Acquisition of the Covalent Quaternary Structure of an Immunoglobulin G Molecule. Reoxidative Assembly in Vitro†

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ABSTRACT: We recently reported results of an investigation of the reoxidation of a human, monoclonal immunoglobulin G, following selective reduction of its interchain disulfides by dithiothreitol (Sears, D. W., et al. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 353). In that work, we described the reoxidative behavior of the molecule under nondissociating conditions. In the present paper, results are presented of the reoxidation of heavy (H) and light (L) chains of this protein alone, or mixed in varying proportions after separation, or mixed with the L chains modified prior to recombination and reoxidation. The overall reoxidative assembly patterns in experiments with H and L separated prior to recombination are similar to those observed when the chains remain noncovalently associated throughout. With equimolar mixtures of H and L, the reoxidation rates also are similar to those of unseparated chains. However, when L chains are present in excess, the overall in vitro rates of covalent assembly are generally diminished, probably indicating transient nonproductive interactions. At the highest molar excesses of L (3:1), the assembly pathways may also be modified. In all experiments with excess L chains, covalent L_2 dimers form at rates which are comparatively slow relative to the H_2L_2 assembly rates. Two kinds of reoxidation experiments with modified L chains are described here for the first time. In the first, the free half-cystine of L is irreversibly blocked by reaction with iodoacetamide, and the alkylated L chains are recombined with reduced H chains. This experiment isolates the reactions in which H_2 disulfides are formed without the accompanying formation of HL bonds. Although the alkylated L chains do not play a direct role in the reoxidation, their presence is required to inhibit aggregation and precipitation of high-molecular-weight products which otherwise ensue; this suggests a possible biological role for excess L in vivo. In the second kind of experiment, covalent L_2 dimers are mixed with reduced H chains. L_2 rapidly disappears with the concurrent appearance of HL, H_2L, and fully assembled H_2L_2. H_2 dimers are also reactive in this process. Special procedures were developed for analyzing the data from these experiments. A complete format is given for the quantitative determination of the concentration of each of the molecular components directly from spectroscopic scans of the gels. The computational methods solve the general analytical problem posed when staining is not proportional to mass and are applicable to a wide variety of systems utilizing gel electrophoresis to study subunit interactions. A theoretical analysis of pathway and kinetic cooperativity in this system is presented in the following paper (Sears, D. W., and Beychok, S. (1977), *Biochemistry 16* (following paper in this issue)).

Although considerable progress has been made toward elucidating the processes through which proteins assume unique, biologically active conformations (see reviews by Anfinsen, 1973; Wetlaufer and Ristow, 1973; Anfinsen and Scheraga, 1975; Baldwin, 1975), this area of protein structure and function is still one of the least well understood. Most of our knowledge concerning protein folding comes from the study of relatively small, single-chain proteins, uncomplicated by the influence of interactions with other polypeptide chains. However, important insights may also be gained from the study of larger multisubunit proteins which require proper subunit assembly at some stage in the folding process.

Immunoglobulins are well suited for studying both the folding and assembly problems. With the availability of large quantities of these proteins, it has been possible to examine extensively the refolding and reassembly of these molecules in vitro from denatured states (see reviews by Dorrington and Tanford, 1970; Cathou and Dorrington, 1975). Moreover, with the establishment of easily maintained tumor cell lines which synthesize and secrete immunoglobulins, it has also been possible to study the intracellular formation of these molecules in considerable detail (see reviews by Scharff and Laskov, 1970; Baumal et al., 1971; and Bevan et al., 1972; Scharff, 1974).

The in vitro studies have, for the most part, focused on the noncovalent assembly of the constituent polypeptide chains of immunoglobulins with irreversibly blocked interchain half-cystines, so that interchain disulfides could not form. In contrast, the intracellular studies have concentrated on the actual covalent assembly process of the chains and thus, indirectly, on the formation of interchain disulfides. Although several investigators (cf. Nisonoff, 1960; Nisonoff and Hong, 1964; Haber, 1964; Fougereau and Edelman, 1964; Whitney and Tanford, 1965; Freedman and Sela, 1966; Stevenson, 1968; Nagy et al., 1971; Jerry and Kunkel, 1972) have made the important observation that a significant degree of native-like structure is recovered in vitro following the reduction and the subsequent reoxidation of disulfides in immunoglobulins, only the initial and final states of the protein were compared by these workers, without analysis of the intervening reoxidation process. Recently, however, in vitro systems for examining the reoxidation kinetics of interchain disulfide bond formation in human immunoglobulins have been developed (Sears, 1974; Petersen and Dorrington, 1974; Sears et al., 1975) and have made possible studies of intermediate states.

†From the Departments of Biological Sciences and Chemistry, Columbia University, New York, New York 10027. *Received October 11, 1976.* Supported in part by grants from the National Institutes of Health (5R01 CA 13014) and the National Science Foundation (BMS74-15008 A02).

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Two phases of our on-going studies of the in vitro assembly of immunoglobulins are covered in this report. First, earlier observations by Sears (1974) and Sears et al. (1975) on the reoxidation of a human IgG1κ are extended and given fuller description. In these "unseparated-chain" reoxidations, nondissociating conditions are employed and the heavy (H)[1] and light (L) chains of the molecule remain noncovalently associated during all stages of the experiments, even after the four interchain disulfides of the molecule are reduced.

The second phase of these studies concerns the results of a series of "separated-chain" reoxidations which are described for the first time. For these experiments, a new protocol is developed for separating H and L chains with reactive interchain half-cystines. This capability provides the opportunity to examine the reoxidation process from several different points of view and, at the same time, to test certain aspects of our understanding of the cellular assembly of immunoglobulins. For example, L chains are usually produced in excess of H chains in cells synthesizing immunoglobulins, although the role of the excess L chains is unclear (Scharff and Laskov, 1970; Baumal and Scharff, 1973). It was possible to probe the effects of such excesses on the in vitro kinetics of reoxidation over the entire range of L/H ratios which have been observed in cellular studies. In the course of these experiments, it was noted that the excess L chains have a pronounced effect in retarding the precipitation of H chains as high-molecular-weight aggregates, and this suggests the possibility of a comparable function intracellularly.

In addition, the excess L intracellularly often converts to L_2 covalent dimers, and it has generally been assumed (Baumal et al., 1971) that L_2, once formed, plays no role in the formation of H_2L_2. By contrast, the in vitro studies reported here show that L_2 can react with H and H_2 to form completely assembled molecules and that the reactivity of L_2 is similar to L in this respect.

Finally, the reoxidation of H chains is studied in the presence of alkylated L chains which have lost their ability to form the inter-HL disulfide. This represents an initial effort toward the systematic investigation of the behavior of each of the disulfides in this molecule.

Materials and Methods

Materials. Whole immunoglobulin and Bence-Jones protein for this study were purified from the plasma and urine, respectively, of a patient (*Fro*) with asymptomatic plasma cell dyscrasia (Osserman, 1971). The plasma and urine were generously donated by Dr. E. Osserman who determined by antigenic typing (unpublished results) that IgG(*Fro*) is an IgG1κ with κ light chains and γ1 heavy chains, and that the Bence-Jones protein is also κ type.

Protein Purification. IgG(*Fro*) was purified as described elsewhere (Sears, 1974; Sears et al., 1975).

Bence-Jones L_2 dimer was isolated from urine by precipitation with 50% saturated ammonium sulfate. Following dialysis and lyophilization, the protein was further purified by DEAE-cellulose chromatography (DE-23) using a salt gradient of 500 mL of 0.02 M Tris-HCl (pH 8.0) and 500 mL of the same buffer containing 0.5 M KCl. Bence-Jones dimer was separated from its monomer and various fragments and polymers by chromatography through a Sephadex G-100 (Pharmacia) column (2.8 × 110 cm) equilibrated with 0.1 M Tris-HCl (pH 8.0). The dimer was shown to be disulfide-linked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis without reducing agent. Under these conditions, it migrated with an apparent molecular weight of 45 000 (A. Kazin, unpublished results). Less than 10% monomer L was detected in the purified dimer fraction.

Separation of H and L Chains. For reoxidation experiments with separated, recombined H and L chains, the procedures of Fleischman et al. (1963) were modified in order to isolate the component polypeptide chains of IgG(*Fro*) with reactive SH groups. Lyophilized protein was dissolved (10–15 mg/mL) in TS (pH 8.1) buffer and reduced for 0.5 to 1 h with a 100-fold (or greater) molar excess of DTT (Calbiochem). The reduced protein mixture was then chromatographed on Sephadex G-100 equilibrated with N_2-aerated 1 M propionic acid (pH 2.3), containing 1 mM EDTA. In some experiments the sample was adjusted with 4.4 M propionic acid to a final concentration of 1 M just prior to chromatography. Both N_2 aeration of the column buffer and the presence of EDTA were necessary to preserve the reduced sulfhydryls during the long chromatography periods.

The eluent from the G-100 column was monitored at 280 nm with an ISCO UA-2 ultraviolet analyzer or a Gilford 250 spectrophotometer. The protein emerged in three absorption peaks, and the material within each peak was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by sulfhydryl measurements (see below). The first peak was excluded by the column and mainly consisted of H chains, presumably in the form of aggregates (Fleischman et al., 1963). The second peak was predominantly H chain, although traces of L, HL, and H_2 were present usually in combined amounts of less than 10% of the total material. The third peak contained only L chains.

H and L from the G-100 column were each pooled in a N_2 atmosphere and separately chromatographed on Bio-Gel P2 (Bio-Rad Laboratories) equilibrated with N_2-aerated 10 mM HOAc (pH 3.2). The column eluent was monitored at 280 nm and approximately two-thirds of the emerging protein peak was collected and stored under N_2 for further use the same day.

When L chains with nonreactive sulfhydryls were required, free SH's were blocked by alkylation with IAAm (Sigma). Following chromatography on G-100, the L chain pool was raised to neutral pH with an equal volume of 4 mM IAAm–2 M Tris-HCl–5mM EDTA (pH 8.7) and incubated for 15 min. IAAm was in approximately a 200-fold molar excess over the L chain SH concentration. Unreacted IAAm was removed by the chromatography step on Bio-Gel P2.

Sulfhydryl Determinations. 1. Measured Sulfhydryl Titers. Sulfhydryl contents were determined by Ellman's method (1959) with modifications introduced by Sears (1974). The SH concentration, (SH), and the SH titer, r, were calculated from the following equations:

$$(SH) = \Delta OD_{412}/\epsilon_M(412) \quad (1)$$

$$r = (SH)/(H_2L_2)_T \quad (2)$$

ΔOD_{412} is the difference in absorption between the sample and a freshly prepared reference containing all but protein; $(H_2L_2)_T$ is the total concentration of protein; and $\epsilon_M(412) = 1.4 \times 10^4$ is the molar extinction coefficient of 5-thio-2-nitrobenzoate (Gething and Davidson, 1972; Collier, 1973). The

[1] Abbreviations used: DTT, dithiothreitol; H, heavy chain; L, light chain; TS (pH 8.1), Tris-saline buffer, 0.15 M Tris-HCl–0.15 M NaCl–2 mM EDTA (pH 8.1); EDTA, ethylenediaminetetraacetic acid disodium salt; SH, sulfhydryl; HOAc, acetic acid; NaOAc, sodium acetate; IAAm, iodoacetamide; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

value of $\epsilon_M(412)$ was confirmed by SH determinations on fully reduced pancreatic RNase.

2. Calculated SH Titers. Although exact SH titers could not be determined from the sodium dodecyl sulfate gel patterns, a minimum–maximum range could be calculated. The H_2, H_2L, and H_2L_2 bands on the gels probably each represent mixtures of molecules with one or two inter-HH disulfide bonds formed. Therefore, if one assumes that all three bands contain only molecules with one HH disulfide formed, the maximum possible number of SH equivalents per mole of total H_2L_2 is:

$$r_{max} = (M_L + 3M_H + 2M_{HL} + 4M_{H_2} + 3M_{H_2L} + 2M_{H_2L_2})/(H_2L_2)_T \quad (3)$$

The numerator here comprises the molar quantities of each intermediate multiplied by their respective SH equivalents, and the denominator is the total possible concentration of H_2L_2. Conversely, if one assumes that all H_2, H_2L, and H_2L_2 molecules have both HH bonds formed, the minimum number of SH equivalents is:

$$r_{min} = (M_L + 3M_H + 2M_{HL} + 2M_{H_2} + M_{H_2L})/(H_2L_2)_T \quad (4)$$

The ratios, $M_L/(H_2L_2)_T$, etc., which define r_{min} and r_{max} were calculated by the quantitative procedures discussed in the next section.

Quantitative Polyacrylamide Gel Electrophoresis. 1. Experimental Procedures. An Ortec Model 4200 electrophoresis system was used with 5% polyacrylamide gel slabs containing 0.1% sodium dodecyl sulfate and 0.05 M sodium phosphate (pH 7.2). Gels were prepared according to the procedures of Weber et al. (1972). Samples for electrophoresis were adjusted to near neutral pH and contained 1% sodium dodecyl sulfate (w/v), 0.09 M IAA, 10% glycerol (v/v), and 0.01% bromphenol blue tracking dye. All samples were heated either for 1 min in boiling water or overnight at 37 °C. The gels were stained for 1 h in 0.25% Coomassie brilliant blue (Schwarz/Mann) dissolved in 45% (v/v) methanol and 9% (v/v) HOAc. Destaining was by diffusion of the dye into several liters of gently agitated 5% (v/v) methanol–7.5% (v/v) HOAc. Two to three days of destaining were required to completely reduce the optical background of the gel to a uniform level of transparency.

2. Identification of Protein Bands. By comparing IgG(*Fro*) with reduced and unreduced, pooled, human 7S IgG on sodium dodecyl sulfate gels, it was concluded that the major band of unreduced IgG(*Fro*) corresponds to H_2L_2, while the two major bands of reduced IgG(*Fro*) correspond to H and L chains. Four other major bands were observed in these experiments. Three were identified as HL, H_2, and H_2L by their migration distances relative to H_2L_2, H, and L. The relative migration distances of these six components on the gels are linearly proportional to the logarithms of their respective molecular weights (Shapiro et al., 1967; Weber et al., 1972); the molecular weights of H and L were estimated to be 51 000 and 23 500, respectively, by averaging the molecular weights of H and L chains with known sequences (Dayhoff, 1972) and assuming 4% carbohydrate for H chains (Smith et al., 1946). The final major band appearing in some of the reoxidations was L_2, as identified by its relative mobility.

Five minor bands were also observed on the sodium dodecyl sulfate gels, each associated with and migrating slightly ahead of a corresponding major H chain-containing band (Sears et al., 1975). The staining intensity of any one minor band rarely exceeded 6% of the corresponding major band. When analyzed separately (Sears, 1974), the minor components show the same general pattern of reoxidation as the major ones and, for the analysis of the gels, it was assumed that the pairs could be treated as one.

3. Densitometry. To measure protein staining intensities, the gel slabs are cut into longitudinal strips along the edges of each well. The 8 × 80 mm strips are placed horizontally into spectral cuvettes containing destaining solution and scanned for absorbance at 570 nm with a Gilford 241 spectrophotometer equipped with a linear gel transport and chart recorder.

4. Quantitation of Protein. In order to determine the amount of each component at various time points in the reoxidation experiments, new procedures were developed for analyzing the densitometer scans. The band intensities are determined from the scans by cutting the individual peaks and weighing them. Fractional intensities are first calculated by dividing each band intensity by the total staining intensity of the gel pattern. This serves to normalize the intensities and, at the same time, eliminates or greatly minimizes problems such as sampling and dilution errors, baseline uncertainties, and variations in staining efficiency.

The H to L molar staining intensity ratio ($F_{H/L}$) is then determined from the fractional intensities (I_L, I_H, etc.). The most direct approach to this problem is to assume that $F_{H/L}$ equals 2, since H has approximately twice the molecular weight of L. However, Coomassie brilliant blue does not stain proteins *strictly* on a weight basis (Fazekas de St. Groth et al., 1963), and a more rigorous approach, as outlined below, is to calculate $F_{H/L}$ for each gel point from the conservation of mass equations. In the present work, the most serious error introduced by the approximation that $F_{H/L}$ equals 2 is in determination of the concentration of L, which is incorrect by as much as 33% in extreme cases; the errors in H and H_2 are as much as 16% (overvaluations) in a few instances, and the errors in HL, H_2L, and H_2L_2 are usually negligible.

In the experiments described, the total molar concentration of L (M_L^T) either equals or exceeds the total molar concentration of H (M_H^T) and the mathematical solution for $F_{H/L}$ differs in the two cases. With $M_L^T/M_H^T = R_{L/H}$, where $R_{L/H} \geq 1$, the general conservation of mass equation is:

$$\frac{I_L}{f_L} + 2\frac{I_{L_2}}{f_{L_2}} + \frac{I_{HL}}{f_{HL}} + \frac{I_{H_2L}}{f_{H_2L}} + 2\frac{I_{H_2L_2}}{f_{H_2L_2}} = \left(\frac{I_H}{f_H} + \frac{I_{HL}}{f_{HL}} + 2\frac{I_{H_2}}{f_{H_2}} + 2\frac{I_{H_2L}}{f_{H_2L}} + 2\frac{I_{H_2L_2}}{f_{H_2L_2}}\right) R_{L/H} \quad (5)$$

The parameters f_H, f_L, etc. are the staining intensities per mole of H, L, etc. I_L/f_L is proportional to the number of moles of L monomer; $2I_{I_2}/f_{L_2}$ is proportional to the number of moles of L in L_2, etc. It is assumed that f_L and f_H are constant regardless of the covalent state of the chain and therefore that $f_{HL} = f_H + f_L$, $f_{H_2} = 2f_H$, $f_{H_2L} = 2f_H + f_L$, and $f_{H_2L_2} = 2f_H + 2f_L$. With these equalities substituted into eq 5, the resulting equation can be solved for the staining intensity ratio of H to L, i.e., $f_H/f_L = F_{H/L}$. When $R_{L/H} = 1$ ($I_{L_2} = 0$), $F_{H/L}$ is the root of a quadratic equation

$$F_{H/L} = [B + (B^2 + C)^{1/2}]/4 \quad (6)$$

where

$$B = [(2I_H + 2I_{H_2} + I_{H_2L})/I_L] - 1 \quad (6a)$$

and

$$C = 8(I_H + I_{H_2})/I_L \quad (6b)$$

When $R_{L/H} > 1$, $F_{H/L}$ is the root of a cubic equation, and in all cases examined, only one real, positive root obtained.

$$F_{H/L} = 2(-a/3)^{1/2} \cos(\phi/3) - (p/3) \quad (7)$$

where

$$\phi = \cos^{-1}[-b/(2(-a^3/27)^{1/2})] \quad (7a)$$

$$a = q - (p^2/3) \quad (7b)$$

$$b = (2p^3 - 9pq + 27s)/27 \quad (7c)$$

$$s = -R_{L/H}(I_H + I_{H_2})/2(I_L + I_{L_2}) \quad (7d)$$

$$p = 3/2 + [2(1 - R_{L/H})(I_{HL} + I_{H_2L_2}) + (1 - 2R_{L/H})I_{H_2L}]/2(I_L + I_{L_2}) + 2s \quad (7e)$$

$$q = p - 1 + s - (1 - R_{L/H})(I_{HL} + I_{H_2L_2})/2(I_L + I_{L_2}) \quad (7f)$$

The calculated values for $F_{H/L}$ vary as the pattern of intermediates changes during the course of the reoxidation. Initially, with 85% or more of the protein in the H and L bands, the average value observed for $F_{H/L}$ was 2.34 ± 0.23 (29 determinations).

Although the actual molar concentrations of each component in the reoxidation mixture could be calculated once $F_{H/L}$ was determined (Sears, 1974), another set of parameters—the fractional moles—proved to be much more useful for comparing levels of intermediates in different experiments. The number of fractional moles (fM) is defined as the observed number of moles of a given component divided by the theoretical total *possible* number of moles of that component in the reoxidation mixture. In order to define the fractional moles in terms of $F_{H/L}$ and $R_{L/H}$, the total intensity was considered in two parts: $I^T = 1 = I_L{}^{X,T} + I_{H_2L_2}{}^T$ where $I_L{}^{X,T}$ is the total contribution to the intensity from all excess L chains—either as monomers or dimers—and $I_{H_2L_2}{}^T$ is the remaining intensity. The latter can be thought of as the total possible intensity of H_2L_2 were all components covalently assembled as such. The following abbreviated equations are found for the fractional moles:

$$fM_{L,L_2} = (F_{H/L} + R_{L/H})I_{L,L_2} \quad (8)$$

$$fM_{H,H_2} = (F_{H/L} + R_{L/H})\, I_{H,H_2}/F_{H/L} \quad (9)$$

$$fM_{HL,H_2L_2} = (F_{H/L} + R_{L/H})\, I_{HL,H_2L_2}/(F_{H/L} + 1) \quad (10)$$

and

$$fM_{H_2L} = 2(F_{H/L} + R_{L/H})I_{H_2L}/(2F_{H/L} + 1) \quad (11)$$

With these equations every component except L and L_2 is represented on a scale of 0 to 1. When $R_{L/H}$ is greater than one, fM_L and fM_{L_2} can exceed one depending on the distribution of L between monomeric and dimeric forms on the gel. With fM_L and fM_{L_2} so defined, the contribution of the excess L chains to the reoxidation can easily be eliminated for the sake of comparing experiments with different $R_{L/H}$ values. Specifically, the observed fM_L and the measured sulfhydryl concentration, (SH), are corrected as follows:

$$fM_L' = fM_L - (R_{L/H} - 1 - fM_{L_2}) \quad (12)$$

and

$$r_{H_2L_2} = (SH)/(H_2L_2)_T - 2(R_{L/H} - 1 - fM_{L_2}) \quad (13)$$

fM_L' is the amount of L which reacts stoichiometrically with H, and $(R_{L/H} - 1 - fM_{L_2})$ is the number of fractional moles of excess L monomers as seen on the gels. (It is always assumed that, when the L_2 levels were low initially, no L_2 takes part in the formation of H_2L_2, and all L_2 is the product of the excess L chains in the reoxidation.)

Reoxidation Experiments. 1. Recombined H and L Chains. Immediately following their isolation, H and L chains, in 10 mM HOAc (pH 3.2) and under a N_2 atmosphere, were recombined in varying molar ratios. The mixture was further incubated under N_2 for 15–30 min before the pH and ionic strength were adjusted to 7.5 and 0.14, respectively, with the slow addition (while stirring) of 0.5 M Tris-HCl–1 M NaCl (pH 8.0) amounting to one-tenth of the final sample volume. The final concentrations were 0.05 M Tris, 0.1 M NaCl, and 1 mM acetate. The temperature varied between 20 and 26 °C. The reaction vessel allowed for a large liquid surface area to permit rapid exchange of N_2 with atmospheric O_2 after raising the pH. At various time intervals, 2-mL aliquots were removed from the reaction mixture for SH measurements. For gel analysis, 1-mL aliquots were removed and immediately alkylated by adding 0.1 mL of freshly prepared 1 M IAAm prior to preparation of the sample for electrophoresis.

Protein concentrations were determined spectrophotometrically at 278 nm using the following molar extinction coefficients (Sears, 1974): $\epsilon_L = 2.35 \pm 0.04 \times 10^4$; $\epsilon_H = 7.2 \pm 0.3 \times 10^4$; $\epsilon_{H_2L_2} = 1.91 \pm 0.06 \times 10^5$. To determine the best value for $R_{L/H}$, the small amount of L, present as HL, H_2L, and free L, which invariably accompanied the H chain preparation, was included in the calculation along with the mixing volumes and OD's of the chain preparations. The total concentration of H_2L_2 in the mixture was found with the following equation:

$$(H_2L_2)_T = OD_{278}/(\epsilon_{H_2L_2} + 2(R_{L/H} - 1)\epsilon_L) \quad (14)$$

$(H_2L_2)_T$ ranged from 2.5 to 5.2 μM in these experiments. In some cases, faint turbidity occurred as the pH was raised, and the measured OD's were, therefore, corrected for light scattering as described by Leach and Scheraga (1960).

2. Unseparated H and L Chains. Some experiments were performed as described by Sears et al. (1975) in which the H and L chains were allowed to reoxidize without prior separation. The procedures for this type of experiment were essentially the same as those above without the chromatography step in propionic acid.

Results

Number of Interchain Disulfides. The number of interchain disulfides in IgG(*Fro*) was determined indirectly by quantitative sulfhydryl measurements on reduced[2] and unreduced protein. Upon partial reduction, L chains contained 1.0 ± 0.2 equiv of SH per mol, H chains 3.0 ± 0.3, and unseparated H_2L_2 8.4 ± 0.2; the value for the unreduced protein was less than 0.2 equiv. The reduction and separation procedures thus yield the expected values with no, or negligible, reduction of any intrachain bonds.

Reoxidation of Unseparated H and L. The reoxidation kinetics of reduced but unseparated H and L chains have been reported previously (Sears, 1974; Sears et al., 1975). However, for the purpose of using the unseparated chain reoxidation experiment as a basis of comparison with other types of reoxidation experiments reported below, additional experiments of this kind were carried out. The experimental conditions were

[2]The term "reduced" as it is used throughout will specifically refer to partially reduced chains with reduced interchain disulfides only, as opposed to fully reduced chains with reduced intra- as well as interchain disulfides.

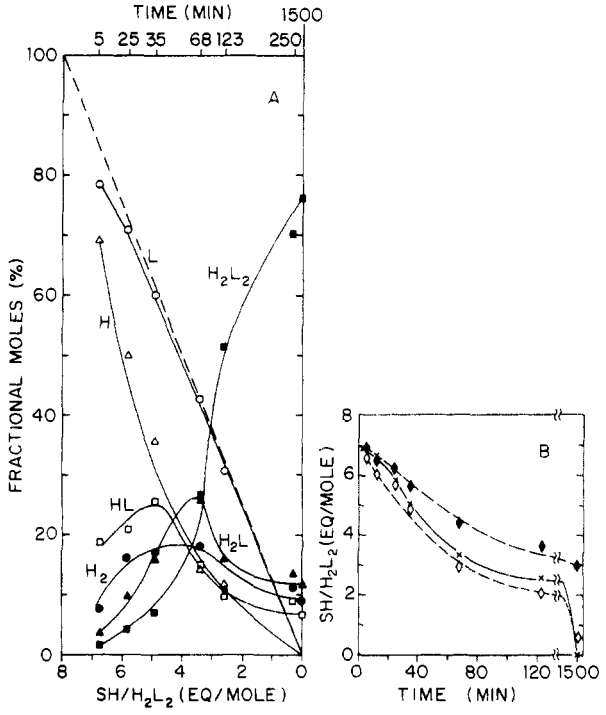

FIGURE 1: Unseparated-chain, 3.2 → 7.5 reoxidation. IgG(*Fro*) at 31.5 μM was reduced by a 100-fold molar excess of DTT for 30 min and then chromatographed on Bio-Gel P2 in 10 mM HOAc (pH 3.2) to remove the reductant. The protein pool from this column was adjusted to reoxidizing conditions of pH 7.5 and $\mu = 0.14$ with a Tris-saline buffer as described in the Materials and Methods section. The total protein concentration, $(H_2L_2)_T$, in the final reoxidation mixture was 3.4 μM. (A) L (○), H (△), HL (□), H_2 (●), H_2L (▲), and H_2L_2 (■) are the fractional moles (in percent) of each component as calculated (see Materials and Methods) from the sodium dodecyl sulfate gel electrophoresis patterns of samples which were removed from the reaction vessel and alkylated with 1.0 M IAAm at the times indicated at the top of the figure. Each component is plotted against the SH titer measured at the time intervals shown in the other panel of this figure. The diagonal dashed line is a calculated line for L (see Sears and Beychok, 1977). (B) The measured (X) SH titer and the calculated minimum (◇) and maximum (◆) SH titers, as determined from the gels (see Materials and Methods), vs. time. In each case, the SH titer, or the number of SH equivalents per mole of H_2L_2, is based on the total possible concentration of H_2L_2, $(H_2L_2)_T$.

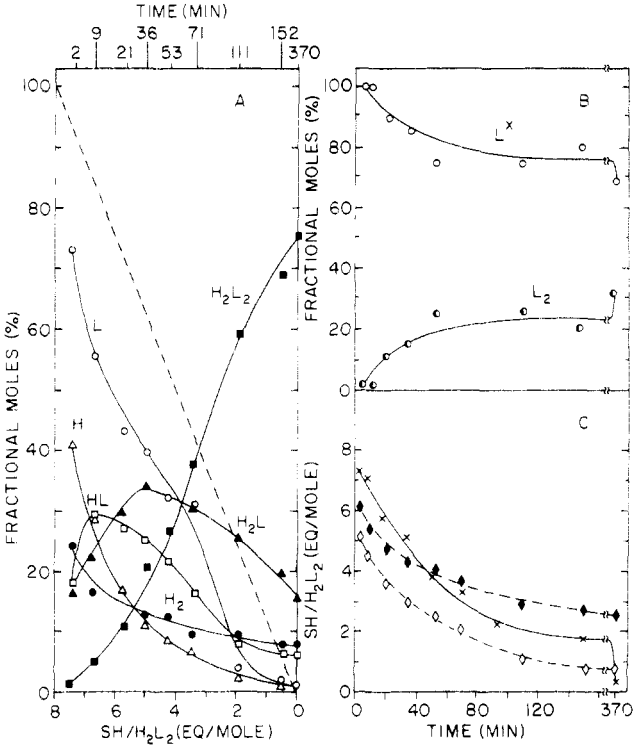

FIGURE 2: Recombined-chain reoxidation; $R_{L/H} = 1.13$. Following reduction of IgG(*Fro*) by DTT, the H and L chains were separated by chromatography on Sephadex G100 in N_2 aerated, 1 M propionic acid–1 mM EDTA (pH 2.3). The L and H pools were separately chromatographed on Bio-Gel P2 in N_2 aerated 10 mM HOAc (pH 3.2), and then mixed in a molar ratio of 1.13 just prior to raising the pH and ionic strength for reoxidation (see Materials and Methods). In the final reoxidation mixture $(L)_T = 6.6$ μM, $(H)_T = 5.8$ μM, and $(H_2L_2)_T = 2.9$ μM. (A) The symbols for the various components and the axes are as defined in the legend of Figure 1. The L line and the measured SH titer (abscissa) are corrected here for the L excess over H as described in Materials and Methods. (B) Fractional moles of the excess L vs. time. On the sodium dodecyl sulfate gels, the excess L appears in two forms: either as a monomer, L (○), or as a covalent dimer, L_2 (◐). The ordinate has been normalized relative to the total concentration of excess L chains ($L^x + 2L_2$) which was 0.75 μM in this case. (C) The measured and calculated SH titers vs. time as in Figure 1B. The SH titers are corrected for the SH contribution of L^x and are determined relative to $(H_2L_2)_T$.

kept as nearly comparable as possible to those used in the previous experiments. Figure 1 shows the results of a "3.2 → 7.5" reoxidation of reduced, unseparated chains following chromatography on Bio-Gel P2 equilibrated with 10 mM HOAc. The profile of intermediates agrees well with those of the earlier work (cf. Table I in Sears et al., 1975). Two aspects of recent experiments differ from findings reported earlier. One is that the maximum peak height reached by H_2 has in some cases exceeded earlier values by as much as 100%. The other difference is that the overall rates of reoxidation, as judged by half-times for sulfhydryl disappearance of H_2L_2 formation, are slower by as much as two- to threefold. Despite this variability (see Discussion), numerous kinetic experiments reveal that the rate differences have no apparent effect on the resulting profiles of the various components when plotted as functions of the SH titer.

The kinetics of disappearance of the measured sulfhydryl are shown in Figure 1B. As one way of evaluating the consistency between measured SH titers and levels of various intermediates on the gels, SH titers are also calculated from the gel patterns. The min–max range for this experiment is plotted in Figure 1B and brackets the experimental SH titer throughout the course of the reoxidation. Although the agreement between the measured and predicted numbers of SH equivalents is not always this close (see below), these two independently determined parameters usually fall to within 10%.

Reoxidation of Recombined Chains: L/H ≃ 1. Figure 2 shows the results of an experiment in which the H and L chains of IgG(*Fro*) were first separated and subsequently recombined prior to establishing reoxidation conditions. The L line in Figure 2A is corrected at each time point for the light chain excess, L^x, in the experiment and all components are plotted as functions of the corrected SH titer (see eq 12, 13, and 14). This procedure allows a direct comparison between Figures 2A and 1A.

The reoxidation profiles and the rates of the unseparated-chain reoxidations and "one-to-one" recombined-chain reoxidations are generally similar. As shown in Table I, values of H_2^{max}, HL^{max}, and H_2L^{max} are found in the same relative order and the average time per unit change in SH titer for the first half of the reoxidation ranges between 15 and 18 min for both the unseparated-chain and recombined-chain reoxidations.

The greatest dissimilarities between Figures 2A and 1A are found in the positions of the various curves. L, HL, and H_2L,

TABLE I: Comparisons between Unseparated-Chain and Recombined-Chain Reoxidations.[a]

$(H_2L_2)_T$[b]	$R_{L/H}$[c]	$fM_{H_2}^{max}$ (r)[d]	fM_{HL}^{max} (r)[d]	$fM_{H_2L}^{max}$ (r)[d]	t_{av}[e]/SH
		Unseparated-Chain Reoxidations			
3.4	1	25 (*f*)	33 (4.8)	28 (3.1)	16
3.5	1	18 (3.4)	26 (4.9)	27 (3.4)	18
		Separated-Recombined-Chain Reoxidations			
2.9	1.13	24 (7.4)	29 (6.7)	34 (5.0)	15
4.7	1.0	27 (6.4)	27 (7.1)	43 (3.9)	17
2.1	2.19	12 (*f*)	31 (*f*)	26 (3.6)	*f*
2.0	2.10	27 (7.5)	20 (5.9)	33 (3.8)	33
1.8	2.28	17 (7.0)	24 (7.0)	28 (5.6)	43
2.2	2.03	26 (*f*)	30 (4.2)	22 (3.4)	42
1.7	3.46	17 (3.9)	30 (4.5)	36 (3.6)	>50
1.7	3.26	32 (7.6)	20 (4.9)	39 (3.2)	19
1.4	3.80	33 (3.9)	25 (5.3)	15 (3.9)	>50
1.7	3.21	25 (*f*)	16 (7.0)	26 (4.1)	>50

[a] All experiments are 3.2 → 7.5 reoxidations (see text). [b] Total possible concentration of H_2L_2 in units of μM. [c] Molar ratio of L to H. [d] Fractional moles, in percent; *r* in parentheses is the corresponding number of SH equivalents per H_2L_2 at the maximum (see text). [e] Average time in minutes per unit change in the measured SH titer from time zero to the time $r = 4$. [f] Insufficient data.

in particular, appear shifted by more than 1 SH unit to the left in Figure 2A as compared with Figure 1A. This apparent shift is mainly attributable to the fact that in the recombined-chain experiment the measured SH titer exceeds the calculated range from the gels in the early stages of the reoxidation, as shown in Figure 2C. The largest discrepancy occurs at the first time point where the measured *r* value (corrected for L^x) is greater than r_{max} by about 1.25 SH units. This discrepancy is directly reflected in Figure 2A by the relatively high levels of reoxidized intermediates at unexpectedly high measured SH titers, thus creating the apparent shift. Generally, measured and calculated SH titers agree closely in unseparated-chain reoxidations but not in recombined-chain reoxidations.

The L chain which is in molar excess over H is analyzed separately in Figure 2B. It is seen that the excess L chain monomers, L^x, reoxidize, albeit very slowly, to covalent L_2 dimers. At any one time the total population of excess light chains appears on the sodium dodecyl sulfate gels as a varying combination of L and L_2. Note that L^x and L_2 have been plotted in Figure 2B against an ordinate scale of 0 to 100. In this particular experiment, L is in total excess of H by about 13%, and, in order to facilitate comparisons with other experiments, the fractional moles of these two species were normalized relative to the 13% excess.

Reoxidation of Recombined Chains: L > H. To test the effect of L chain excesses on H_2L_2 reoxidation, H chains were mixed with up to 3.5-fold molar excesses of L. The results of such an experiment are shown in Figure 3 where L exceeds H by approximately 2.25-fold. As discussed with reference to Figure 2A, the fractional moles of L and the SH titer in Figure 3A have been corrected for excess L chains. Therefore, one can directly compare Figure 3A with Figures 1A and 2A. Qualitatively, all three are similar. H_2^{max}, HL^{max}, and H_2L^{max} are in the same relative ratios, and L behaves similarly. Again, the excess L chains slowly reoxidize to covalent L_2 dimers as indicated in Figure 3B. In Figure 3C the measured SH titer (corrected for L^x) is compared with the min–max range calculated from the gels.

Comparable results were obtained for other excess chain reoxidations as outlined in Table I in the sense that even large excesses of L chains, more than three to one, do not markedly change the assembly pathway. However, there are occasional discernable differences in the maximum values achieved by

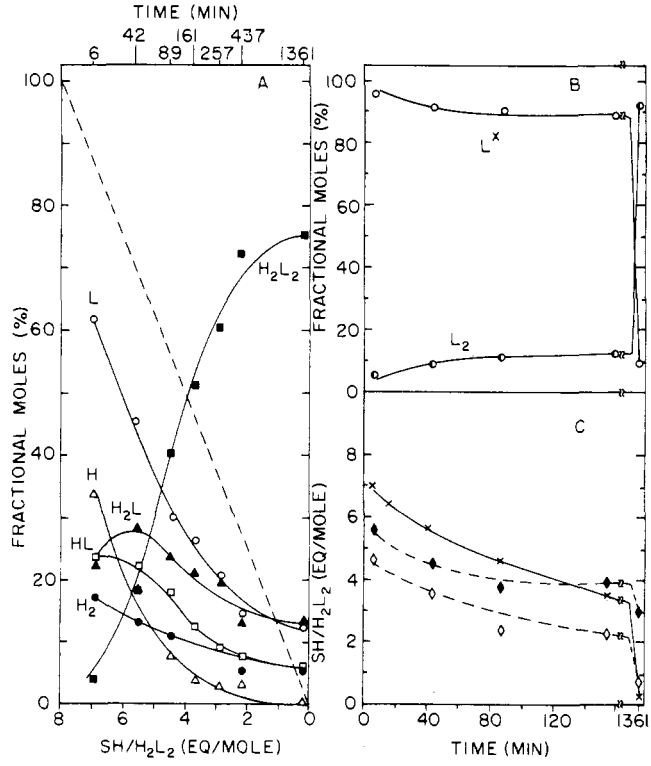

FIGURE 3: Recombined-chain reoxidation; $R_{L/H} = 2.28$. This experiment is exactly as that described in the legend of Figure 2 except that $(L)_T$ = 10.5 μM, $(H)_T$ = 4.6 μM, and $(H_2L_2)_T$ = 2.3 μM.

the intermediates and in their relative orders[3]. This was especially obvious when the initial values of H_2 were high, but other factors may also influence the actual and relative intensities.

The last column of Table I reveals that, with one unexplained exception, the rate of sulfhydryl disappearance in excess L experiments was slower than that in the "one-to-one" experiments.

[3] In two out of 13 such experiments, H_2^{max} exceeded HL^{max} but this discrepancy was judged to be the consequence of abnormally high levels of H_2 at the initial stage of the reoxidation.

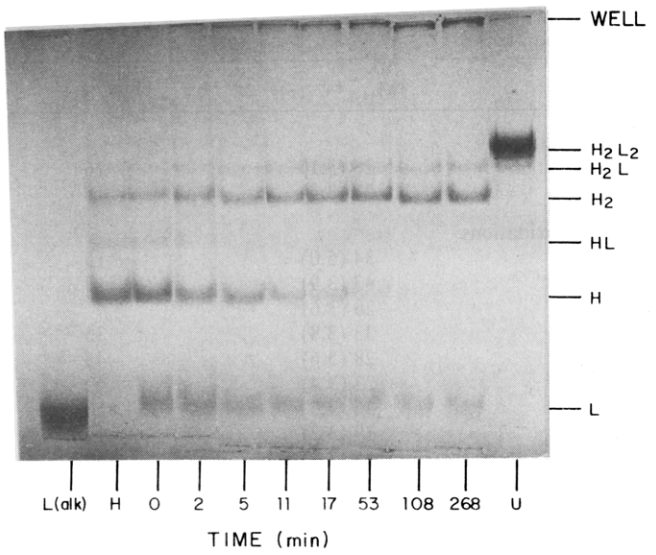

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gels of the reoxidation of H chains mixed with prealkylated L chains, L(alk); $R_{L/H} = 1.5$. The samples in the gels designated L(alk) and H were taken from the respective pools just prior to their being mixed for reoxidation. The gel designated by U is that of an unreduced sample of IgG(*Fro*), included for reference. In the final reoxidation mixture $(L)_T = 5.5\ \mu M$, $(H)_T = 3.7\ \mu M$, and $(H_2L_2)_T = 1.85\ \mu M$.

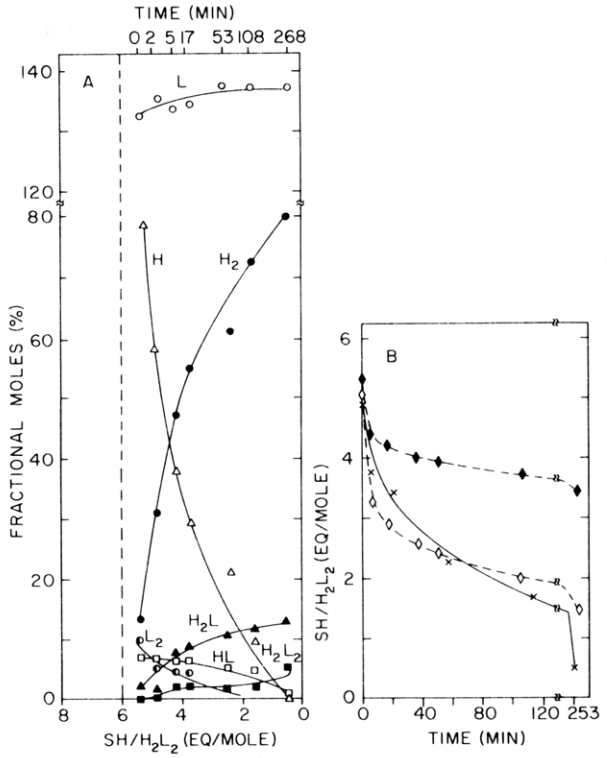

FIGURE 5: Analysis of the gels of the recombined-chain reoxidation with prealkylated L shown in Figure 4; $R_{L/H} = 1.5$. The fractional moles of each component vs. SH (A) and the change in SH titer vs. time (B) are shown using the same symbol designations as in Figure 1. Note that the ordinate in A exceeds 100% here because the excess L over H was not subtracted from the total amount of L seen on the gels. The vertical dashed line in A represents the theoretical upper limit on the SH titer if all L chains are alkylated.

Reoxidation of the Individual Chains. H and L chains were also reoxidized individually after their separation. As expected from the foregoing reoxidation experiments with L chain excesses, L is found to reoxidize slowly to covalent L_2 dimers.

H chains are not as well-behaved when alone in the reoxidizing buffer. In addition to the expected formation of H_2 dimers, higher molecular weight covalent aggregates also readily form. This behavior of H is consistent with the observations of Stevenson and Dorrington (1970) and Bjork and Tanford (1971a), who found that the H chains of IgGs are usually soluble at acid pH but insoluble near neutral pH, in solution which are approximately physiological in ionic strength. The formation of aggregates is too rapid in these experiments to allow any conclusions to be reached about the reoxidative behavior of the inter-HH disulfides of the H chains in the absence of L.

Reoxidation of H Combined with Alkylated L. Because H chains require the presence of L in the reoxidizing buffer to remain soluble and unaggregated, one straight-forward way of studying the reoxidation of the inter-HH disulfides—in the absence of inter-HL bond formation—is to block the SH groups of the L chains irreversibly with IAAm before mixing them with H. Figures 4 and 5 show the results of such a reoxidation where L chains with alkylated interchain sulfhydryls, L(alk), are combined in approximately 50% molar excess over H chains with reactive sulfhydryls. The sodium dodecyl sulfate gels of this experiment (Figure 4) clearly illustrate the fact that the level of L does not change significantly over the entire course of the experiment as expected. However, H_2 dimers form rapidly. Although some aggregates (presumably from the H chains) do eventually form, the degree of aggregation is only a small fraction of that which occurs when H chains are alone.

The quantitative analysis of this reoxidation is shown in Figure 5. The predominant event in this reaction is clearly the covalent conversion of H to H_2. However, residual levels of other species are also formed as indicated in the lower portion of the figure. These arise because small amounts of HL and L are invariably present in the H chain pooled fractions from the Sephadex G-100 column and also because the procedure for alkylating the L chains results in the conversion of some L to L_2. Small amounts of L and HL are available then for reoxidation and, as discussed in the next section, L_2 is also capable of reoxidizing with H.

The SH titer is well-behaved for this reaction, the measured value falling consistently within the range calculated from the gels, is indicated in Figure 5B. Because the L SH groups are blocked, the maximum measured number of SH equivalents per mole of H_2L_2 should not exceed 6, and this is consistent with the results. The vertical dashed line in Figure 5A delineates this boundary on the reoxidations by marking the theoretical beginning of the reaction on the SH scale. If no incorrect disulfides are formed, the reaction should in principle end at a sulfhydryl titer of 2, where the two inter-HL half-cystines of the H chains would remain unreoxidized. The sulfhydryl titer does, in fact, level off near or just below this value. At longer times it drops significantly below two, indicating either that some of the inter-HL bonds of the H chain do eventually form with the residual reactive L or that the SH groups are oxidized to other inactive forms (perhaps even inter-HH bonds).

Reoxidative Formation of H_2L_2 by H and H_2 Combined with L_2. As illustrated in Figure 6A, when urinary Bence-Jones, when covalent dimer, L_2, is mixed with partially reduced H and H_2 under reoxidizing conditions, covalent H_2L_2 molecules are reoxidatively generated with the concurrent reduction of L_2. There are a number of interesting points to note in this experiment. First, the initial level of H_2 is extremely high—

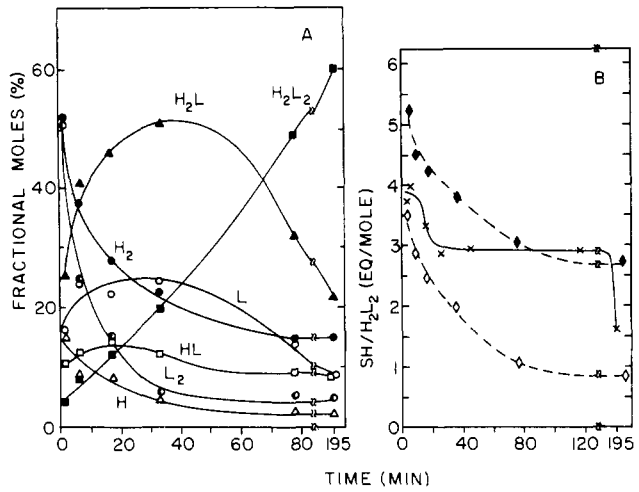

FIGURE 6: Reoxidation of H chains combined with L_2; $R_{L/H}$ = 1.14. Reduced H chains, isolated from reduced IgG(*Fro*), and Bence-Jones L_2 covalent dimers were mixed in the following amounts: $(L_2)_T$ = 4.0 μM and $(H)_T$ = 7.0 μM to give for the final reoxidation mixture, $(H_2L_2)_T$ = 3.5 μM. The total molar excess of L chains equalled 1.0 μM. The fractional moles of each component vs. time (A) and the change in the SH titer vs. time (B) are shown using the symbol designations described in the legends of Figures 1 and 2.

much higher in fact than H—and this is probably a result of H reoxidation occurring during a long period of standing at acid pH before mixing with L_2. In any event, the predominant initial reaction is the formation of H_2L and L from H_2 and L_2 as indicated by the sharp drop in H_2 and L_2 and the sharp rise in H_2L and L at the beginning of the reoxidation. The rise in HL also indicates that L_2 reacts with H to generate L and HL. A final point to note is that the overall rate of the reaction is similar to those found in the reoxidations discussed in preceding sections. The time for H_2L_2 to reach 50% fractional moles, for example, is approximately 80 min.

Thus, the H chains are able to reduce the L_2 interchain bond and subsequently incorporate L into the form of H_2L_2. The mechanism for this reaction is disulfide exchange, as indicated in Figure 6B, where the measured and calculated SH titers are plotted. After an initial drop, the measured SH concentration remains fixed through the middle time period of the reoxidation before again dropping to lower values. Thus, while the levels of the various species undergo dramatic changes from 20 to 120 min after the start of the reoxidation, the SH concentration remains constant. It is because of this constancy in SH concentration that the reaction is plotted against time in Figure 6A rather than against the SH titer as was the case in preceding figures.

Discussion

Our basic objective in this and prior studies (Sears, 1974; Sears et al., 1975) has been to examine the acquisition of the covalent quaternary structure of an immunoglobulin molecule in vitro. The formation of the interchain disulfides of the molecule was studied by reducing these bonds and establishing conditions for their reoxidation. Several essential features of this process were noted: (1) The rate and pattern of covalent chain assembly was qualitatively the same whether the reduced H and L chains remained associated or were separated by propionic acid and recombined prior to establishing reoxidation conditions. (2) Molar excesses of L over H chains—up to threefold—did not have a marked effect on the pattern of covalent H_2L_2 assembly, although at mole ratios of greater than 3:1, the values of $fM_{H_2}^{max}$ were increased (in three of four experiments) and the overall rates of sulfhydryl disappearance were diminished, suggesting the possible occurrence of nonproductive transient intermediates. (3) The basic pattern of covalent assembly appears to be characteristic of human IgG1 proteins in general since Petersen and Dorrington (1974) and, very recently, Kishida et al. (1976) found qualitatively similar reoxidation patterns in studies of other myeloma proteins. (4) Reduced H chains readily reoxidize to H_2 dimers in the presence of alkylated L chains, which have irreversibly lost their capacity to form inter-HL disulfide bonds. (5) A disulfide bond between L chains in the form of L_2 dimers does not prevent the formation of inter-HL disulfide bonds; on the contrary, mixtures of reduced H chains and covalent L_2 dimers readily reoxidized to fully assembled H_2L_2 molecules with concurrent disappearance of L_2 through disulfide exchange.

From the first two observations above, one can draw the broad conclusion that the covalent chain assembly of IgG(*Fro*) in vitro proceeds in a characteristic fashion which, under the conditions of these experiments, at least, is qualitatively independent of whether the chains are physically separated prior to reoxidation and whether L exceeds H in concentration. The basic similarity between the unseparated-chain and recombined chain reoxidations is probably accounted for by the very strong, essentially irreversible, noncovalent affinities between chains which maintain H and L in the assembled H_2L_2 form in both types of experiment. Sears et al. (1975) described sedimentation velocity experiments which indicated that at every stage of the unseparated-chain reoxidation experiments, and particularly during reoxidation, IgG(*Fro*) remains tetrameric with little if any dissociation. This observation is in accord with numerous observations from other laboratories (Mannik, 1967; Stevenson and Dorrington, 1970; Bjork and Tanford, 1971b; Zimmerman and Grey, 1972; Azuma et al., 1975), including the observations of Bigelow et al. (1974) and Azuma and Hamaguchi (1975), who estimated from kinetic data that the dissociation half-time of reduced and alkylated human IgG is on the order of 10^4 to 10^5 h, or greater, at pH 5. In the separated-chain reoxidation, chain reassociation into tetramers is expected to be rapid. Preliminary sedimentation studies (A. Kazin, unpublished results) indicate that, while alkylated L and H chain mixed together in 10 mM HOAc (pH 3.2) do not rapidly reassociate, they do so almost immediately after the buffer is adjusted to reoxidizing conditions. Other studies also show the reassociation process to be relatively slow at acid pH (Azuma et al., 1975) and increasingly more rapid—a few minutes (Bigelow et al., 1974; Azuma et al., 1975) or even a few seconds (unpublished results of Bellon, Halbwachs, Delaage, and Fougereau, as cited in de Préval and Fougereau, 1976; Friedman, Yang, and Bevchok, unpublished results)—as the pH approaches neutral values. Thus, disulfide formation is the rate-limiting step in the separated-chain reoxidations and it takes place for the most part in the preassembled tetramers thereby accounting for the similarity between this type of experiment and the unseparated-chain reoxidations.

The fact that the H and L chains in these experiments exist primarily as noncovalently assembled tetramers with varying numbers of covalent interchain disulfide bonds formed between them, depending on the extent of reoxidation, simplifies the theoretical interpretation of these results, as discussed in the following paper (Sears and Beychok, 1977). There it is demonstrated that under the conditions of these experiments the disulfides do not reform randomly, but do so in a cooperative fashion: as compared with the inter-HH disulfides, the inter-HL disulfides are almost twice as likely to reoxidize in the

first half of the reoxidation, but reoxidize with an equal likelihood during the last half of the reoxidation.

Because the bonds do not form independently of one another and the pathway analysis is very complex, it is essential to dissect the process into its individual steps wherever possible. As an initial approach toward this objective, we isolated the reactions in which H_2 disulfides are established by examining the reoxidation of H chains either alone or in the presence of L chains containing alkylated, irreversibly blocked interchain half-cystines. The results of the former experiments were essentially uninterpretable, because of the strong tendency of the H chains to aggregate in the absence of L. However, the latter experiments showed that inter-HH disulfides do rapidly form even when covalent HL bond formation is prevented.

A different result is obtained in the H chain reoxidations if the L chain inter-HL half-cystine is reversibly blocked by a disulfide bond between L chains. H chains and H_2 dimers readily react with L_2 dimers to form fully reoxidized and assembled H_2L_2. Through disulfide exchange, the H chain half-cystines reduce the disulfide of the L_2 dimer, as is apparent from the rapid loss of L_2 and the concurrent appearance of L, HL, and H_2L. This process is also evident in the time course of the SH titer which plateaus early in the reaction, while the levels of the various intermediates change relative to one another. These results could not have been anticipated from previous studies (Gally and Edelman, 1964; Stevenson and Dorrington, 1970; Jerry and Kunkel, 1972) which reported the failure of alkylated H chain preparations to react with covalent L_2 dimers to form H_2L_2 molecules.

Several aspects of the reoxidation studies of IgG(*Fro*) need further clarification. One is whether the half-cystine pairing in the reoxidized molecule is that of the native molecule in each case. Off-diagonal peptide mapping, now in progress, should establish this. Another unresolved problem is the source of the quantitative variation between experiments. No two experiments showed identical levels of the various components nor identical rates of reoxidation. One source of variation in the levels and positions of HL^{max}, H_2L^{max} and, particularly, H_2^{max}, is their partial dependence on the initial levels of these components. Another stems from the apparent shift in profiles caused by discrepancies between the measured SH titers and the calculated range from the gels (see Figures 2C and 3C). Generally, the measured and calculated SH titers closely agreed in the unseparated-chain reoxidations but for unknown reasons not nearly as well in the recombined-chain reoxidations.

The variation in rates is even more complex because the kinetics of sulfhydryl oxidation to disulfides are subject to catalytic control by various factors, of which the concentration of Cu^{2+} and other metal ions is the best known (Takagi and Isemura, 1963; reviewed in Wetlaufer and Ristow, 1973). This complexity makes studies of the rates of disulfide formation exceedingly sensitive to trace metal contaminants in all reagents and in water. Various methods have been attempted to circumvent the problems (see Wetlaufer and Ristow, 1973), but none of these has proved feasible or desirable thus far in our studies.

It is partly because of the difficulty in controlling the rates of this process that we adopted the procedure of presenting the reoxidation intermediates as functions of the sulfhydryl titer rather than time. As discussed in the following paper, this procedure has a number of additional advantages. For example, it makes possible an approach to fundamental questions concerning the reactivity of particular half-cystine residues during various stages of the reoxidation process, irrespective of how long it takes to make the bonds. The unaccounted and troublesome kinetic variability has accordingly not been an insurmountable obstacle to our basic objectives in these studies.

With respect finally to the relationship of in vitro reoxidation results and corresponding cellular processes, there are still too few cases where the same protein has been studied to allow definitive comparisons. The present work, taken together with other investigations on both human and mouse immunoglobulins (Baumal et al., 1971; Zolla et al., 1970; Petersen and Dorrington, 1974; Sears et al., 1975; Kishida et al., 1976; Percy et al., 1976; Margulies et al., 1976; Weitzman and Scharff, 1976; Baumal and Scharff, 1976), suggests that the kinetics and pathways of covalent assembly are influenced by both intrinsic protein and environmental factors. Thus while the data are still very limited, available information leads to a tentative conclusion that the pattern of covalent chain assembly, whether in vitro or intracellular, is characteristic of the subclass, but the patterns of in vitro and intracellular assembly may not always resemble one another for the same protein.

Acknowledgments

We are grateful to Dr. E. Osserman, for his generosity in providing plasma, and for invaluable guidance and discussion, to Dr. M. D. Scharff and to D. H. Margulies, for their suggestions through much of this work and for critical reading of the manuscript, to Dr. M. E. Percy, who sent two manuscripts prior to publication, and to Drs. J. Greer and M. N. Pflumm, for helpful discussions and comments on the manuscript.

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