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A Kinetic Study *In Vitro* of the Reoxidation of Interchain Disulfide Bonds in a Human Immunoglobulin IgG1κ. Correlation Between Sulfhydryl Disappearance and Intermediates in Covalent Assembly of H_2L_2

(Ellman's reagent/quantitative polyacrylamide gel electrophoresis/random reoxidation/ pathways of assembly in the mouse)

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ABSTRACT **After reduction by dithiothreitol and removal of the reductant by molecular sieve chromatography, the four interchain disulfide bonds of the human IgG1κ protein *Fro* reoxidize in the presence of oxygen and trace metal ions. The six molecular components of the reoxidation—L (light chain), H (heavy chain), HL, H_2, H_2L, H_2L_2—are quantitatively determined from polyacrylamide gels containing sodium dodecyl sulfate and the time-dependent sulfhydryl titer is measured with 5,5′-dithiobis-(2-nitrobenzoic acid).**

The rates of H_2L_2 covalent assembly depend on pH in an unexpected way: If the reduced protein is chromatographed at pH 3.2 and then adjusted to pH 7.5 (25°, ionic strength = 0.14), H_2L_2 formation proceeds rapidly, with half-times ranging between 20 and 40 min. In contrast, if chromatography is carried out at pH 5.5 before adjusting to the same final conditions, the half-times for H_2L_2 formation are considerably longer (120–180 min). The half-times in the former case approach the somewhat faster rates of H_2L_2 assembly observed in pulse-chase experiments with various types of mouse, IgG-producing cells [Baumal, *et al.* (1971) *J. Exp. Med.* 134, 1316–1334].

To facilitate comparison of experiments and models, we plot the concentrations of the six components against the corresponding number of sulfhydryl equivalents per mole of *Fro*. The respective plots for the pH 3.2 → 7.5 and 5.5 → 7.5 experiments are very similar despite the rate differences. Moreover, these plots differ significantly from the calculated plot for a hypothetical random reoxidation in which the intrinsic probability for formation of each correct HL and H_2 disulfide bond is assumed equal and independent. It is concluded that the *in vitro* reoxidation of *Fro* (i) is other than random; (ii) involves a pathway or pathways with HL, H_2, and H_2L precursors; and (iii) involves at least some kinetic cooperativity in bond formation, since no model based solely on independent bond formation adequately accounts for the results. The models were used also to examine the cellular assembly pathways of mouse IgG proteins.

As part of a long-term effort to understand the process of self-assembly of multi-chain proteins, we have undertaken a series of investigations of the factors that control the conformation of immunoglobulin subunits and their covalent assembly into functional antibody molecules. While the immunoglobulins represent an excellent group of proteins for such studies in several respects, the most important for the purpose of the present paper is that they are the only class of proteins for which, thus far, *in vivo* assembly pathways have been established in specific cases.

Abbreviations: H, immunoglobulin heavy chain; L, light chain; SH, sulfhydryl; NaOAc, sodium acetate; HOAc, acetic acid.

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The cellular processes by which heavy (H) and light (L) chains covalently assemble into whole immunoglobulin molecules have been investigated extensively and recently reviewed by Scharff and Laskov (1, 2) and Bevan *et al.* (3). In the most thorough studies to date, Scharff and his colleagues (2), using mouse myeloma cells, have presented impressive evidence which demonstrates a relationship between the H chain subclasses of this species and the major covalent precursors of H_2L_2 assembly. Specifically, proteins of subclasses IgG1 and IgG2a assemble primarily through H_2 and H_2L precursors, whereas, those of subclass IgG2b assemble mainly through HL. This correlation between subclass and assembly is interesting in terms of protein structure, since it is known that the arrangement of interchain disulfide bonds is distinctly different for each subclass (4, 5). The four human subclasses differ also in their pattern of interchain bonding (6) but only one example of cellular assembly, an IgG1λ protein, has been reported (7).

As a counterpart to these cellular systems, we report here an *in vitro* system designed for studying the sequential, covalent formation of H_2L_2 after reduction of all interchain disulfide bonds, along with the corresponding SH loss that occurs in the course of reoxidation. The long-term aim of this work is to isolate, monitor, and control various aspects of the reoxidation process, including the domain interactions involved in the assembly, and to examine in detail the concept of a pathway.

In this report we examine the reoxidation of human IgG1κ with its four interchain disulfide bonds reduced. The conditions employed in the present work permit the chains to remain *noncovalently* associated throughout, so that the results focus on the kinetics and pathway of reoxidation. To determine which disulfide bonds have formed at any time during reoxidation, samples are withdrawn for alkylation and sodium dodecyl sulfate gel electrophoresis. During the latter process only, noncovalently associated chains become dissociated. (In different experiments, reported elsewhere, conditions are established to separate the chains after reduction and prior to reoxidation, so that the effects of prior dissociation on subsequent covalent assembly may be analyzed.)

When the present work was nearing completion, Prof. K. Dorrington kindly made available to us, prior to publication, a preprint of a paper (8) reporting studies of the *in vitro* reoxidation kinetics of other human IgG1 proteins. While the experimental approaches and several of the experimental parameters being sought in the two laboratories differed considerably, there was sufficient agreement in many respects

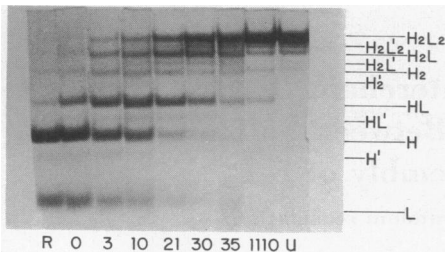


FIG. 1. Strips cut from 5% sodium dodecyl sulfate–polyacrylamide gel slabs stained with Coomassie brilliant blue. [Each well (top) was loaded with 24 μg of protein.] The various components are identified at the right (see *text*). The wells labeled U and R represent an unreduced sample and a reduced and alkylated sample taken prior to reoxidation. The remaining strips contain samples alkylated at various times (indicated in minutes at the bottom) in a pH 3.2 → 7.5 reoxidation of reduced *Fro* at a concentration of 4.6 μM. The zero time sample was taken immediately after the protein emerged from the Bio-Gel P2 column (see *Materials and Methods*). (In many instances the primed components were discernible only in the densitometer scans.)

to encourage the belief that the study of *in vitro* reassembly processes will shed light on the underlying basis for class and subclass distinction in cellular self-assembly.

MATERIALS AND METHODS

Protein Purification and Characterization. Dr. E. F. Osserman kindly provided plasma from a patient with the clinical symptoms of a plasma cell dyscrasia. The monoclonal IgG *Fro* was purified from the plasma by procedures described by Edelman *et al.* (9), with an additional final purification step on Bio-Gel P200. Concentrations were determined from extinction coefficients referred to dry weight measurements: at 278 nm, $A^{1\%}_{1\,cm} = 12.8 \pm 0.4$ and $\epsilon = 1.91 \pm 0.06 \times 10^5$ M^{-1} cm^{-1} (assuming the molecular weight = 149,000 and 2.9% carbohydrate). *Fro* is an IgG1κ (E. F. Osserman, unpublished results).

Reduction and Reoxidation. Lyophilized protein was reduced for 35–60 min at room temperature in Tris–sal. ıe buffer at pH 8.1 using Cleland's reagent, dithiothreitol (10), in exactly 25-fold molar excess over interchain disulfides. The protein was separated from reductant and brought either to pH 5.5 or 3.2 by passage through Bio-Gel P2 (50–100 mesh, Bio-Rad) equilibrated with 10 mM NaOAc buffer or 10 mM HOAc, respectively. After separation from reductant, under N_2, the pooled peak fractions were immediately transferred to wide bottomed vessels, for exposure to air and adjustment of pH with Tris buffer. The final conditions for reoxidation were: 0.1 M NaCl, 0.05 M Tris·HCl, 1 mM acetate at pH 7.5, ionic strength 0.14.

Sulfhydryl Determinations. Sulfhydryl titers were determined at specified times during the course of reoxidation by the method of Ellman (11), using 5,5′-dithiobis-(2-nitrobenzoic acid) (Sigma, Calbiochem) and a revised extinction coefficient (12, 13).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. At intervals during the reoxidation samples were removed and alkylated in 0.1 M iodoacetamide (Calbiochem). The samples and the six-well, 5% polyacrylamide gel slabs were prepared for electrophoresis (model 4200 Electrophoresis System, Ortec), stained with 0.25% Coomassie brilliant blue (Schwarz/Mann), and destained according to procedures described by Weber *et al.* (14).

Quantitation of the Reoxidation Intermediates. The six wells of the destained (minimum 48 hr) gel slabs were cut into straight strips 8–9 mm wide and scanned at 570 nm using a Gilford 241 spectrophotometer with a linear transport attachment. The intensity of each intermediate was determined from the area under the trace. Control gels established that the *relative* but not *absolute* quantities of intermediates could be routinely determined from the gels by first calculating the fractional staining intensities of the intermediates (relative to the total intensity of the well) and then converting to molar values using correction factors based on the ratio of molar staining intensities for H and L (f_H/f_L). (Additional analytical details and equations for calculating f_H/f_L—which varied between 2 and 3, generally increasing for the more reoxidized samples—will be published elsewhere.)

Ultracentrifugation. Sedimentation experiments were carried out at 20° in a Spinco model E analytical ultracentrifuge with a four-hole AnF rotor and an RTIC temperature control.

RESULTS

Ultracentrifugation

The following $s_{20,w}$ values were obtained for *Fro* under various conditions: 5.47 S (unreduced, 10 mM HOAc, pH 3.2); 5.13 S (reduced and alkylated, 10 mM HOAc, pH 3.2); 6.40 S (unreduced, 10 mM NaOAc, pH 5.4); and 6.29 S (unreduced, 10 mM NaOAc pH 6.8). At pH 3.2 the reduced alkylated sample (reduction was verified on a dodecyl sulfate gel) has a slightly lower sedimentation coefficient than the unreduced protein in the same buffer, perhaps indicating that a small fraction of the reduced molecules may be in a dissociated state. This is found to be the case with other IgG1κ proteins under similar conditions (15). Virtually no dissociation is expected at pH 5.5, however, since the $s_{20,w}$ values for reduced and unreduced protein (at this pH and ionic strength) are found to be identical in human (16) and rabbit (17) immunoglobulins. The constancy of the $s_{20,w}$ value at pH 5.5 and 6.8 as well as the magnitude and direction of the drop in $s_{20,w}$ at pH 3.2 are both consistent with earlier observations concerning changes in the sedimentation coefficient accompanying the acid denaturation of immunoglobulins between pH 4.2 and 3.4 (18).

Reoxidation Studies

pH 3.2 → 7.5 Reoxidation. In Fig. 1 a typical reoxidation experiment is shown. H_2L_2 is identified in the right-most strip, containing unreduced *Fro*. H and L are identified on the left-most strip, containing protein immediately alkylated after reduction. The remaining major reoxidation components, HL, H_2, and H_2L, were identified by their migration distances relative to L, H, and H_2L_2, which are linearly proportional to their molecular weights plotted on a logarithmic scale (19). On such a semi-log plot, all six major components fell on a straight line when we used 23,500 and 50,900 for the molecular weights of L and H, respectively, as determined from the available sequence data on other κ and γ1 chains (20).

Five minor components, labeled H′, HL′, H_2′, H_2L′, and H_2L_2′, are also identified in Fig. 1. Except for H′, these usually appeared as faster-migrating shoulders on the major bands in the gel scans; their entire contribution to the total intensity of a scan varied between 4 and 27% (average 15% in 48 scans), whereas the intensity of any one primed component seldom exceeded 6% of the total intensity. From the semi-log plot of the mobilities, the estimated molecular weights of the primed components fluctuated between 9,000 and 13,000 less

than their unprimed counterparts. In the results reported below, the primed and unprimed pairs are treated as one by summing the intensities for each primed–unprimed pair. In so doing, we assume that the interchain half-cystines behave similarly, which appears to be the case when the primed components are separately analyzed.

In order to analyze the sequential order of bond reoxidation, the intensities of the bands in the gel scans were converted to fractional moles, which are defined here as the ratio (in percent) of the moles of a given component (M_I) to total moles of that component (M_I^{TOTAL}) which are possible from the starting amount of IgG. All intermediates can then be represented on a scale of 0–100.

Fig. 2 shows such an analysis. The fractional moles are plotted versus time (Fig. 2B) and versus the number of sulfhydryl equivalents per mole of *Fro* (Fig. 2A). The latter quantity is also plotted against time in Fig. 2B (right ordinate). These results are representative of those experiments where the reduced protein is chromatographically separated from dithiothreitol at pH 3.2 (10 mM HOAc) just before the pH is raised to 7.5.

The scale of 0 to 8 SH equivalents per mole of *Fro* was based on the following evidence: (*i*) the observed maximum SH titer frequently exceeded 7 but never 8; (*ii*) Ellman determinations on isolated L and H chains gave 1 SH per mole L and approximately 3 SH per mole H, respectively (unpublished results); and (*iii*) Ellman determinations on unreduced *Fro* gave no indication of free SH, that is, <0.2 SH equivalents per mole of unreduced protein.

In Fig. 2A, the SH titer at zero-time (when the pH is raised from 3.2 to 7.5) is somewhat less than that of the reduced sample (SH calculated from the gel). Typically, one-half to one SH equivalent was lost in the 15–20 min interim in which the protein was chromatographically separated from dithiothreitol. Moreover, the unreduced sample is not 100% H_2L_2. Only 80–95% of the possible H_2L_2 ever existed as such, with the remaining material found in the various intermediate states.

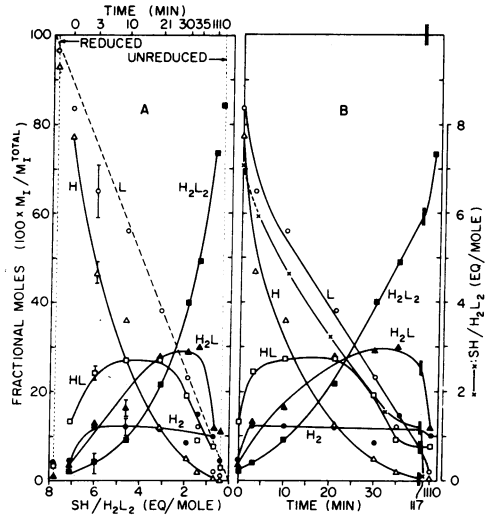
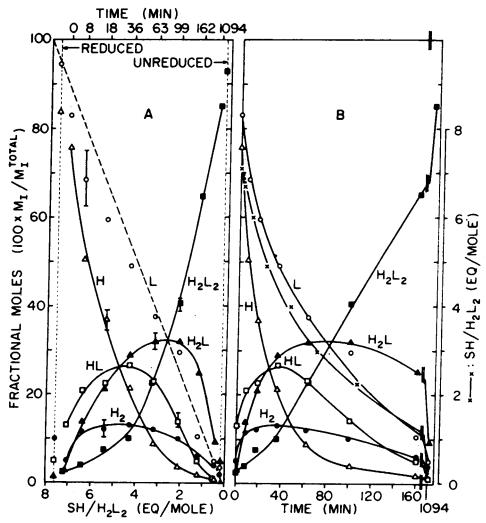


FIG. 2. Analysis of the pH 3.2 → 7.5 reoxidation gels of Fig. 1. The left ordinate for panels A and B refers to the fractional moles of each intermediate (see *text*): L (○); H (△); HL (□); H_2 (●); H_2L (▲); and H_2L_2 (■). The levels of reoxidation intermediates are plotted as functions of (A) the SH equivalents/mole of total protein and (B) time. In A, the points for L (○) are not joined and instead, a broken diagonal line is drawn for comparison to the L-line for a random reoxidation (see Fig. 4B). The vertical error bars do not represent standard deviations but *relative* errors as calculated by statistical formulae for the propagation of experimental errors (manuscript in preparation). In B, the SH titer versus time is also plotted (×; right ordinate); because the zero-time value of SH was calculated from the pattern of intermediates on the gel, it is joined by a broken line to the first measured value.

pH 5.5 → 7.5 Reoxidation. Fig. 3 shows the results of one experiment in which the reduced protein is chromatographically separated from dithiothreitol at pH 5.5 (10 mM NaOAc) before reoxidation at pH 7.5. Although the reoxidation rate was much slower here as compared to the 3.2 → 7.5 experi-



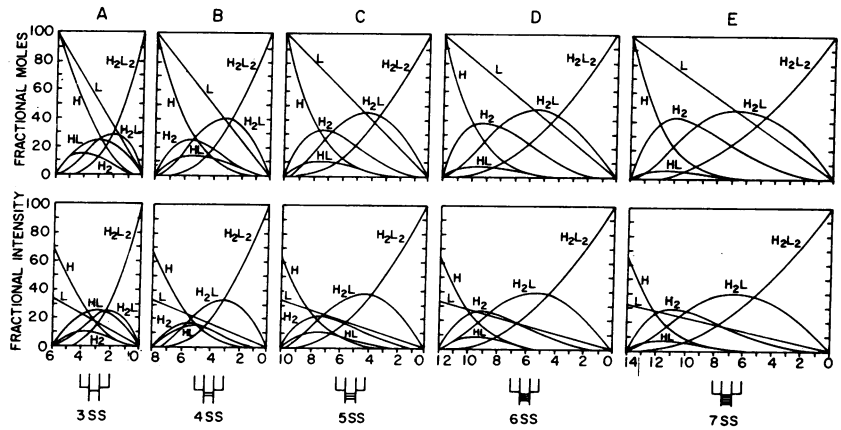


FIG. 3 (*left*). Analysis of a pH 5.5 → 7.5 reoxidation (protein concentration, 3.3 μM). Symbols and axes are the same as in Fig. 2 except for the expanded time scale of the abscissa of B.

FIG. 4 (*right*). The hypothetical restricted random reoxidation curves for various patterns of interchain bonding schematically illustrated at the bottom. Each HL and HH bond is assumed to form independently and with the same probability. In the upper panels the components are represented in terms of fractional moles. In the lower panels they are represented in terms of fractional intensities, where intensity here means any conserved property of the H and L chains such as staining intensity, radioactive label, etc. In converting the upper curves to the lower ones, it was assumed that, whatever the property is, it manifests itself in exactly a 2:1 ratio for H and L chains.

TABLE 1. *Comparisons between pH 3.2 → 7.5 and pH 5.5 → 7.5 reoxidation experiments and the restricted random model*

C*(μM)	H_2L_2 (%)† at t_0	SH‡ at t_0	$t_{1/2}$§ (min)	SH‡ at $t_{1/2}$	HL^{max} (%)¶	H_2^{max} (%)¶	H_2L^{max} (%)¶
				3.2 → 7.5			
4.7	6	7.2	40	2.3	30 (4.0)	10 (5.0)	34 (2.5)
2.4	6	7.2	20	—	26	14	23
4.6	2	7.1	36	1.3	27 (4.0)	12 (4.5)	30 (2.0)
		Average	32	1.8	28 (4.0)	12 (4.8)	29 (2.2)
				5.5 → 7.5			
2.6	3	6.3	180	1.4	25 (4.3)	17 (4.3)	33 (2.5)
5.2	3	6.5	122	1.3	32 (4.5)	18 (4.4)	34 (2.3)
3.3	2.5	7.1	124	1.7	27 (4.4)	13 (4.7)	32 (2.7)
		Average	142	1.5	28 (4.4)	16 (4.5)	33 (2.5)
				Restricted random model			
				2.2	15 (5.4)	25 (5.7)	40 (3.0)

* Concentration of protein in the reoxidation mixture.
† Fractional moles in percent at time zero.
‡ Sulfhydryl equivalents per mole of protein.
§ The half-time in minutes for the appearance of 50% H_2L_2.
¶ Peak fractional moles, in percent; in parentheses, the corresponding SH titer, (r).

ment, the profiles of the intermediates were very similar when plotted as functions of SH equivalents. The most distinct differences here are (*i*) a slightly broader HL curve and (*ii*) a narrower, less symmetrical H_2L curve in the 3.2 → 7.5 experiment.

Further comparisons between these two types of experiments and other experiments of the same type are found in Table 1. The average half-time ($t_{1/2}^{ave}$) for the formation of H_2L_2 in the 5.5 → 7.5 reoxidations is 4.4 times longer than $t_{1/2}^{ave}$ tabulated for the 3.2 → 7.5 experiments. Yet, the average peak magnitudes for HL, H_2, and H_2L and their average positions on the SH scale are remarkably similar for the two cases, suggesting that the sequential order of H_2L_2 formation is essentially the same in both.

Reoxidation Models. The calculated curves for the restricted random reoxidation models for five patterns of interchain disulfide bonding are illustrated in Fig. 4. These curves derive from the assumptions that (*i*) the intrinsic probabilities for the formation of any HL or HH disulfide bond are independent and equal and (*ii*) that the half-cystines yield only one possible arrangement of disulfides depicted at the bottom, corresponding to the following immunoglobulins: A, rabbit (21); B, human IgG1 and IgG4 (7); C, mouse IgG1 (4) and IgG2a (5); D, mouse IgG2b (5) and human IgG2 (7); and E, human IgG3 (7). It is interesting to note that the maximum levels of HL and H_2 decrease and increase, respectively, with increasing numbers of interchain bonds. In addition, L always decreases linearly with sulfhydryl.

A comparison between the two experimental cases (Figs. 2A and 3A) and the corresponding model (Fig. 4B, upper panel) reveals significant differences, as partially summarized in Table 1. The observed HL^{max} is 70–110% greater than predicted by the model, whereas H_2^{max} and H_2L^{max} are less by 30–60% and 20–40%, respectively. Moreover, the points for L, while not connected in Figs. 2A and 3A (see legend of Fig. 2), fell below the random diagonal line (dashed line, Figs. 2A and 3A) at higher SH values and on or near the line at intermediate and lower SH values in all experiments.

Taken together, these results suggest that the probability of forming an HL bond is greater than that for forming an HH bond, at least initially. This information was incorporated into another model where the bonds were still assumed to reoxidize independently of one another but HL formation was made one and one-half times more likely than the formation of any HH bond. Although this model brings improved agreement with the experimental values of HL^{max}, H_2^{max}, and H_2L^{max}, serious discrepancies arise in the profiles of all six components. Further variations of the relative probabilities revealed that in no case would a model with completely independent bond formation fully account for the experimental results; for example, the L-line remains linear in all cases but its intercept with the abscissa moves significantly away from zero as the HL/HH probability ratio is varied from 1.

DISCUSSION

Based on the arrangement of the interchain disulfides in immunoglobulin molecules, three pathways for sequential covalent chain assembly have been proposed (1):

$$\text{I.} \quad H + L \longrightarrow HL \xrightarrow{+H} H_2L \xrightarrow{+L} H_2L_2$$

$$\text{II.} \quad H + L \longrightarrow HL \xrightarrow{+HL} H_2L_2$$

$$\text{III.} \quad H + H \longrightarrow H_2 \xrightarrow{+L} H_2L \xrightarrow{+L} H_2L_2$$

Although any one pathway would be evident by the absence of certain intermediates and the presence of others (appearing with the expected relationships in time), the situation is usually more difficult to interpret. In the 14 mouse tumors and two cultured myeloma cell lines studied by Baumal *et al.* (2), one major and one or more minor pathways appear to be operative in each case. An important complication arising in the interpretation of the events in a system which appears to involve multiple pathways is that a random reoxidation—which by definition represents the lack of any pathway—also gives the *appearance* of multiple pathways (see Fig. 4). We propose that one necessary criterion for establishing multiple

pathways in a system is that the profiles of the H_2L_2 precursors deviate from those expected from the particular restricted random model for that system.

Upon re-examining the assembly data (2, 22) on MOPC 141 (IgG2b), MOPC 31 (IgG1), and Adj PL-5 (IgG2a) from this point of view, we conclude that: (*i*) all three assembly patterns deviate to some extent from the corresponding random models (Fig. 4, lower panels), although the deviations are less pronounced for the latter two proteins; (*ii*) assembly is therefore via one or more pathways; (*iii*) the major assembly pathways in these three instances have been correctly identified as III for MOPC 31 and Adj PC-5 (2, 22) and correctly limited to I and/or II for MOPC 141 (2). A comparison of our theoretical curves with the published experimental results—adjusted to fractional radioactive "intensities"—was possible in these cases because the cells synthesize equal amounts of H and L chains, whereas many do not (23), and the models assume a molar ratio of L/H = 1.

With respect to the relationship between *in vivo* and *in vitro* assembly, several points are worth noting. First, the reoxidation rates found in our 3.2 → 7.5 experiments indicate that H_2L_2 can form in physiologically significant times (cf. ref. 2) *in vitro* even in the absence of a thiol–disulfide interchange system such as the Saxena-Wetlaufer glutathione system (24) utilized by Petersen and Dorrington, who found its presence greatly accelerated H_2L_2 formation. The 4- to 5-fold difference in H_2L_2 half-time observed between the 3.2 → 7.5 and 5.5 → 7.5 experiments, which are the same in terms of levels of intermediates at any stage of reoxidation, is probably related to the numerous changes in physical properties accompanying the denaturation of immunoglobulins below pH 4 (18, 25, 26).

The second point is that comparison of the *in vitro* reoxidation curves of *Fro* with those in Fig. 4B (upper panel) reveals that the reoxidation deviates significantly from the restricted random model. As noted above, no model in which the bonds are independently formed will adequately account for the observed results and the reoxidation then must involve a pathway or pathways with some cooperativity in bond formation.

Where similar experimental procedures allow comparisons, our results are in good qualitative agreement with those of Petersen and Dorrington (8), who studied the reoxidation kinetics of three other IgG1 proteins. An important finding in that work is that IgG1 and IgG4 exhibit different assembly pathways, although the number of interchain disulfides is the same. This suggests that subclass specificity does not arise solely from this source.

Because *in vitro* assembly studies in other proteins customarily involve chain dissociation followed by reassociation, it is important to emphasize that the present studies examine the process of reformation of disulfide bonds in molecules that had all interchain disulfides reduced but remained noncovalently associated as tetramers. However, the presence of noncovalently assembled tetramers prior to covalent disulfide formation must not be taken to imply that the nature and extent of interactions between chains are the same at pH 3.2 and 5.5, or for that matter in the finally assembled molecule. Indeed, the differences in kinetics of reoxidation at pH 7.5 after exposure to pH 3.2 and 5.5, respectively, suggest that the conformation of the chains and the relative spatial relations between chains prior to restoration of reoxidizing conditions is critical to the kinetics of formation of the covalent bonds, if not to the pathway. Moreover, the kinetics of reoxidation, after chain separation in stronger acid, may be quite distinctive from those observed here.

Future papers will accordingly deal with reoxidation of previously dissociated chains, with conformational comparisons of native and reoxidized proteins, with descriptions of models involving cooperativity in disulfide bond formation, and with comparisons of cellular and *in vitro* rates and pathways in mouse proteins.

We would like to thank Dr. E. Osserman for the generous supply of immunoglobulin used in this study and for invaluable advice and guidance throughout the course of this work. We are grateful to Dr. Y. K. Yip for running the sedimentation experiments reported here and to Drs. M. Scharff, B. Birshtein, and S. G. Nathenson for a very stimulating discussion of some of the work presented here. Finally, we wish to thank Dr. K. Dorrington for providing us with his paper prior to publication, and for several invaluable discussions. D.W.S. is a trainee of the National Institutes of Health.

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