reaction or kinetic phase; BPTI, bovine pancreatic trypsin inhibitor.

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proteolytic fragment has a structure which differs significantly from its conformation in the native chain. In the case of pFc', however, it has recently been shown that the three-dimensional structure of guinea pig IgG1 pFc' retains the same general features of $C_\gamma 3$ structure observed in intact human Fc (Johns Hopkins University crystallography group; P. Phizackerley, personal communication). This is an important observation since it demonstrates that the overall conformation of the $C_\gamma 3$ region is not altered by removing an adjacent domain, thereby further justifying the use of an isolated immunoglobulin domain as a model for protein folding in immunoglobulin chains. The in vitro folding kinetics of this fragment were monitored via the large changes in tryptophan fluorescence which accompany these processes.

Materials and Methods

Proteins. Human IgG1 was isolated from ACD-treated myeloma plasma by chromatography on QAE-Sephadex A-50 (Pharmacia) by the method of Joustra & Lundgren (1969). The peptic Fc' fragment (pFc') was made by incubating with pepsin (Worthington Biochemicals Corp.) at an enzyme to substrate ratio of 1:100 (w/w) for 18 h at 37 °C and pH 4.5 (Turner & Bennich, 1968). The digestion was terminated by raising the pH to 9.0, and the mixture was subjected to gel filtration on a column of Sephadex G-100 superfine (3.0 × 90 cm) equilibrated in 0.01 M Tris-HCl, 0.15 M NaCl, pH 8.2, containing 2 mM EDTA (TBS–EDTA). Under these conditions, the pFc' fragment eluted as a well-resolved peak requiring no further purification. Homogeneity was assessed by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ and by immunochemical methods. Fc fragment was prepared from IgG1 by trypsin digestion as previously described (Ellerson et al., 1976). Radiolabeling of Fc or pFc' with ^{125}I was accomplished by using the lactoperoxidase procedure of Marchalonis (1969). Protein concentrations were determined spectrophotometrically by using as $E_{280}^{1\%}$ the value of 15.0 for pFc'.

Acrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis in 11.2% polyacrylamide gels was performed according to the method of Fairbanks et al. (1971). Electrophoresis in acid–polyacrylamide gels (7%) in the presence of 6 M urea was performed by using the procedure of Panyim & Chalkley (1969).

Static Fluorescence Measurements. Fluorescence emission spectra were recorded on a Perkin-Elmer MP44A spectrofluorometer equipped with a thermostated cell compartment at 23 ± 0.5 °C. Excitation was at 280 nm (band width 3 nm), and the emission spectra were recorded between 290 and 460 nm (collection band width 10 nm). All spectra were recorded in the ratio mode in order to correct for variations in light source intensity. The nominal absorbance of the protein at 280 nm ranged between 0.01 and 0.1.

Determination of the Equilibrium Denaturation Curve. The equilibrium in the system was determined by measurement of fluorescence intensity at 350 nm as a function of guanidine hydrochloride (Gdn·HCl) concentration. Solutions were prepared 18 h prior to fluorescence measurements by diluting into TBS–EDTA, pH 8.2, appropriate amounts of protein stock solution and Gdn·HCl (Schwarz/Mann, Ultrapure) stock solutions of known concentrations. Reversibility was assessed by diluting protein stock solutions inititally at higher Gdn·HCl concentrations to lower ones followed by an overnight equilibration prior to fluorescence measurements.

Kinetic Fluorescence Measurements. The unfolding or refolding of pFc' was initiated by dilution of the protein equilibrated at the initial Gdn·HCl concentration into the final desired concentration of denaturant. The fluorescence changes accompanying these processes were monitored at the wavelength of maximal change in the MP44A fluorometer where the cell compartment had been modified so as to permit injection of the sample into a cuvette containing the diluent. Mixing was achieved by means of a motor-driven glass propeller capable of fully mixing the contents within 1 s. Following injection and mixing, the stirrer was stopped in order to reduce noise due to bubble formation. The response time of the instrument was 0.3 s. The temperature for all kinetic measurements was 23 °C. Unfolding or refolding curves were converted to digital values with a Biomation Model 802 transient recorder equipped with an external variable time base for slow sampling. In experiments displaying multiphasic kinetics, data were collected at two or more different sampling rates within the same run so as to assure a sufficient number of data points in each range. The data were analyzed by fitting the curves to the theoretical model by using a nonlinear least-squares program based on the Marquardt (1963) or Powell (1971) algorithms. In most analyses, time constants, their respective amplitudes, and the infinite time fluorescence intensity value were taken as free parameters. In those cases where extremely slow kinetics were observed, the value of the infinite time fluorescence intensity was fixed at the value determined from the equilibrium denaturation curve (Figure 2). In this way, the effect of machine drift on the calculated values of the time constants and amplitudes for very slow transitions was minimized. Computations were performed on the IBM 370 facility of the Weizmann Institute Computing Centre.

Reduction and Reoxidation Procedures. The reduction of the intrachain disulfide bond of pFc' was performed with dithiothreitol (DTT) in the presence of 4 M Gdn·HCl. We have found that removing the reducing agent by dialysis always resulted in significant reoxidation taking place despite attempts to maintain an anaerobic environment. Gel filtration desalting procedures, while considerably more rapid, resulted in an overdilution of the protein. During the subsequent concentration procedure, the protein would often partially reoxidize. In order to circumvent these problems, a procedure was adopted in which the DTT concentration was lowered prior to reoxidation by rapid dilution. Accordingly, a small volume of protein of relatively high concentration was reduced with minimal amounts of DTT. Typically a solution containing 4×10^{-4} M disulfide was quantitatively reduced by 10^{-3} M DTT within 1 h. The completeness of reduction under these conditions was assessed by electrophoretic analysis in acid–urea polyacrylamide gels (Panyim & Chalkley, 1969). In this technique, advantage is taken of the property that the electrophoretic mobility of a reduced domain is retarded relative to the corresponding unreduced domain (D. E. Isenman, K. J. Dorrington, and R. H. Painter, unpublished observations), presumably reflecting conformational differences assumed by these two species in the acid–urea medium. Reoxidation was initiated by diluting the solution 100 times into a suitable reoxidation buffer made 0.5 M in Gdn·HCl. This nondenaturing concentration of Gdn·HCl was required in order to keep the reduced protein in solution. Disulfide bond formation was facilitated by a disulfide interchange system where mixtures of oxidized and reduced glutathione act as catalysts (Saxena & Wetlaufer, 1970; Hantgan et al., 1974). Alkylation of the remaining reduced protein at any stage of the oxidation procedure was achieved by the addition of iodoacetamide to a final concentration of 0.1 M. This high concentration of iodoacetamide assured fast alkylation.

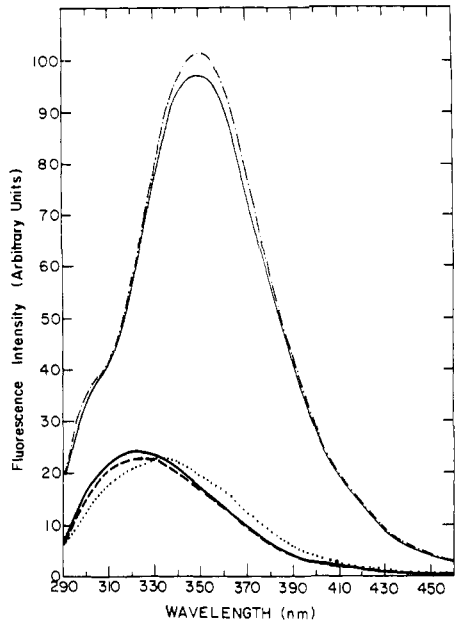


FIGURE 1: Fluorescence emission spectra of the native, denatured, and renatured forms of pFc'. Excitation in all cases was at 280 nm. Symbol key: disulfide intact pFc' in 0.5 M Gdn·HCl (—), in 4.0 M Gdn·HCl (–·–), and in 0.5 M Gdn·HCl after an initial equilibrium at 4.0 M (---). Reduced and alkylated pFc' in 0.5 M Gdn·HCl (—). Initially reduced pFc', reoxidized in the presence of 1 mM GSSG, 1 mM GSH, in TBS–EDTA, pH 8.2, containing 0.5 M Gdn·HCl (···).

Results

Equilibrium Fluorescence Spectra. The fluorescence emission spectra of pFc' under a number of solvent conditions are shown in Figure 1. In 0.5 M Gdn·HCl the protein displays a typical tryptophan emission profile with no evidence of a significant tyrosine contribution. The fluorescence intensity of pFc' relative to an equimolar concentration of *N*-acetyltryptophanamide (at their respective emission maxima) is 20%. The wavelength of maximum emission for pFc' is 325 nm, a value slightly lower than the range reported for most proteins (328–340) (Teale, 1960). This spectrum is indistinguishable from that obtained at zero Gdn·HCl concentration (not shown, but see also Figure 2). Unfolding of the protein by 4 M Gdn·HCl resulted in a 4.2-fold increase in fluorescence intensity and a red shift of 25 nm in the emission maximum. In addition, there was a shoulder at 305 nm presumably arising from a tyrosine contribution to the emission spectrum of the denatured protein. It has previously been reported that protein denaturation significantly increases the yield of fluorescence from tyrosine (Teale, 1960). At the λ_{max} of the unfolded protein (350 nm), the fluorescence intensity is 5.9 times greater than it is for the native protein at the same wavelength. The fluorescence intensity is 88% that of *N*-acetyltryptophanamide (NATA) and the wavelength of maximal emision is coincident with that of this model compound. Thus at this wavelength, the internalization of tryptophan residues accompanying protein folding provides a large signal change through which this process may be followed. Reduction and alkylation of the protein's intrachain disulfide in the presence of 4 M Gdn·HCl did not alter the emission spectrum from that observed for the disulfide-bonded species in the same solvent. Lowering of the Gdn·HCl concentration from 4.0 M to 0.5 M in the disulfide bonded species resulted in the restoration of the native spectrum (Figure 1). In contrast, removal of the Gdn·HCl from the reduced and alkylated species resulted in a spectrum which was not significantly different from that observed in 4.0 M Gdn·HCl. These observations are consistent with previous

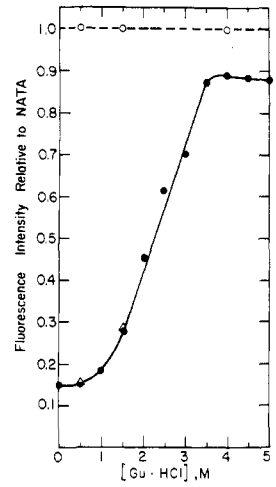


FIGURE 2: Equilibrium measurements of the fluorescence intensity at 350 nm of disulfide intact pFc' as a function of Gdn·HCl concentration. Points obtained in the unfolding direction starting from the native protein are represented by ●. The open triangles (Δ) shown at 0.5 M and 1.5 M Gdn·HCl represent renaturation points where the protein was initially at 4.0 M and 3.0 M Gdn·HCl, respectively. The fluorescence intensity at 350 nm of NATA at a concentration equal to that of the protein's tryptophan content is also shown as a function of Gdn·HCl concentration (○).

data obtained for this domain by using circular dichroism to monitor the conformation of the protein (Isenman, 1976). Reoxidation of reduced, but not alkylated, pFc' (catalyzed by 1 mM GSSG, 1 mM GSH in the presence of 0.5 M Gdn·HCl at pH 8.2, 24 h) resulted in an emission spectrum which closely resembled that of the native protein. This finding is corroborated by a restoration of the spectral bands in both the near- and far-ultraviolet regions of the reoxidized proteins circular dichroism spectrum. Slight deviations from the spectrum of the original native material which we observed in both techniques probably reflect a contribution from a small amount of material which has not reoxidized.

Equilibrium Denaturation Curve. The shift in equilibrium between native and denatured states was measured via the change in fluorescence intensity at 350 nm as a function of Gdn·HCl concentration (Figure 2). No significant change occurs below 1.0 M Gdn·HCl. Between 1.0 M and 3.5 M Gdn·HCl, the protein underwent what appeared to be a cooperative conformational transition, the midpoint being near 2.5 M. Figure 2 also illustrates the reversibility of the system in terms of fluorescence changes; the fluorescence of samples initially at 4.0 M and 3.0 M Gdn·HCl fall on the transition curve when the denaturant concentration is lowered to 0.5 M and 1.5 M, respectively. This observation does not rule out the possible kinetic irreversibility of the system (see below). The fluorescence intensity of NATA at the same concentration as the protein's tryptophan content is also shown as a function of Gdn·HCl concentration. Clearly the quantum yield of tryptophan fluorescence in the unfolded protein approaches that of free tryptophan in aqueous solution.

The Kinetics of Protein Folding and Unfolding with the Intrachain Disulfide Initially Intact. Since the prime objective of this study was to investigate the overall folding processes of pFc' in going from the completely unfolded state to the native state, a considerable effort was invested in determining the rates of the transition from 4.0 M to 0.5 M Gdn·HCl as precisely as possible. However, in order to mechanistically interpret these experiments it was also necessary to measure the rates of both the folding and unfolding processes at several points within the transition zone, as well as at points which begin or end outside of this zone (Hagerman, 1977). All these

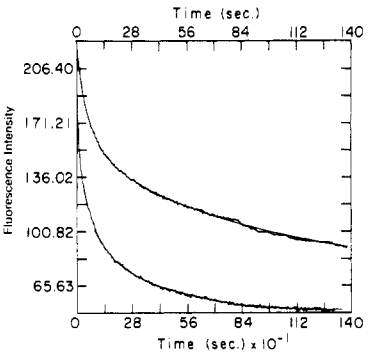


FIGURE 3: Kinetics of the 4.0–0.5 M Gdn·HCl transition for disulfide intact pFc′. The upper curve (and upper time scale) represents an expansion of the first 10% of the recorded data. The lower curve (and lower time scale) shows the complete data set. The fluorescence intensity scale is in Biomation units. The noisy curve depicts the raw data, while the smooth curve is calculated by using the fit parameters derived from a sum of exponents analysis of the data. The sampling interval was 0.2 s for the first 80 s and 2.0 s for the remainder of the reaction. The protein concentration was 1.5×10^{-5} M, the reaction temperature was 23 °C, the wavelength of excitation was 280 nm, and the wavelength of emission monitored was 350 nm.

Table I: Time Constants and Amplitudes for Complete Refolding Experiments[a]

Gdn·HCl transition (M)	protein concn (M)[b]	τ_1 (s)	A_1	τ_2 (s)	A_2	τ_3 (s)	A_3
4.0 → 0.5	1.53×10^{-5}	5.5[c]	0.33	52	0.35	346	0.32
4.0 → 0.5	3.06×10^{-6}	4.6	0.24	39	0.32	311	0.44
4.0 → 0.5	6.12×10^{-7}	ND	ND	46	(0.47)	265	(0.53)
3.0 → 0.5	2.6×10^{-6}	7.3	0.32	43	0.32	232	0.36

[a] Temperature, 25 °C, pH 8.2 TBS-EDTA buffer. [b] Protein concentration is calculated as mol/L of monomer chain. [c] The error in the kinetic parameters here, as well as in subsequent fluoresence-detected refolding and unfolding experiments, is estimated to be 10–20%, as judged from the variance in replicate experiments. ND, not determined; see text.

processes were monitored fluorometrically at 350 nm.

Shown in Figure 3 is a representative folding curve for the transition from 4.0 M to 0.5 M Gdn·HCl. Analysis of this curve in terms of a sum of exponentials revealed three well-separated first-order processes having time constants (τ) of about 5, 50, and 350 s and roughly equal amplitudes (see Table I). Attempts to fit the same data to two exponents resulted in a poor fit, the residual sum of squares being six times larger than for the three exponent fit.[2] These values were determined at a protein concentration of 1.53×10^{-5} M calculated on the basis of monomer chain. Since we found that, on a calibrated Sephadex G-50 column, in 0.5 M Gdn·HCl ^{125}I-labeled pFc′ chromatographs as a dimer even at a concentration as low as 10^{-10} M, it was necessary to examine whether second-order dimerization kinetics were superimposed upon the first-order folding processes. If a second-order process was involved, one would expect to see a concentration dependence in the folding kinetics. As can be seen in Table I, a 25-fold decrease in protein concentration

[2] In general, the improvement in the goodness of fit with increasing number of exponents tends to converge, so that fits to n and $n + 1$ exponents will be equally good. We therefore adhered to the following criteria. First, there must be a significant improvement in "goodness of the fit" as reflected by a factor of 2 or more difference in the residual sum of squares, before choosing the fit to $n + 1$ exponents as the correct one. Second, the principle of separability must hold; that is, the observed τ values in the fit to the larger number of exponents should be separated by a minimum of a factor of four before this curve is chosen as being the correct one.

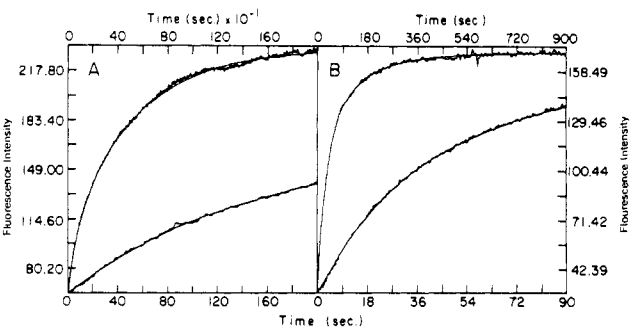


FIGURE 4: Kinetics of the complete unfolding transitions for disulfide intact pFc′ at 23 °C. (A) The 0.5–4.0 M Gdn·HCl transition; (B) 0.5–5.0 M Gdn·HCl transition. The lower curves represent expansions of the first 10% of the collected data. In all other respects, the collection and presentation of data are similar to those described in Figure 3.

Table II: Time Constants and Amplitudes for Experiments of Unfolding into Guanidine Concentrations above the Transition Zone[a]

Gdn·HCl transition (M)	τ_1 (s)	A_1	τ_2 (s)	A_2
0.5 → 4.0	55	0.2	500	0.8
0.5 → 5.0	25	0.5	130	0.5
1.5 → 4.0			570	1.0
0 → 4.0	166	0.3	780	0.7
0 → 4.0[b]	146	0.3	925	0.7

[a] Temperature, 23 °C, pH 8.2 TBS-EDTA buffer. [b] The protein sample used in this guanidine-jump was initially equilibrated in 4.0 M Gdn·HCl and was then reequilibrated into Tris-EDTA buffer by dialysis.

did not lead to any significant change in any of the kinetic constants. The data from refolding experiments done at the lowest protein concentration (i.e., 6.1×10^{-7} M) were extremely noisy and unreliable for the first 5–7 s following injection. This was due to mixing turbulence which becomes significant at the amplifer sensitivity required for this low concentration of protein. By giving zero weight in the fit procedure to the points collected during the first 10 s after injection, a good fit was obtained to the remainder of the curve when analyzed in terms of two exponents. As can be seen in Table I, the values obtained correspond quite closely to the τ_2 and τ_3 values obtained in the three exponential analysis done at higher protein concentration. The absence of a concentration dependence is consistent with there being no fluorescence change accompanying the dimerization process. Refolding the protein from 3.0 M Gdn·HCl resulted in similar kinetics (Table I).

Data for the corresponding unfolding transition (i.e., 0.5–4.0 M Gdn·HCl) are shown in Figure 4A. This transition is biphasic having τ values of approximately 50 and 500 s, the latter time accounting for 80% of the change (Table II). Shown in Figure 4B is a similar unfolding experiment to 5.0 M Gdn·HCl (i.e., even further into the base line of the unfolded species). This transition was also biphasic, although considerably faster, and it had different relative amplitudes from the unfolding experiment to 4.0 M Gdn·HCl (Table II). Unfolding experiments beginning at 1.5 M Gdn·HCl and ending at 4.0 M Gdn·HCl display single exponential kinetics with τ values which agree well with the slow time of the 0.5–4.0 M Gdn·HCl transition.

Also listed in Table II is an experiment which shows that the unfolding kinetics of previously refolded pFc′ are the same as those of the native protein. This kinetic finding further

Table III: Time Constants and Amplitudes for Unfolding or Refolding within the Transition Zone[a]

Gdn·HCl transition (M)	τ_1 (s)	A_1	τ_1 (s)	A_2
3.0 → 1.5	270	0.5	1 180	0.5
0.0 → 1.5			10 500	1.0
4.0 → 2.0	680	0.2	10 000	0.8
0.0 → 2.0	2070	0.5	10 000	0.5
4.0 → 3.0	700	0.2	13 000	0.8
1.5 → 3.0	690	0.2	5 350	0.8
1.5 → 0.5[b]	190	0.6	980	0.4
1.5 → 0.5	170	0.4	>15 000[c]	0.6

[a] Temperature, 23 °C, pH 8.2 TBS-EDTA buffer. [b] Sample initially at 3.0 M Gdn·HCl and was reequilibrated at 1.5 M Gdn·HCl. [c] Here we were not able to follow the reaction to completion and the value is obtained by extrapolation.

corroborates the equilibrium evidence that the final state of refolding is the native one.

Table III presents data for denaturant jumps, with equal final Gdn·HCl concentrations in each pair. This type of experiment is intended to check kinetic reversibility (Hagerman, 1977). It may be seen that, within each pair, time constants and amplitudes for refolding and unfolding are not equal. This observation is most clearly illustrated by comparing the folding transition from 3.0 to 1.5 M Gdn·HCl with the corresponding unfolding transition from 0 to 1.5 M Gdn·HCl. The kinetics of these transitions are plotted on the same time scale in Figure 5. The refolding transition is relatively fast and biphasic, while the unfolding transition is extremely slow and apparently monophasic (Table III). These results differ strikingly from those reported previously for proteins of comparable size (Ikai et al., 1973; Hagerman & Baldwin, 1976) in that in our case kinetic reversibility is not strictly observed.

Further insight into the apparent kinetic irreversibility of our system is obtained from experiments where the refolding of the protein from 1.5 to 0.5 M Gdn·HCl is followed in two samples which have experienced different histories. In one case, the native protein was initially equilibrated overnight at 3.0 M Gdn·HCl and then at 1.5 M Gdn·HCl, while in the second case the native protein was directly equilibrated at 1.5 M Gdn·HCl. In both cases, the initial fluorescence intensity relative to NATA was the same (Figure 2). However, as can be seen in Table III the kinetic behavior of these two samples was quite different. Whereas the sample which had experienced a prior equilibration at 3.0 M Gdn·HCl displayed biphasic kinetics which were quite similar to those observed in going from 3.0 to 1.5 M Gdn·HCl, the sample which was equilibrated directly at 1.5 M displayed a much longer τ value for the slower time. This may suggest the presence of a hysteresis in the system, namely, that in the two cases structurally different, although fluorometrically indistinguishable species exist at 1.5 M Gdn·HCl.[3]

The Kinetics of Protein Folding with an Initially Cleaved Intrachain Disulfide Bond. As shown above (Figure 1), in the absence of the intrachain disulfide bond the molecule exists in an unfolded state even at the nondenaturing concentration of 0.5 M Gdn·HCl. Since it was also shown that the protein could refold if disulfide bond formation is allowed to occur, studying the kinetics of the simultaneous reoxidation and refolding could provide a useful in vitro approximation to the folding processes which occur in vivo in the nascent chain.[4] The study of the in vitro process consisted of two complementary experimental approaches. In the first stage, the kinetics of disulfide bond formation were studied under optimal catalytic conditions. Then, under identical conditions, the concomitant reoxidation–refolding processes were monitored.

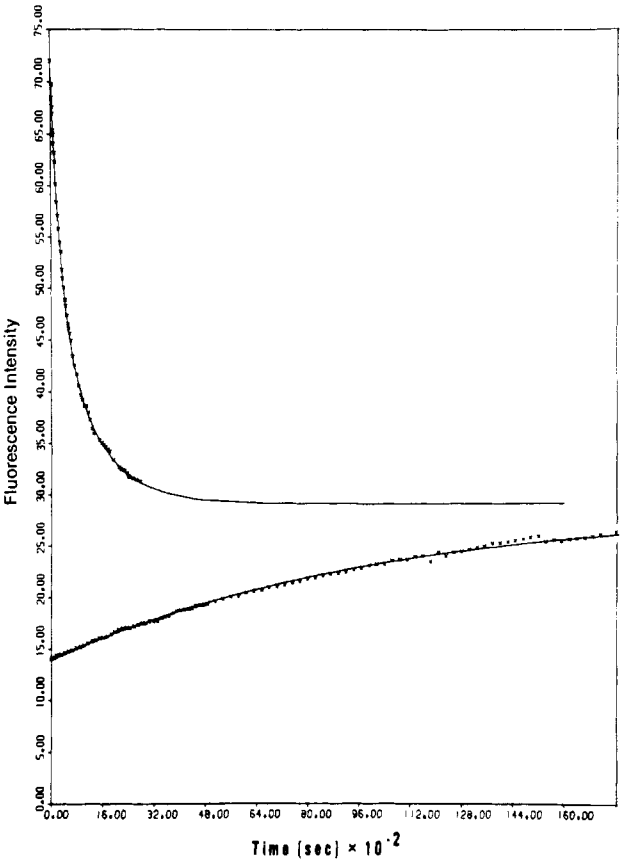


FIGURE 5: Kinetics of the 3.0–1.5 M Gdn·HCl refolding transition (upper curve) and of the 0–1.5 M Gdn·HCl unfolding transition (lower curve) at 23 °C. The data sets were hand measured from strip chart recordings, and the fluorescence intensity values were normalized to the same scale as was used in the plot of the equilibrium transition curve. As before, the smooth curves are calculated from the fit parameters of an exponential analysis.

(*1*) *The Kinetics of Disulfide Bond Formation.* The reoxidation of a sample of pFc′ reduced as described in the Materials and Methods section was initiated by dilution into a TBS–EDTA, pH 8.2, buffer that contained 0.5 M Gdn·HCl, 1 mM GSSG, 1 mM GSH and a trace amount of ^{125}I-labeled Fc fragment. The final concentration of protein chain in the reoxidation medium was 3×10^{-6} M. The reaction was allowed to proceed at 23 °C and, at various time intervals, aliquots were withdrawn and further reoxidation was stopped by the addition of a large excess of iodoacetamide (0.1 M final concentration). All samples were then allowed to stand at 23 °C for a minimum of 18 h during which time that fraction of the protein which had reoxidized could fold to its equi-

[3] The possibility existed that the slow refolding time observed in the case of direct equilibration at 1.5 M Gdn·HCl was due to hindered refolding of aggregated material which might form under these conditions but be absent when 1.5 M Gdn·HCl is approached from an initial equilibration point of 3 M Gdn·HCl. Accordingly, a sample of radioiodinated pFc′ equilibrated directly in 1.5 M Gdn·HCl was chromatographed on a calibrated Sephadex G-50 column in the same buffer. The protein eluted as a single symmetrical peak in a position corresponding to that of chymotrypsinogen (mol wt 25 700) and showed no evidence of higher molecular weight species.

[4] Clearly, the absolute validity of this approximation depends on how independently the folding of each domain proceeds from that of its neighboring domain(s) in vivo.

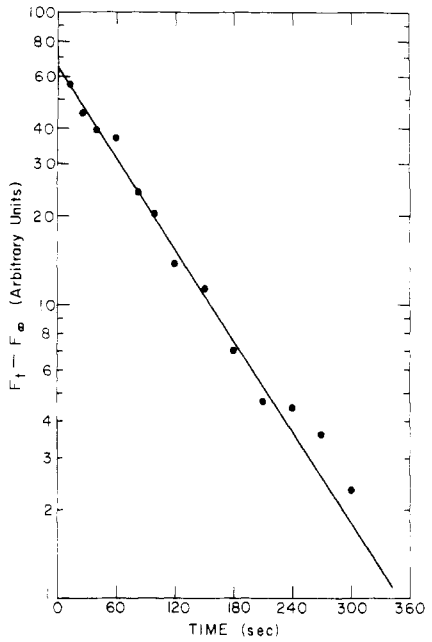


FIGURE 6: A first-order plot of the kinetics of disulfide bond formation. F_t and F_∞ refer to the fluorescence intensities of the protein after reoxidation has been allowed to proceed for a given time (t) and infinite time, respectively. The experimental conditions are as follows: the reoxidation buffer was TBS–EDTA, pH 8.2, containing 0.5 M Gdn·HCl, 1 mM GSSG, and 1 mM GSH. The protein concentration was 3×10^{-6} M and the reaction temperature was 23 °C. The solid line is calculated from the fit parameters as before. The time constant for disulfide bond formation in this particular experiment is 90 s.

librium state. Since we found that only the reoxidized form can fold to a state in which the tryptophan fluorescence is quenched, the fluorescence intensity of a given sample also becomes a measure of the concentration of the remaining reduced species. However, because the large excess of iodoacetamide results in serious inner filter and fluorescence quenching effects, low molecular weight reagents were first removed by gel filtration of each time course sample on a column of Sephadex G-25 equilibrated in TBS–EDTA containing 0.5 M Gdn·HCl. The fluorescence at 350 nm of each sample was then measured and the values obtained were normalized to the same protein concentration value based on counting data of ^{125}I-labeled Fc which acted as an internal concentration marker.

Although the reoxidation of the intrachain disulfide by oxidized glutathione is at least in part a bimolecular process, the large excess of the latter reagent over the concentration of protein SH ensures that pseudo-first-order kinetics will prevail throughout the reaction. Accordingly, the time dependence of the remaining fluorescence was found to fit a single exponent. The results obtained for one such experiment are displayed semilogarithmically in Figure 6. The calculated τ value for the formation of the disulfide bond varies between 80 and 200 s. Considering the complex handling sequence of rather dilute solutions inherent in this procedure, this degree of experimental scatter is not unexpected. Under similar oxidation conditions, but at 4 M Gdn·HCl, a very slow rate of reoxidation was observed. At the end of 2.5 h, the longest time studied, approximately one-third of the material had reoxidized. By extrapolation we estimate that reoxidation was at least two orders of magnitude slower in 4 M Gdn·HCl than at 0.5 M, strongly suggesting that reoxidation is directed by a folding nucleation event.

(*2*) *Fluorescence Monitoring of Refolding Simultaneous to Disulfide Bond Formation.* Experiments were performed

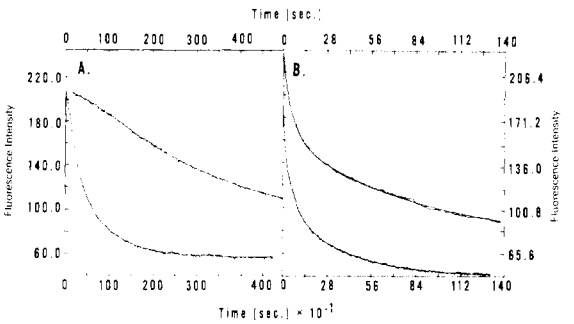


FIGURE 7: (A) Kinetics of the fluorescent changes observed when reoxidation is carried out under the same conditions as were described in Figure 6. The collection and presentation of the data are similar to what were described in previous kinetic plots with the exception that the smooth curve is calculated from fit parameters derived from a numerical treatment of the data in terms of the mechanism illustrated in Scheme III. (For further details, see text and Appendix.) (B) Reanalysis of the same experimental data set shown in Figure 3 for the 4.0–0.5 M Gdn·HCl refolding transition in terms of the mechanism illustrated in Scheme III not including the reoxidative steps.

in which the folding of pFc′ was monitored fluorometrically simultaneous to the reoxidation procedure. The experimental curve shown in Figure 7A depicts the fluorescence changes observed when reoxidation is carried out under the same conditions as described in the previous section. A prominent feature of this curve is the "lag time" observed in the rate of fluorescence change at the onset of the folding process. This observation is consistent with a sequential first-order mechanism in which two of the macroscopically observed rate constants are of similar magnitude.

The role of reduced glutathione as an agent required to initiate the reshuffling of wrongly formed disulfides has previously been described (Saxena & Wetlaufer, 1970; Hantgan et al., 1974; Creighton, 1975a). In its absence, the first part of the curve remained essentially unaltered. However, at longer times there was an apparent decrease in rate leading to a final fluorescence intensity which was 10–15% above the expected time infinity level. This effect is presumably due to the formation of mixed disulfides with both half-cystine residues, which in the absence of significant levels of reduced glutathione cannot reoxidize and, therefore, are unable to refold. Addition of reduced glutathione (1 mM) to such a prematurely aborted reoxidation mixture indeed resulted in a reinitiation of the oxidative process as detected by the decrease in fluorescence intensity to the expected level.

Discussion

(*a*) *Fluorescence Properties.* The 4.2-fold increase in fluorescence intensity displayed by pFc′ upon denaturation indicates that the tryptophan fluorescence in the native molecule is highly quenched. This degree of enhancement in quantum yield is more than twice as large as was found in a number of proteins examined by Teale (1960) and by Teipel & Koshland (1971). One of the most efficient quenchers of tryptophan fluorescence in proteins is the disulfide bond. It has been shown by Cowgill (1970) that, in order for this group to efficiently quench tryptophan fluorescence, the encounter distance must be less than 7 Å during the lifetime of the excited state. It is well known from the X-ray crystallographic analysis of immunoglobulin domains that the conserved tryptophan of each domain (Trp-381 in $C_\gamma 3$) is located in close proximity to the intrachain disulfide bond and, thus, one would expect it to be largely quenched in the native molecule. The other tryptophan (Trp-417) of the $C_\gamma 3$ domain is far removed from the interdomain contact region and is in an area which

is close to the surface (Deisenhofer et al., 1976). Judging from the guinea pig protein, the second tryptophan residue is probably also quenched as it is found to be in close proximity to phenylalanyl and arginyl residues (P. Phizackerley, personal communication). Both may cause quenching, the former by charge-transfer interaction and the latter by its electrostatic charge. Thus, the low level of fluorescence of the native molecule cannot be assigned unequivocally to either of the two tryptophan residues alone and probably encompasses contributions from both.

(*b*) *Folding Kinetics of Disulfide Bond Intact pFc'.* Comparing the time constants of the various refolding and unfolding experiments performed with pFc' with the values obtained with proteins of similar size such as ribonuclease (Garel et al., 1976; Hagerman & Baldwin, 1976), lysozyme (Tanford et al., 1973), and cytochrome *c* (Ikai et al., 1973; Tsong, 1976) reveals that both processes are one to two orders of magnitude slower in the case of pFc'. While the three fast folding proteins contain significant amounts of α helix (Dickerson & Geis, 1969; Dickerson & Timkovich, 1975), pFc', by contrast, has been shown to contain none of this structure (Deisenhofer et al., 1976). Rather, its dominant secondary structure is the anti-parallel β-pleated sheet. It has been suggested that the formation of α helices or β structure could serve as nuclei in protein folding (Baldwin, 1975). Even if this is not the case, clearly the rate of final structure acquisition would be dependent on the rates of formation of these secondary structures. While the pH-dependent, coil-to-helix transition in synthetic polyaminoacids is an extremely rapid process (e.g., the completion of this process in poly(L-glutamic acid) requiring less than 10 μs (Schwartz, 1965, 1968; Zana, 1975)), the formation of β structure from helical poly(L-lysine) is a much slower process requiring times in the range of 10^1 to 10^3 s for completion (Snell & Fasman, 1973; Hartman et al., 1974). Clearly the formation of the complex β structure present in the native form of pFc' would be a major contributing factor to the relatively long times required for the folding process to occur.

Brandts et al. (1975) and Lin & Brandts (1978) have suggested a mechanism to account for the slow phase observed in the refolding kinetics of several low molecular weight proteins. According to their hypothesis, the slow phase (10–50 s) is due to the cis–trans isomerization of proline peptide bonds in the denatured state. Only that form of the denatured state having the native configuration of the proline residues is capable of complete folding. Indeed it has recently been shown by this group that the refolding of a proline devoid protein (carp parvalbumin) does not display this slow phase (Brandt et al., 1977; Lin & Brandts, 1978). In this context, it is noteworthy that pFc' contains nine proline residues per chain.

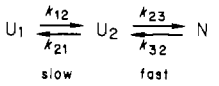
We have interpreted the absence of any concentration dependence in the folding rates as indicating that there is no fluorescence change accompanying the dimerization process. This interpretation is consistent with the X-ray structure of human Fc (Deisenhofer et al., 1976) which shows that there are no tryptophan residues involved in the $C_\gamma 3$ dimer interface. Although the dimerization process may be spectroscopically undetected, one would expect that kinetic coupling would lead to a concentration dependence if the dimerization process occurred before the folding of the individual chains was complete. One cannot completely rule out the possibility that the concentration-independent kinetics result from dimerization being a very rapid early process which is spectroscopically invisible. However, the intricate nature of the contacts involved at the dimer interface argues against this latter hypothesis and, indeed, suggests that chain folding would have to be complete before dimerization could occur.

Rowe & Tanford (1973) have previously investigated the folding kinetics of a human immunoglobulin light chain and reported that both the folding and unfolding processes displayed multiphasic kinetics. The slow response time of their instrumentation limited their analysis to the slow phase of the process. This slow phase was found to have τ values in the range of 150–360 s depending on the initial and final Gdn·HCl concentrations. Where it was checked, kinetic reversibility of the slow phase was observed. Rowe and Tanford have interpreted their findings in terms of a two-state transition occurring at different rates in each of the two domains comprising the L chain. They further concluded that no kinetic intermediates are observed within the individual domains. Clearly such interpretation cannot apply to the multiphase kinetics observed in our single domain system. We cannot at this time reconcile the kinetic reversibility of the slow phase reported by Rowe and Tanford with the apparent kinetic irreversibility observed by us.

(*c*) *The Question of Apparent Kinetic Irreversibility.* For reversible, multistep processes initiated by jumping from one set of conditions to another, the observed time constants are functions of rate and equilibrium parameters of the final conditions and are independent of the direction or extent of the jump. In contrast, the fractional amplitudes may also depend on the initial conditions if these conditions affect the relative concentration of each species (Garel & Baldwin, 1975a; Hagerman & Baldwin, 1976). Hagerman has pointed out that apparent kinetic irreversibility need not necessarily indicate that the pathways of refolding and unfolding proceed via different intermediates. Rather, it is possible that the amplitudes of all fast phases have gone to zero and that a very slow rate which had negligible amplitude in one direction becomes dominant in the reverse direction. For example, the very slow process seen in the 0–1.5 M Gdn·HCl transition (τ = 10 500 s) could also be present as a third kinetic phase in the 3.0–1.5 M Gdn·HCl transition, but have negligible amplitude and therefore not be detected. While we cannot rigorously rule out this explanation of our observed kinetic irreversibility, in view of the hysteretic behavior of the 1.5–0.5 M Gdn·HCl transitions described above, we tend to favor the alternative explanation that, in this particular protein, the nature of at least some of the intermediate species through which the protein passes in transitions of either direction is not dependent solely on the final denaturant conditions.

(*d*) *The Rate of Disulfide Bond Formation.* In the case of all proteins thus far studied, facilitated disulfide bond formation per se is a relatively fast reaction. The half-times are in the range of 1 to several minutes, and the exact time is a function of the molar excess of oxidant. However, the initial pairings usually are not the native ones and the relatively slow acquisition of functional activity by these proteins reflects the time taken for the incorrectly paired disulfides to reshuffle to the native ones. The rate of this process is considerably enhanced by the presence of a disulfide interchange catalyst such as reduced glutathione (e.g., Saxena & Wetlaufer, 1970; Hantgan et al., 1974). In the case of BPTI, Creighton (1975a–c, 1977a,b) has shown that only one of the initial pairings is consistently the native one in all of the trapped intermediates, the others being relatively random. In the presence of 6 M Gdn·HCl, the rate of disulfide bond formation in BPTI was unaffected; however, under these conditions, the initial pairings were completely nonnative, there being a statistical preference for primary sequence nearest-neighbor

Scheme I



pairings. Furthermore, only upon removal of the denaturant did reshuffling occur to the native pairings.

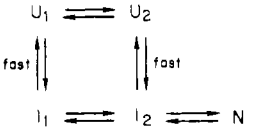
Since each chain of reduced pFc′ contains only two cysteine residues, the complication of wrongly paired intrachain disulfide bonds does not arise. In reoxidizing, the two cysteines must either form the correct intrachain bond or form interchain disulfides leading to oligomerization. The extremely low protein concentrations used in the reoxidation experiments make the latter event an unlikely occurrence. Indeed, electrophoretic analysis in acid–urea polyacrylamide gels of ^{125}I-labeled partially reoxidized pFc′ did not reveal the presence of any oligomeric species.

The rate of s–s reoxidation of pFc′ in 4 M Gdn·HCl is found to be much slower than the corresponding rate in BPTI (Creighton, 1977b). This may be accounted for by assuming that in both cases the process of s–s formation at high Gdn·HCl concentrations is a statistically controlled random event, the differences arising from the larger separation of half-cystines in pFc′ (57 residues). Under nondenaturing conditions, the reoxidation rate of pFc′ becomes much faster, suggesting that it is directed by early refolding events. This is in contrast to the case of BPTI, where, although refolding directs the selection of s–s formation, its rate remains the same as under denaturing conditions. For example, the most distant cysteine pairing in the primary structure of BPTI does not occur at all when the reoxidation is carried out in 6.0 M Gdn·HCl, while being a prominent pairing when the reoxidation takes place in the absence of denaturant (Creighton, 1977b).

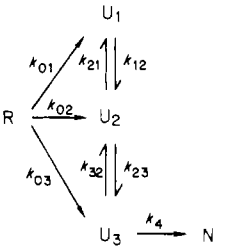
(*e*) *The Overall Folding Process from the Reduced State.* One of the primary aims of our studies on this isolated domain of IgG was to determine if it is possible to account for the overall in vitro folding process from the reduced state in terms of the folding rates observed with disulfide bonds initially intact and the rate of disulfide bond formation. In order to perform this kind of analysis, a mechanistic scheme had to be chosen. We had originally hoped that the unfolding and refolding experiments done within the transition zone with the disulfide bonded protein would enable us to choose between the various schemes which were to be found in the literature (e.g., Tsong, 1976; Hagerman & Baldwin, 1977; Ikai et al., 1973; Rowe & Tanford, 1973). All of these schemes, however, required kinetic reversibility, a criterion which is apparently not met in our system. Therefore, any scheme which we propose will not be valid in the general case; it may, however, be valid if it is restricted to describing one given type of transition. The transition of interest in terms of analyzing the overall process is the 4.0–0.5 M Gdn·HCl transition.

A growing body of evidence had begun to accumulate (see Hagerman, 1977) which suggests that the extended two-state model proposed for ribonuclease (Garel & Baldwin, 1973, 1975a,b; Hagerman & Baldwin, 1976) could account for the folding kinetics of a number of small molecular weight proteins. In this scheme (Scheme I), the only species of appreciable concentration present during folding are the native protein and multiple forms of the unfolded protein which are isomers differing only in chain configuration. It was suggested by Brandts et al. (1975) that the slow $U_1 \rightleftarrows U_2$ interconversion was governed by a cis–trans proline isomerization. Indeed Schmid & Baldwin (1978) have recently shown that, in unfolding conditions, the kinetics, the catalytic effect of strong acids, and the enthalpy of activation of the $U_1 \rightleftarrows U_2$ interconversion in ribonuclease were consistent with the rate-limiting step being proline isomerization. However, Nall et al. (1978) found that the slow phase of ribonuclease folding, when measured under refolding conditions, displayed kinetics which were inconsistent with the simultaneous validity of both the extended two-state model and rate-limiting proline isomerization. They currently favor a more complex scheme (Scheme II) involving a square mechanism in which factors in the initial as well as the final conditions of a transition could determine which pathway is followed. While the $U_1 \rightleftarrows U_2$ interconversion would be rate limited by proline isomerization in the unfolding direction, under refolding conditions, partial folding would rapidly occur before isomerization of all prolines to their native configuration is complete. Indeed a scheme involving partially folded species with intermediate physical properties is needed to account for the biphasic kinetics we observe in the complete unfolding transitions (cf. Figure 4 and Table II).

Scheme II



Scheme III



While Scheme I did not survive the rigorous testing involved in the study of Nall et al. (1978), it did adequately explain most of the kinetic behavior observed for ribonuclease. The need to allow for a third kinetic phase in the complete refolding transition in addition to a reoxidation step will complicate our mechanism to the point where attempts to develop it within the framework of the newly proposed Scheme II would be premature. A scheme involving partially folded species with intermediate spectral properties introduces additional variables, namely, the molar fluorescence intensities of these intermediates. In view of these complications, we have chosen to base our analysis of the overall folding process from the reduced state on an elaboration of the extended two-state hypothesis as is shown in Scheme III. We believe that analysis in terms of this minimal scheme is capable of determining whether a mechanism having the essential features of a nucleation–reoxidation step, followed by the same folding processes as those observed with the intrachain disulfide bond initially present, can semiquantitatively account for the observed kinetics of the overall process.

R represents the reduced form of the unfolded protein with k_{01}, k_{02}, k_{03} being the rate constants for nucleation–reoxidation events which result in the formation of the three conformational isomers of the reoxidized–nucleated but still largely unfolded form of the protein U_1, U_2, and U_3. In order to minimize the number of parameters, as a first approximation we have made this step irreversible, the rationale being that the oxidative conditions used in these experiments strongly favor reoxidation over reduction. We cannot rule out the reverse reaction entirely. The extent of back reduction of the reoxidized–nucleated species U_1, U_2, or U_3 would depend on

Table IV: Rate Constants Derived from an Analysis of the 4.0 → 0.5 M Gdn·HCl Transition in Terms of an Extended Two-State Mechanism[a]

Gdn·HCl transition (M)	× 10^3 s^{-1}			× 10^3 s^{-1}		× 10^2 s^{-1}		s^{-1}
	k_{01}	k_{02}	k_{03}	k_{12}	k_{21}	k_{23}	k_{32}	k_4
4.0 → 0.5 s–s intact				3.3	2.0	1.7	1.4	0.16
4.0 → 0.5 s–s initially reduced	3.8	3.7	1.7	1.6	0.9	2.1	10.6	0.2

[a] Temperature, 23 °C, pH 8.2 TBS–EDTA buffer.

both the accessibility to the solvent of the disulfide bond in the reoxidized and nucleated species and on the relative rate of reduction compared with reoxidation and refolding. If significant back-reduction does occur, the absolute rate constants k_{01}, k_{02}, and k_{03} become apparent rate constants which encompass the reverse direction as well. The three largely unfolded species with an intact disulfide bridge U_1, U_2, and U_3 are in conformational equilibrium. In the case of the denatured protein with disulfide bonds intact, this equilibrium may either be present in 4.0 M Gdn·HCl or a random structure present at 4.0 M Gdn·HCl may very rapidly distribute into this equilibrium mixture when the Gdn·HCl concentration is lowered to 0.5 M. The formation of these species in 0.5 M Gdn·HCl from the initially reduced form may result in a different ratio. This ratio would depend on the relative magnitudes of the three k_{0i} values and may reflect a distribution in R or it may result from preferential nucleation. The U_3 to N conversion in the 4.0–0.5 M Gdn·HCl transition must be essentially irreversible since the final Gdn·HCl concentration is in the base line of the pretransition zone.

An ultimate test for the validity of this scheme would be to try to obtain a fit to a consistent set of values for its parameters by using the data from the following three different types of experiments: (1) refolding of the chain with an initially intact disulfide bridge (Figure 3); (2) reoxidation of the SH groups to form a disulfide bridge (Figure 6); (3) the combined process of reoxidation and refolding (Figure 7A). This has been accomplished by using a nonlinear best parameter fit procedure (see Appendix). As a preliminary stage of this procedure, the data set shown in Figure 3, originally analyzed phenomenologically in terms of exponents, was reanalyzed using Scheme III not including the reoxidative steps. The fit obtained is shown in Figure 7B and is judged to be equally good by the residual sum of squares. The values of the rate constants obtained are shown in Table IV. The rate constants of the three kinetic phases differ in relative magnitudes by approximately the same factor as was previously determined by the sum of exponentials method. As was the case in the sum of exponents analysis, attempts to fit the data by using only two species of U were unsuccessful.

Figure 7A shows the fit obtained to the combined reoxidation–refolding experiment by using the full Scheme III. Clearly the proposed mechanism can successfully account for the experimentally observed "lag time" in the folding process. The values of the nucleation–reoxidation rate constants and of the various refolding rate constants are shown in Table IV. Dealing first with the three nucleation–reoxidation rate constants, one of the assumptions inherent in the model is that part of the folding process is limited by disulfide bond formation. This provides an important check of the model since the reciprocal of $(k_{01} + k_{02} + k_{03})$ should predict the experimentally observed τ value for disulfide bond formation (τ_{s-s}).[5] The calculated value of 109 s agrees well with the directly determined τ_{s-s} values of 80–200 s. We feel that the slight preference shown for the formation of U_1 and U_2 over U_3 is not significant since this difference was not observed in the analysis of another data set which gave a predicted τ_{s-s} value of 190 s, a value still within the variation of the directly determined values.

Comparison of the rate constants for the three consecutive folding steps observed in the initially reduced protein with those of the initially disulfide bonded protein reveals that semiquantitative agreement is generally observed, the only exception being k_{32}. In view of the fact that numerically fitting the overall process involves ten free parameters and also keeping in mind the sensitivity of the procedure to quite small changes in the experimental data or initial guesses, we feel that agreement within such limits is satisfactory and should be taken as an indication of rough consistency with a model which is known to be an oversimplification.

Thus, the essential features of the in vitro folding process starting from a reduced immunoglobulin domain may be described in terms of a scheme with the basic elements of rate-limiting nucleation–reoxidation events followed by folding processes which are very similar to the ones observed in renaturation experiments where the disulfide bonds are initially intact. The exact details of Scheme III may not be correct. It might also be possible to obtain an equally good fit by using an analogous elaboration of Scheme I, where multiphase folding kinetics are accounted for by intermediately folded species rather than by conformational isomers of the unfolded protein. Thus, further analysis of the folding pathways of pFc′ may prove worthy. Finally, an extension of these studies to other immunoglobulin domains and to a multidomain chain (e.g., L chain or Fc) should provide useful insights into the effect of structural variability and of possible longitudinal cooperativity in domain folding processes.

Acknowledgments

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Appendix

Analysis of the data in terms of the mechanism proposed in Scheme III was done by using the following curve fitting technique. The least-squares fitting procedures used by us here as well as in the exponent analysis are iterative, where, at each iteration, a set of parameters (initial guesses or improved guesses) is assumed and used to find the value of the calculated function at each time point. The deviation from the experimental curve serves as a criterion for the best parameter search minimization routine. In the case of the exponent analysis all 1000 time-sweep data points were analyzed in this

[5] In the direct reoxidation experiment, one measures the combined $R \rightarrow U_i$ τ reactions, which may be treated as a single step reaction since the U_i's are indistinguishable in its scope. The experimentally determined value of τ_{s-s} would also encompass contributions from any back-reduction which occurs.

fashion simultaneously. In contrast, in the case of the mechanistic analysis, each iteration comprised a simulation of the reaction where consecutive time points were generated by using rate difference equations. For the particular mechanism used here (Scheme III), these were as shown by eq 1–5. Δt was always 0.001 of the total time sweep. The

$$\Delta R(t_{i+1}) = -(k_{01} + k_{02} + k_{03})R(t_i)\Delta t \quad (1)$$

$$\Delta U_1(t_{i+1}) = [k_{01}R(t_i) + k_{21}U_2(t_i) - k_{12}U_1(t_i)]\Delta t \quad (2)$$

$$\Delta U_2(t_{i+1}) = [k_{02}R(t_i) + k_{12}U_1(t_i) + k_{32}U_3(t_i) - (k_{21} + k_{23})U_2(t_i)]\Delta t \quad (3)$$

$$\Delta U_3(t_{i+1}) = [k_{03}R(t_i) + k_{23}U_2(t_i) - (k_{32} + k_4)U_3(t_i)]\Delta t \quad (4)$$

$$\Delta N(t_{i+1}) = k_4U_3(t_i)\Delta t \quad (5)$$

normalized concentrations at $t = 0$ were taken to be $R = 1$ with all others being zero for the reoxidation–refolding. For the refolding, an equilibrium mixture of U_1, U_2, and U_3 (with their sum equal to 1) was calculated according to the parameters used in the particular iteration. The concentration of species X at each time point was then calculated by using eq 6. In the calculation of the computed trace F_N and F_U,

$$X(t_i) = X(t_{i-1}) + \Delta X(t_i) \quad (6)$$

the normalized molar fluorescences of the native form N and of the unfolded forms U_1, U_2, U_3, and R, respectively, were also used as free parameters. The algorithm of Powell (1971) was used for the error minimization, and a typical run consumed 20 s of CPU time on an IBM 370/155.

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