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Relative Susceptibilities of the Interchain Disulfides of an Immunoglobulin G Molecule to Reduction by Dithiothreitol†

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ABSTRACT: The reduction by dithiothreitol (DTT) of the four interchain disulfides of a human IgGlκ immunoglobulin has been studied by two methods: variation of the concentration of DTT relative to the protein concentration (incremental reduction); and variation of the time of reduction at fixed levels of DTT and protein (kinetic reduction). In both cases, the results depend on whether the reduction is carried out aerobically or anaerobically. Under aerobic conditions, the relative levels of intermediates (HL, H_2, and H_2L) which are generated as native molecules (H_2L_2) are converted to reduced heavy (H) and light (L) chains depend on the concentrations of protein and DTT as well as on the exposure time to DTT; no stable equilibrium is reached between reduced and oxidized states and conditions gradually revert from those favoring reduction to those favoring reoxidation. By contrast, anaerobic reduction is independent of protein concentration or time of exposure to DTT, beyond about 30 min, indicating that an equilibrium between partially reduced and oxidized states is achieved. The distribution of intermediates observed under anaerobic conditions has been analyzed according to theoretical models (Sears, D. W., and Beychok, S. (1977), *Biochemistry 16* (second in a series of three articles in this issue)). Within experimental error, both kinds of anaerobic experiments resemble a random reduction process wherein the four disulfides are equivalent and independent of each other with respect to rate and extent of reduction by D. It is concluded that there are no readily detected pathways in the process, as would occur if the intrinsic reactivities of the bonds were distinct, and no marked cooperativity between the four reaction sites, as would be observed if reduction of one bond materially facilitated or hampered reactivity at another site. Both of these characteristics of the reduction are in direct contrast to those of the reoxidative process, which is marked by the initial preference for formation of a bond between heavy and light chains, and by kinetic cooperativity in bond formation during the course of the reaction (Sears, D. W., et al. (1977), *Biochemistry 16* (first in a series of three articles in this issue); Sears, D. W., and Beychok, S. (1977), *Biochemistry 16* (second in this series)).

Previous studies from this laboratory (Sears et al., 1975, 1977) described in detail the reoxidation kinetics of the interchain disulfides of a human IgGlκ immunoglobulin. Analysis of the intermediates[1]—HL, H_2, and H_2L—which form as L and H chains combine covalently to produce fully assembled H_2L_2 reveals that some degree of cooperativity is manifested between reoxidizing bonds in this molecule (Sears and Beychok, 1977). An interesting question arising from these studies is whether the "reverse" process—the reduction of the interchain disulfides—also exhibits cooperativity.

In order to probe this question, two approaches are taken to determine the relative susceptibilities of the four interchain disulfides of this immunoglobulin to reduction by DTT. In the first approach (incremental reduction), which is essentially the method introduced by Palmer and Nisonoff (1964) in their study of rabbit IgG disulfides, the protein is exposed for a fixed period of time to various concentrations of DTT in molar excesses which vary in small increments. In the second approach (kinetic reduction), reduction is monitored with time after mixing DTT with the protein. The effects of reducing under aerobic as compared with anaerobic conditions are tested in both types of experiment. The intermediates and products of the reduction experiments are analyzed as described previously (Sears et al., 1977).

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[1] Abbreviations used: H, heavy chain; L, light chain; DTT, dithiothreitol; EDTA, ethylenediaminetetracetic acid disodium salt; IAAm, iodoacetamide; SH, sulfhydryl; r, SH titer or number of SH equivalents per mole of protein; r_{max} and r_{min}, maximum and minimum calculated r assuming that all H_2, H_2L, and H_2L_2 molecules contain either one or two inter-HH disulfides, respectively; $(H_2L_2)_T$, total protein concentration; fM, fractional moles or the number of moles of a given component present in a gel band divided by the total number of moles possble in that band if all protein were converted to the appropriate form.

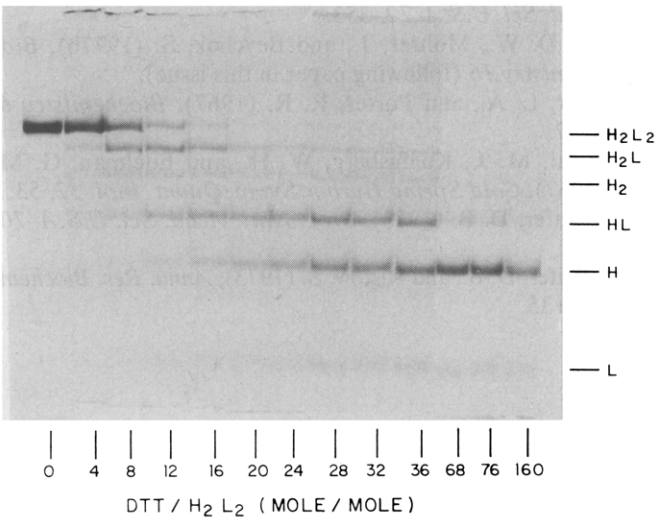

FIGURE 1: The pattern of sodium dodecyl sulfate gel bands for an incremental reduction experiment conducted under aerobic conditions. As indicated at the bottom of the figure, increasing amounts of DTT were added to protein in fixed concentration of 30 μM. Reduction was at room temperature in open vessels and was stopped after 60 min by the addition of enough IAAm to achieve a 20-fold or greater molar excess over the DTT concentration. The bands were identified by their relative mobilities as described by Sears et al. (1977).

The essential finding of this study is that the reduction of the interchain disulfides of this protein closely approximates a kinetically and thermodynamically random process. Thus, the reduction process does not directly reflect the reoxidation process.

Materials and Methods

Materials. The monoclonal, human immunoglobulin IgG(*Fro*) was purified as described by Sears et al. (1977) from the serum of a patient with asymptomatic plasma cell dyscrasia (Osserman, 1971). The serum was generously donated by Dr. E. Osserman, who determined by antigenic typing (unpublished results) that IgG(*Fro*) is an IgG1κ with γ1 heavy chains and κ light chains. The protein was stored at -20 °C in lyophilized form. For each experiment a fresh stock solution was prepared by dissolving some of the lyophilized protein in 0.15 M Tris-HCl–0.15 M NaCl–2 mM EDTA (pH 8.0). The protein concentration was determined using the molar extinction coefficient, $\epsilon_M(278) = 1.91 \times 10^5$ (Sears, 1974).

The interchain disulfides of the protein were reduced with dithiothreitol (DTT; Calbiochem) added from a freshly prepared stock solution of DTT dissolved in water. The concentration of reduced DTT in the stock was determined by measuring its SH concentration with Ellman's (1959) reagent, 5,5′-dithiobis(2-nitrobenzoic acid), according to the following equation:

$$(\text{DTT}) = (\text{SH})/2 = \text{OD}_{412}/2\epsilon_M(412) \qquad (1)$$

where $\epsilon_M(412) = 1.4 \times 10^4$ (Gething and Davidson, 1972; Collier, 1973).

Incremental Reductions. For the incremental reduction experiments, a stock solution of protein (9–50 μM in concentration) was divided into 1-mL aliquots, and 0.1 mL of DTT, in varying concentrations, was added to each sample. The molar ratio of DTT to protein ranged in increments from 0 to as high as 400. In some experiments, the reduction mixtures were kept open to air, whereas in others reduction was within the confines of a glove box purged of O_2 with N_2. Reduction was carried out for 1 h at room temperature and was terminated then by the addition of freshly prepared 0.1 M IAAm. The excess of IAAm over the DTT concentration was always tenfold or greater.

Kinetic Reduction. In the kinetic experiments, reduction was achieved by adding approximately a 30-fold molar excess of DTT to a stock solution of IgG(*Fro*). The reduction mixture was incubated at room temperature, and at selected time intervals 1-mL aliquots were removed and the reaction was immediately terminated by the addition of 0.1 mL of 0.1 M IAAm. As in the case of the incremental reductions, experiments were carried out in either air or in N_2 atmosphere.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The methods for preparing, running, and analyzing the samples by sodium dodecyl sulfate–polyacrylamide gel electrophoresis are described by Sears et al. (1977). Also given in that paper are the equations for calculating the relative quantities of the six components—L, H, HL, H_2, H_2L, and H_2L_2—seen on the gels. Each component is represented in units of fractional moles (fM), this being the number of moles of a given component actually present in a given gel band divided by the total possible number of moles which would be found in the same band if all the protein were converted to the appropriate form.

Calculation of the SH Titer. As previous work has shown (Sears, 1974; Sears et al., 1977) IgG(*Fro*) contains four interchain disulfides connecting the four chains of the molecule in a symmetrical LHHL arrangement. It was also shown that DTT exclusively reduces the interchain disulfides under mild reducing conditions used here, leaving the intrachain disulfides intact. Thus, at maximal reduction one expects the SH titer, or the number of SH equivalents per mole of H_2L_2, not to exceed eight. No free SH groups are found in the unreduced molecule, so the minimal value for r is zero.

The presence of DTT prevents direct measurement of the protein SH concentration. However, one can calculate a minimum–maximum SH titer range from the levels of the various components on the gels as described by Sears et al. (1977).

$$\text{Average SH}/(H_2L_2) = r_{av} = \tfrac{1}{2}(r_{min} + r_{max})$$

Results

Incremental Reductions. An example of the sodium dodecyl sulfate gel patterns which result when IgG(*Fro*) is exposed to a range of DTT concentrations is shown in Figure 1. Clearly, as the DTT concentration increases relative to the protein concentration (from left to right in the figure), H_2L_2—the unreduced, native form of the molecule—gradually converts to the end products, H and L, by the way of three intermediates—H_2L, H_2, and HL. Because the intrachain disulfides are not reduced in the buffer conditions employed in these experiments (Sears et al., 1977), the behavior of the protein in Figure 1 is entirely accounted for by the reduction of the two inter-HL disulfides and the two inter-HH disulfides of this molecule.

With four disulfides involved in the reduction process, the SH titer of the protein is expected to range between 0 and 8 SH equiv per mol of IgG(*Fro*). Although DTT precludes direct SH measurements, the minimum–maximum range of the SH titer can be calculated from the distribution of the six molecular components on the sodium dodecyl sulfate (Sears et al., 1977) and they can then be plotted as functions of the average of r_{min} and r_{max} (eq 2). The purpose of representing the data in this fashion is that it allows the reduction experiments to be directly compared with earlier reoxidation experiments (Sears

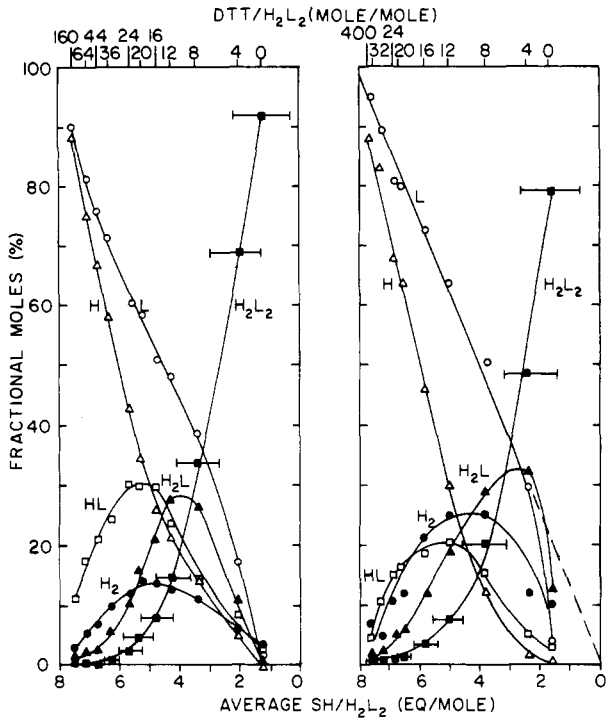

FIGURE 2: Quantitative analysis of the sodium dodecyl sulfate gels following the incremental reduction of IgG(*Fro*). The fractional moles (in percent) of L (○), H (△), HL (□), H_2 (●), H_2L (▲), and H_2L_2 (■) are plotted as functions of the average SH titer. The corresponding DTT/H_2L_2 molar ratios are indicated at the top of the figure. The horizontal bar through each H_2L_2 point represents the uncertainty in both the r value and the positioning of the points as discussed in the text. (A) Reduction under aerobic conditions. This figure is the analysis of the gels shown in Figure 1. (B) Reduction under anaerobic conditions. The protein concentration was 31 μM and reduction was at room temperature for 60 min under a N_2 atmosphere.

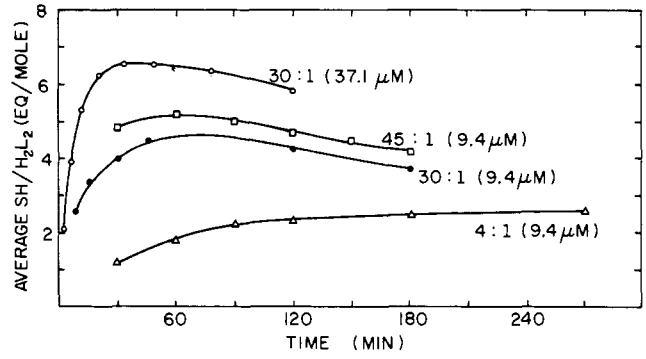

FIGURE 3: The change in the calculated average SH titer with time during reduction under aerobic conditions. IgG(*Fro*) was reduced by adding DTT (time zero on the abscissa) and incubating at room temperature in open vessels. The molar ratio of $(DTT)/(H_2L_2)_T$ and $(H_2L_2)_T$ are indicated at the right of each curve: (○), 30:1 (37.1 μM); (□), 45:1 (9.4 μM); (●), 30:1 (9.4 μM); (△), 4:1 (9.4 μM).

et al., 1977) and certain theoretical models (Sears and Beychok, 1977).

The corresponding plot for the incremental reduction in Figure 1 is shown in Figure 2A. The DTT to protein ratio in this figure increases from right to left. The quantity of each component is represented (ordinate) in terms of fractional moles. The possible error in the calculated sulfhydryl titer for each distribution of components (on a vertical line), at a given DTT/H_2L_2 point, is defined by left and right extremes of the bars, r_{max} and r_{min}, respectively, with H_2L_2 and the accompanying points positioned at r_{av}, the center of the bar. In principle, the set of points associated with the error bar can lie on a vertical line anywhere between the lateral bounds it defines. Increasing levels of reduction result in a decreased plotting error because fewer H_2, H_2L, and H_2L_2 molecules exist at higher DTT concentrations, thereby minimizing the uncertainty in r_{av} created by the unresolvable subpopulations of molecules having either one or two inter-HH disulfide bonds. The curves tend to appear skewed to the left at the lower DTT concentrations. This results from the fact that the lowest possible value of the SH titer is not zero, as it should be for the unreduced protein, but is one, because r_{av} is derived on the assumption that half of all H_2L_2, H_2L, and H_2 molecules have one broken inter-HH disulfide.

Because the techniques are essentially identical with those of the reoxidation experiments, the errors in fractional moles are of the order given in Table I of Sears and Beychok (1977).

Reduction experiments carried out under aerobic conditions (i.e., in the presence of atmospheric O_2, as was the case for Figure 2A) give variable profiles depending on the protein concentration and the length of time reduction was allowed to proceed. The concentration dependence is illustrated in Table I and Figure 3, which also illustrates the time dependence of the reductions conducted under aerobic conditions. As expected, r_{av} initially rises, but fails to achieve a stable plateau. The value instead declines in three of the four experiments, indicating that protein SH groups are reoxidizing. Reoxidation is also evidenced by the behavior of the components after very long times: in some experiments the levels of H and L, after reaching maximum values, began showing small gradual declines with a concomitant increase in H_2L_2. The complex concentration and time dependence of these experiments thus appears to be the result of two opposing processes occurring simultaneously: DTT reduction *and* air reoxidation of the protein disulfides.

In order to examine the reduction of the protein under conditions in which the reoxidation process is completely eliminated, incremental reductions were also carried out in a N_2 atmosphere. The resulting profiles for one such experiment are shown in Figure 2B. In contrast to the experiments described above, incremental reductions under anaerobic conditions show little dependence on the protein concentration (cf. columns 3 and 4 of Table I) or on the length of time of reduction after 20–30-min exposure to DTT. The levels of intermediates and the value of r_{av} stabilize after this time.

Kinetic Reduction. The second type of experiment was designed to examine the kinetics of reduction of the interchain disulfides of IgG(*Fro*). Examples of kinetic reductions carried out under aerobic and anaerobic conditions are shown in Figures 4A and 4B, respectively, and summarized in Table I. Again, just as in the case of the incremental reductions, the results of the kinetic reductions under aerobic conditions (columns 5 and 6 of Table I) appear to be strongly dependent on the protein concentration, in direct contrast to the reductions carried out in a N_2 atmosphere (columns 7 and 8 of Table I).

Theoretical Random Reduction. In the preceding paper, Sears and Beychok (1977) developed a general theoretical format for analyzing the reoxidative behavior of the interchain disulfides of immunoglobulins. Although the emphasis in that paper was on reoxidation, the theory is essentially independent of whether the disulfides are in the process of forming or breaking, and it is therefore equally applicable to the reduction properties of the disulfides. In fact, the assumptions on which

TABLE I: Comparisons between Incremental and Kinetic Reductions, and the Theoretical Random Reduction.

	Incremental Reductions[c]				Kinetic Reductions[d]				Random Reduction[e]
	Aerobic		Anaerobic		Aerobic		Anaerobic		
$(H_2L_2)_T$[a]	30	46	31	46	38	41	26	52	
fM_{HL}^{max} (r_{av})[b]	31	25	20	18	≥25	14	20	24	15
	(5.7)	(6.2)	(5.0)	(5.2)	(5.3)	(4.8)	(5.0)	(5.3)	(5.3)
$fM_{H_2}^{max}$ (r_{av})[b]	16	25	25	22	22	≥32	22	24	25
	(5.2)	(3.9)	(5.0)	(4.9)	(3.7)	(5.5)	(5.0)	(5.3)	(5.7)
$fM_{H_2L}^{max}$ (r_{av})[b]	28	21	33	33	32	37	30	36	40
	(4.3)	(3.9)	(2.4)	(3.5)	(2.5)	(3.2)	(4.0)	(3.4)	(3.1)

[a] Total protein concentration in μM. [b] Maximum fractional moles in percent at the r_{av} value in parentheses. [c] Values achieved after 60-min reduction. [d] Values achieved during 35 min of reduction. [e] Values taken from Sears and Beychok (1977).

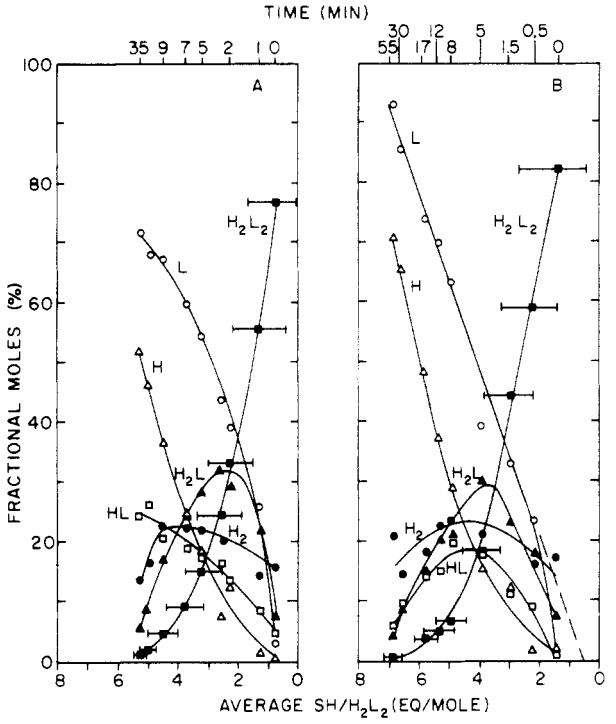

FIGURE 4: Reduction kinetics of IgG(*Fro*) by DTT. The notation and symbols are as defined in the legend of Figure 2. The protein was incubated with DTT at room temperature and samples were removed from the reduction mixture at times indicated at the top of the figure, alkylated, and prepared for sodium dodecyl sulfate gel electrophoresis. The sample at time zero was taken just prior to the addition of DTT. (A) Reduction under aerobic conditions. The protein concentration was 38 μM and the molar ratio $(DTT)/(H_2L_2)_T$ = 30:1. (B) Reduction under anaerobic conditions. The protein concentration was 26 μM and $(DTT)/(H_2L_2)_T$ = 30:1.

the theoretical model is based are less stringent in the case of reduction because no assumptions need be made about the noncovalent state of the molecule and whether the disulfides are forming correctly. The only basic assumption for the application of the theory then is that DTT reduces only the interchain and not the intrachain disulfides, as appears to be the case in this system (see above).

One special case of the theory which is of particular interest for these studies is the random case. Figure 2A in Sears and Beychok (1977) shows the expected profiles for L, H, etc. as functions of the SH titer, either for random reoxidation *or*, when viewed in the opposite sense, for random reduction of the interchain disulfides. For purposes of comparison, the characteristic features of the random curves are the relative maximum values and positions of HL, H_2, and H_2L (see column 9 of Table I) and the straight-line behavior of L. The curves for the incremental and kinetic reductions carried out under anaerobic conditions are very similar to those of the random case. HL^{max}, H_2^{max}, and H_2L^{max} are present in the same relative amounts and L increases linearly as a function of r_{av}. The L line departs from the theoretical only where the r_{av} method has a large error. A linear extrapolation to zero sulfhydryl of the straight line portion of the experimental points falls on the calculated line (Figures 2 and 4) well within the error bars. Moreover, the H_2L_2 curve is virtually indistinguishable from the random model in both anaerobic experiments. The agreement is not exact, however. Quantitatively, the largest differences between the experimental and theoretical plots are HL^{max}, H_2L^{max}, and r_{av} at H_2^{max}, although the differences are within experimental error, except for H_2L^{max}.

Discussion

The relative susceptibilities of the four interchain disulfides of IgG(*Fro*) to reduction by DTT vary according to the conditions of the experiment. The pattern of intermediates generated under aerobic conditions depends not only on the molar ratio of DTT to protein but also on the concentration of the protein itself. In addition, there is a particularly complex time dependence in these experiments. Although EDTA was included in the reaction mixtures as an inhibitor of reoxidation (Saxena and Wetlaufer, 1970), it was known from previous studies (Sears, 1974) that the interchain disulfides can reoxidize in the presence of 1 mM EDTA, albeit very slowly (half times for H_2L_2 formation of several hours). Therefore, the observed time dependence can be explained in terms of two competing and simultaneously occurring processes: reduction and reoxidation of the disulfides. Reduction, the primary reaction, is expected to be rapid and essentially complete due to the thermodynamic stability of the oxidized DTT product which forms during disulfide exchange between the SH groups of the protein and reduced DTT (Cleland, 1964). However, because the excess DTT is limited relative to the protein concentration, reduced DTT is soon depleted by the initial reduction of bonds and then further by the re-reduction of reoxidized bonds. Eventually reoxidation becomes the predominant reaction.

In contrast, reductions under anaerobic conditions give a uniform result which is independent of the protein concentration or the exposure time to DTT after approximately 30 min. By eliminating O_2 from the reaction mixture, the competing process of reoxidation is also eliminated and the reduction reaction goes to equilibrium. The pattern of intermediates which is generated in these experiments is very similar to (but not exactly identical with) that which is theoretically

predicted for random reduction of the interchain disulfides (Sears and Beychok, 1977).

The incremental and kinetic reductions provide different kinds of information about the chemical properties of the disulfides. The incremental reductions measure the distribution of intermediates at equilibrium. Because the distribution approaches the random distribution, the intrinsic redox potentials of the four interchain disulfides are equivalent in this protein. The kinetic reduction experiments probe the relative kinetic accessibilities of the disulfides to DTT before equilibrium is reached. Again, the fact that this process resembles the random case indicates that the four interchain disulfides are equally exposed to solvent.

In summary, there appears to be no significant difference in the relative susceptibilities of the interchain disulfides of IgG(*Fro*) to reduction by DTT as judged by two criterion: the redox potentials and the kinetic accessibility to DTT. Furthermore, no cooperativity is exhibited between reaction sites, as would occur if reduction of one bond facilitated or hampered reactivity at another site. This contrasts to the cooperativity of the reoxidative process (Sears and Beychok, 1977) and demonstrates that reduction is not a reversal of the reoxidation pathways.

The reductive properties of other human IgGl proteins have been studied previously by Williamson and Askonas (1968) and by Virella and Parkhouse (1973). Our results disagree with those of the former study and partially agree with those of the latter. Williamson and Askonas did not find HL half-molecules in their studies whereas HL is clearly a product of the reduction of IgG(*Fro*). Virella and Parkhouse also found significant levels of HL, along with H_2 and H_2L, as intermediates in the incremental reductions of several other human IgGl proteins, and they suggest the failure of Williamson and Askonas to find HL may have been the result of incomplete denaturation of the protein prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis.

In a general sense, our findings with IgG(*Fro*) contrast an earlier observation by Bevan et al. (1972) who found (referring to aerobic reductions) that "the relative stability to reduction of the disulfide bonds correlates exactly with the order of formation of these disulfide bonds during biosynthesis" for a number of immunoglobulins of different species, classes and subclasses. For the in vitro processes described here, the reduction does not correlate with the reoxidation. There are, however, cases of a clear-cut relationship between differences in the susceptibility to reduction and the preference for oxidation of the disulfides of the immunoglobulins. For example, the relative resistance of the inter-HL disulfide of human IgG4 proteins to reduction (Virella and Parkhouse, 1973) does appear to correlate with the strong preference for the reoxidation of this bond in vitro (Petersen and Dorrington, 1974). Also, Weitzman and Scharff (1976) have shown that the differences in assembly of two mouse immunoglobulins—one derived from a myeloma cell line and the other from a mutagenized subclone of the same line—show correlations with the differences observed in the kinetic reduction properties of these two molecules.

Numerous investigations, moreover, of the more complex IgA and IgM molecules (Morris and Inman, 1968; Beale and Feinstein, 1969; Kownatzki, 1973; Chapuis and Koshland, 1974; Hauptman and Tomasi, 1975; Scharff and Laskov, 1970; Bevan et al., 1972) appear to be consistent with the notion that the differential susceptibilities of the disulfides to reduction do play a role in assembly because the intersubunit bonds are the most labile to reduction, and the last to form. It is thus possible that different classes of disulfides within one immunoglobulin molecule may serve, by their differing susceptibilities to reduction, the biological role of ensuring that the correct half-cystine pairs are formed at the proper time and the proper site during folding and assembly. This idea warrants further investigation in efforts to understand the formation of structures in these molecules.

Acknowledgments

We are grateful to Dr. E. F. Osserman for generous provision of plasma and for guidance and discussions and to Dr. M. N. Pflumm for advice on many aspects of these investigations.

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