Acquisition of the Covalent Quaternary Structure of an Immunoglobulin G Molecule. Theoretical Reoxidation Models†

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ABSTRACT: A theoretical format, suitable for analyzing diverse complex kinetic systems where reaction pathways may exhibit cooperativity, is developed to account for the in vitro kinetics of air reoxidation of a human IgG1κ immunoglobulin, in which the four interchain disulfide bonds have been reduced (Sears, D. W., et al. (1977), *Biochemistry 16* (preceding paper in this issue)). The equations relate experimentally determined concentrations of the reactants, product, and macroscopic intermediates to the probability of occurrence of any of 12 distinct microscopic states. The concentrations of the macroscopic species—light chains (L), heavy chains (H), covalently associated intermediates HL, H_2, H_2L, and product H_2L_2—are also explicitly related to an observable function of those concentrations, the sulfhydryl titer, r. Since values of r can be calculated for any microscopic state of the system, the theory is in principle capable of a complete description of the experimental reaction course. In practice, limited experimental information precludes a unique solution of the equations at present, and it was not judged worthwhile to attempt curve fitting or approximation of probability terms with adjustable parameters of unknown physical significance. Certain special cases of the theory are, however, readily and exactly solved. These include models in which reoxidation is a random process and others in which the probabilities for inter-HL and inter-HH bond formation are different but independent of one another. The experimental results in the case of the IgG1κ studied here clearly depart from the predicted behavior in either of these models. The initial probability for formation of a bond between a heavy and a light chain is 1.5 to 2 times greater than for a bond between heavy chains. From the fact that this ratio changes as the reaction proceeds, and from the pattern of variation in concentration of intermediates during the reaction, it is concluded that the reoxidation process is not random, and that the bonds do not form independently, exhibiting instead kinetic cooperativity. The results are discussed in terms of assembly pathways in this and related systems. A novel feature of the theory is that it eliminates time as an explicit variable in the treatment of the kinetic process. This makes it especially useful for discerning whether the formation of one bond influences the reaction probability of another in a system where several similar or intrinsically identical reactions occur, and where kinetic order is difficult to establish. While the theory here is formulated in terms of the reoxidation reactions, it is, for example, equally applicable to the reduction process described (Sears, D. W., et al. (1977b), *Biochemistry 16* (following paper in this issue)).

Experimental methods of disulfide intermediate analysis have recently been developed to study the folding or subunit assembly of proteins in vitro (cf. Ristow and Wetlaufer, 1973; Sears, 1974; Petersen and Dorrington, 1974; Creighton, 1974; Hantgan et al., 1974). These methods are based on the identification of specific disulfide bond intermediates which form as molecules pass from reduced, nonnative structures to some final native-like structure. In this way one is able to single-out and characterize a discrete subset of intervals in a process which is usually very complex when viewed in its entirety.

Although these methods have the potential for elucidating certain aspects of the protein folding problem, the interpretation of the experimental data from such studies is frequently difficult. In our studies of the covalent chain assembly of a human IgG molecule (Sears et al., 1975, 1977a), the various interchain-disulfide-bond intermediates which form as the H and L chains[1] reoxidize to fully assembled H_2L_2 molecules can be resolved and identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. However, the resulting profiles of intermediates in this process do not provide obvious answers to several relevant questions: What defines a pathway for this system and are there distinct pathways leading from the reduced state of the molecule to the fully assembled state? Do the bonds form independently or does the presence, or absence, of one bond preferentially influence the formation of another? What are the intrinsic reaction probabilities of the eight reduced cysteine residues under reoxidizing conditions near neutral pH?

The objectives of this paper are to outline a theoretical approach to answering such questions and to report the results of the application of this theory. Some of the results are summarized in Sears et al. (1975), without accompanying details, and are more fully described below. Although the theoretical description of the experimental results is still incomplete, the theoretical models developed thus far do provide a useful starting point for understanding the complex process of disulfide bond formation in antibody assembly.

Results

Generalized Four-Bond Reoxidation Model. The formation of covalent H_2L_2 molecules from the individual H and L chains

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[1] Abbreviations used: H, heavy chain; L, light chain; SH, sulfhydryl; fM_H, etc., fractional moles of L, H, etc., respectively; $P(\bar{l}h_1h_2\bar{l})$, etc., probability of finding the molecular species designated in the parentheses; l and $\bar{l}$, an inter-HL disulfide either oxidized or reduced, respectively; $h_1(h_2)$ or $\bar{h}_1(\bar{h}_2)$, an inter-HH disulfide either oxidized or reduced, respectively, with subscripts 1 and 2 designating the two nonidentical HH bonds in the molecule; $P(HL)_{av}$ or $P(HH)_{av}$, respective average probabilities for finding an inter-HL bond or an inter-HH bond in the reoxidized state.

of immunoglobulins is essentially determined by two processes and their interrelationships: (1) the chains must make noncovalent contact with one another; and (2) interchain disulfides must form between H and L chains and between H chains themselves. Experimental systems designed for studying the in vitro reoxidation kinetics favor very rapid, complete, and essentially irreversible noncovalent association of H and L into H_2L_2. As discussed by Sears et al. (1977a), disulfide bond formation proceeds much more slowly and is the rate-limiting step in covalent assembly. Accordingly, it has been assumed for all that follows that interchain disulfides form exclusively within preassembled tetrameric H_2L_2 molecules maintained by noncovalent interchain bonds and the first process above is eliminated from further consideration. As regards the second process, two additional assumptions are made: reoxidation proceeds without disulfide interchange; and only one and the same final configuration of interchain disulfides is attained in the reoxidized molecule and this corresponds to the disulfide arrangement found in IgG1κ proteins in general (Frangione and Milstein, 1967; Steiner and Porter, 1967; Waxdal et al., 1967).

The generalized reoxidation scheme for an immunoglobulin with four interchain disulfides is illustrated in Figure 1. Shown are all possible routes of disulfide bond formation which connect different possible microscopic states of the molecule. Ideally, if one could determine the distribution of microstates at all times during reoxidation, one would know the order of disulfide formation in the molecule. However, this information is not explicitly available from the experiments. What is available, as discussed in the preceding paper, is the time-dependent sulfhydryl concentration and the total, time-dependent levels of six macroscopic components—L, H, HL, H_2, H_2L, and H_2L_2—which are separated and identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The macroscopic and microscopic components are related by the following conservation of mass equations:

$$\mathrm{fM_L} = P(\bar{l}\bar{h}_1\bar{h}_2\bar{l}) + P(\bar{l}h_1\bar{h}_2\bar{l}) + P(\bar{l}\bar{h}_1h_2\bar{l}) + P(\bar{l}\bar{h}_1\bar{h}_2l) + P(\bar{l}h_1h_2\bar{l}) + P(\bar{l}h_1\bar{h}_2l) + P(\bar{l}\bar{h}_1h_2l) + P(\bar{l}h_1h_2l) \quad (1)$$

$$\mathrm{fM_H} = P(\bar{l}\bar{h}_1\bar{h}_2\bar{l}) + P(\bar{l}\bar{h}_1\bar{h}_2l) \quad (2)$$

$$\mathrm{fM_{HL}} = P(\bar{l}\bar{h}_1\bar{h}_2l) + P(l\bar{h}_1\bar{h}_2l) \quad (3)$$

$$\mathrm{fM_{H_2}} = P(\bar{l}h_1\bar{h}_2\bar{l}) + P(\bar{l}\bar{h}_1h_2\bar{l}) + P(\bar{l}h_1h_2\bar{l}) \quad (4)$$

$$\mathrm{fM_{H_2L}} = 2[P(\bar{l}h_1\bar{h}_2l) + P(\bar{l}\bar{h}_1h_2l) + P(\bar{l}h_1h_2l)] \quad (5)$$

$$\mathrm{fM_{H_2L_2}} = P(lh_1\bar{h}_2l) + P(l\bar{h}_1h_2l) + P(lh_1h_2l) \quad (6)$$

$$1 = \sum_{i=1}^{16} P_i \quad (7)$$

Each fM term on the left is the subscripted component in fractional moles.[2] Each term on the right is the probability for the microstate in parentheses (See Figure 1). Note that the two HL bonding pairs in a molecule are assumed to be intrinsically equivalent and indistinguishable by virtue of symmetry thereby reducing the 16 possible microstates to 12 unique states, 4 of which are degenerate (e.g., $P(\bar{l}\bar{h}_1\bar{h}_2l) = P(l\bar{h}_1\bar{h}_2\bar{l})$). Equation 7 follows from the definition of probability and contains all probability terms counting degenerate states twice.

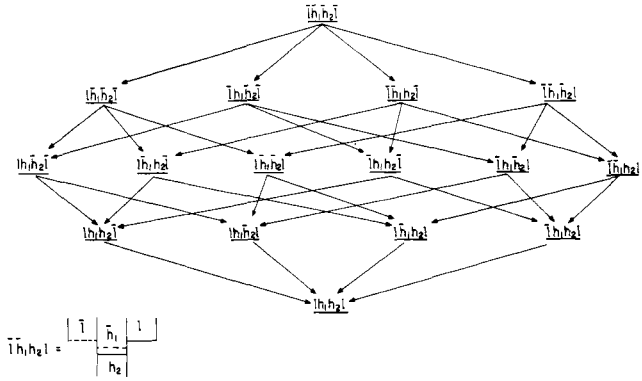

FIGURE 1: Theoretical reoxidation pathways for an immunoglobulin molecule with four interchain disulfide bonds. A four-letter code is used to indicate the arrangement of closed and open disulfides in each species as illustrated by the schematic in the lower left. Specifically, each letter itself corresponds to one of the four disulfides in the molecule and the absence or presence of a bar over the letter indicates that the bond is either oxidized or reduced, respectively. The horizontal solid and dashed lines of the immunoglobulin in the schematic indicate that a particular bond is either oxidized or reduced, respectively. Because the molecule is presumably symmetrical, no distinction is made between the two bonds involving L. The two inter-HH bonds are distinguishable, however, h_1 being proximal to and h_2 being distal to the N-terminal end of the H chain. The molecule in the inset, $\bar{l}h_1h_2l$, is one in which (reading from left to right) one HL and one HH bond are reduced and the other two disulfides are oxidized. Note that this prticular molecular intermediate would dissociate into L and H_2L after exposure to sodium dodecyl sulfate in preparation for sodium dodecyl sulfate gel electrophoresis.

The probability terms in these equations can be further partitioned into conditional probabilities, as described in any standard treatise on probability. In the language of probability theory, each probability term represents the intersection of probabilities. For example

$$P(\bar{l}\bar{h}_1h_2l) = P(\bar{l} \cap \bar{h}_1 \cap h_2 \cap l)$$

where $P(\bar{l} \cap \bar{h}_1 \cap h_2 \cap l)$ is the probability for the simultaneous occurrence of $\bar{l}$ and $\bar{h}_1$ and h_2 and l; the oxidation or reduction state of each bond is considered as a separate event. Thus, each probability term can be written as a series of conditional probabilities as illustrated in the following example:

$$P(\bar{l}\bar{h}_1h_2l) = P(\bar{l})P(\bar{h}_1|\bar{l})P(h_2|\bar{l}\bar{h}_1)P(l|\bar{l}\bar{h}_1h_2) \quad (8)$$

where

$$P(\bar{l}) = 1 - P(l) = P(\bar{l}\bar{h}_1\bar{h}_2\bar{l}) + P(\bar{l}h_1\bar{h}_2\bar{l}) + P(\bar{l}\bar{h}_1h_2\bar{l}) + P(\bar{l}\bar{h}_1\bar{h}_2l) + P(\bar{l}h_1h_2\bar{l}) + P(\bar{l}h_1\bar{h}_2l) + P(\bar{l}\bar{h}_1h_2l) + P(\bar{l}h_1h_2l) \quad (9)$$

$$P(\bar{l}h_1|\bar{l}) = 1 - P(h_1|\bar{l}) = P(\bar{h}_1 \cap \bar{l})/P(\bar{l}) \quad (10)$$

$$P(h_2|\bar{l}\bar{h}_1) = P(h_2|\bar{l} \cap \bar{h}_1) = P(h_2 \cap \bar{l} \cap \bar{h}_1)/P(\bar{l}\bar{h}_1) \quad (11)$$

$$P(l|\bar{l}\bar{h}_1h_2) = P(l|\bar{l} \cap \bar{h}_1 \cap h_2) = P(l|\bar{l} \cap \bar{h}_1 \cap h_2)/P(\bar{l} \cap \bar{h}_1 \cap h_2) \quad (12)$$

Note that the given order of the bonds within the parentheses of these expressions is inconsequential and it can be equally written according to any one of the 24 permutations of the four letters. The most interesting terms in the above expressions are the four-bond conditional probabilities since these refer to single molecular states. For example, $P(l|\bar{l}\bar{h}_1h_2)$ is the probability that the inter-HL bond is formed in a molecule given that the other inter-HL is reduced, that one inter-HH is reduced, and that the other inter-HH is oxidized.

[2] By definition, the fractional moles (fM) is the number of moles of a component actually observed at a given time on the gel divided by the total *possible* number of moles that could be derived from all the protein in the sample; the total possible L, for example, is the sum of L in all L-containing components on the gel. This parameter effectively normalizes each of the six components to a scale of 0 to 1.

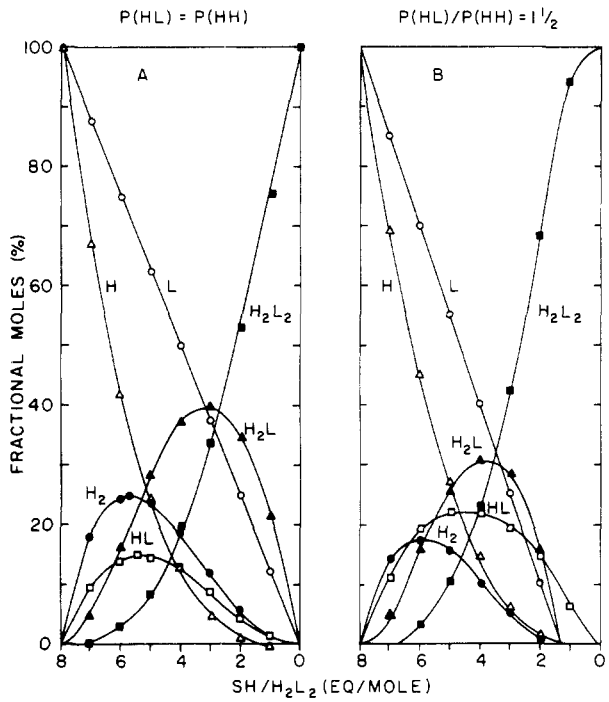

FIGURE 2: Theoretical reoxidation profiles for noncooperative disulfide bond formation. The fractional moles of L (○), H (△), HL (□), H_2 (●), H_2L (▲), and H_2L_2 (■) were calculated as functions of the SH titer according to the equations developed in the text for the reaction scheme illustrated in Figure 1. (A) Random reoxidation. The four interchain disulfides are assumed to reoxidize with equal and independent probabilities, i.e., $P(HL) = P(HH)$. (B) Nonrandom reoxidation with independent but unequal probabilities for HL and HH formation. This hypothetical reoxidation model was derived assuming that $P(HL)/P(HH) = 1.5$ for all values of $P(HL) < 1$. When the SH titer drops into the range between 1.4 and 0, all inter-HL disulfide bonds are formed (i.e., $fM_L = 0$ and $P(HL) = 1$ by definition) and the value of $P(HH)$ is itself directly proportional to the SH titer (see footnote 4).

The experimentally determined SH titer, r (i.e., number of SH equivalents per mole of H_2L_2), provides an additional relationship:

$$r = 4P(\bar{l}) + 2P(\bar{h}_1) + 2P(\bar{h}_2) = 4(P(\bar{l}) + P(\bar{h})_{av}) \quad (13)$$

Because the inter-HH bonds are not yet experimentally distinguishable, an average probability is substituted:

$$P(\bar{h})_{av} = \tfrac{1}{2}(P(\bar{h}_1) + P(\bar{h}_2)) \quad (14)$$

Even though these equations represent a kinetic process, an explicit relationship to time has not been given. By relating the levels of components to the SH concentration, time is eliminated as an explicit variable. As evidenced below, this greatly simplifies comparison between theory and experiment since no assumptions need be made about the kinetic order of each possible reaction. Unfortunately, without additional experimental information of additional assumptions, the foregoing equations are not sufficient for a complete solution of the four-bond conditional probabilities. Despite this limitation, however, certain special cases of the general theory are readily solved and provide a number of insights into the reoxidation mechanism of the protein described in the preceding paper. Two important cases are treated below because they form the basis for deciding whether the reoxidation process exhibits kinetic cooperativity.

Random Reoxidation. In a random reoxidation, all routes to assembled H_2L_2, as denoted by arrows in Figure 1, would be equally likely and mutually independent of each other. One can predict how the distribution of components would appear if the reoxidation were random by setting the following condition of randomness: the probability that any one bond forms at any given time must be equal to and independent of the probability that any other bond also forms at the same time.[3] Thus, following the example in eq 8-12

$$P(l|\bar{l}\bar{h}_1h_2) = P(l) \quad (15)$$

$$P(\bar{h}_1|\bar{l}) = P(\bar{h}_1) \quad (16)$$

$$P(h_2|\bar{l}\bar{h}_1) = P(h_2) \quad (17)$$

$$P(l) = P(h_1) = P(h_2) = P \quad (18)$$

$$P(\bar{l}) = P(\bar{h}_1) = P(\bar{h}_2) = \bar{P} \quad (19)$$

$$P(\bar{l}\bar{h}_1h_2l) = P(\bar{l})P(\bar{h}_1)P(h_2)P(l) = (\bar{P})^2(P)^2 \quad (20)$$

$\bar{P}$ and P are determined directly from the SH titer, eq 13 and 14, and are then used to calculate the fractional mole terms. The resulting reoxidation profiles are shown in Figure 2A. The independence and identity of the disulfide bonds are indicated in another way at the top of Figure 2A by the equation, $P(HL) = P(HH)$, which states that the probability of forming any inter-HL bond is always equal to the probability of forming any inter-HH bond.

Comparing Figure 2A with the experimental results of Sears et al. (1975, 1977a) which were plotted on exactly the same coordinate scales, it is possible to decide directly whether the molecule reoxidizes randomly. The most sensitive indicators are HL^{max}, $H_2{}^{max}$, and H_2L^{max}. These comparisons are given in Table I where it is evident that, in the random reoxidation, $H_2{}^{max}$ would exceed HL^{max} by about twofold, which is just opposite to the situation found experimentally. Another difference, not indicated in Table I, is the behavior of L. In Figure 2A, L is linear, whereas L in the experiments shows straight line behavior between $r = 4$ and $r = 0$, but not between $r = 8$ and $r = 4$. It is concluded from such comparisons (Sears, 1974; Sears et al., 1975) that, if the basic assumptions at the beginning of the preceding section are correct, the mode of disulfide bond formation in this molecule is definitely not random. Thus, it must follow that the disulfides form through one or more preferred pathways, because, a random process by definition must correspond to the absence of any pathway.

TABLE I: Maximum Intermediate Levels in Experimental and Theoretical Reoxidations.

	(HL^{max}) $(r)^a$	$(H_2{}^{max})$ $(r)^a$	(H_2L^{max}) $(r)^a$
Unseparated-chain reoxidations[b]	27 ± 4 (4.6 ± 0.7)	18 ± 8 (5.7 ± 1.4)	29 ± 4 (2.8 ± 0.7)
Random reoxidation, $P(HL)/P(HH) = 1$[c]	15 (5.3)	25 (5.7)	40 (3.1)
Independent bond reoxidation $P(HL)/P(HH) = 1.5$[c]	31 (4.5)	16 (6.0)	30 (4.0)

[a] Maximum fractional moles at the SH titer, r, indicated in parentheses. [b] Average of five experiments in which the H and L chains were not separated after reduction and prior to reoxidation (see Sears et al., 1977a). [c] See text for definition of probabilities.

[3] Strictly speaking, a random reoxidation implies that *any* two SH groups in the molecule can form a disulfide bond with equal likelihood. However, the final disulfide arrangement has been restricted to only one native arrangement out of 21 theoretically possible arrangements.

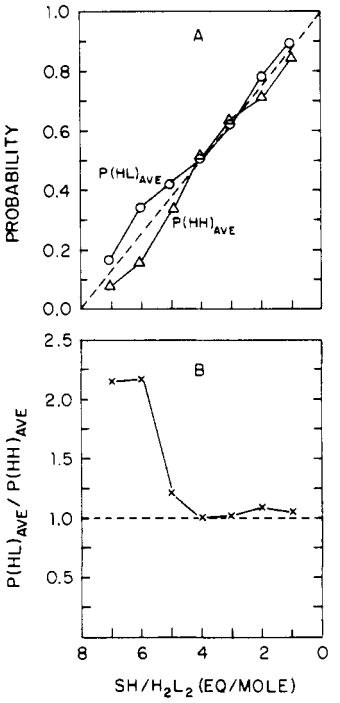

FIGURE 3: Disulfide bond formation probabilities for an unseparated-chain, 3.2 → 7.5 reoxidation (see Sears et al., 1975, for experimental details). (A) The average probabilities for forming either an inter-HL bond (O—O) or an inter-HH bond (Δ—Δ) were determined from the experimental reoxidation profiles for L, H, etc. and the corresponding SH titers. The diagonal dashed line is the corresponding probability expected for both of these bonds if the reoxidation where random as depicted in Figure 2A. (B) The ratio (X—X) of the average probabilities shown in panel A. The horizontal dashed lines again refers to the random case.

Nonrandom Reoxidation: Independent Bond Formation. Another type of model to test against experiment is one in which the disulfides reoxidize independently, as in the random model, but with unequal probabilities. In this case, eq 15–17 still obtain but eq 18 and 19 are replaced by

$$P(1) = P(\bar{HL}) \neq P(h)_{av} = P(HH)_{av} \qquad (21)$$

and the ratio, $P(HL)/P(HH)_{av}$, is a constant not equal to one.[4] The fact that the experimental value of HL^{max} exceeds H_2^{max}, in contrast to the random case, suggests that the inter-HL disulfide bonds are more likely to reoxidize than the inter-HH disulfide bonds, at least initially. This concept is incorporated into the independent bond model by assuming that $P(HL)/P(HH)_{av} > 1$.

Figure 2B shows the reoxidation profiles which would result if this ratio is 3/2. The intermediate maxima of this model are listed in Table I and it is evident that the "fit" to experimental results is greatly improved. However, three major features of this model still differ significantly with experiment: (1) the position of H_2L^{max} in the model lies to the left on the SH scale; (2) the intercept on the SH scale for both H_2L and L in the model is $r = 1.33$ (here $P(HL) = 1$; see footnote 4), whereas the same two components intercept near $r = 0$ in the experimental reoxidation; (3) L in the model is linear throughout. Increasing or decreasing the probability ratios for this model does not substantially improve the fit to experiment; as the probability ratio approaches one, the intercept of the L line and H_2L on the abscissa improves but the levels of H_2^{max} and HL^{max} approach random values. Moreover, L is linear for all independent probability ratios.

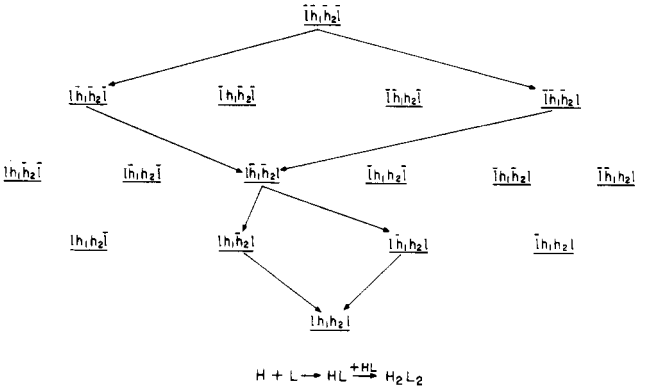

FIGURE 4: One pathway involving absolute cooperativity which is identifiable by sodium dodecyl sulfate-polyacrylamide gels. Depicted here are the allowed (arrows) intermediate stages which follow if both HL bonds necessarily precede the formation of any HH bonds. The equation at the bottom corresponds to the temporal appearance of the components as they would be identified on the gels following the disruption of noncovalent bonds by sodium dodecyl sulfate. The symbolic representation for each molecular species is described in the legend of Figure 1.

Figure 3 shows further evidence that the experimental $P(HL)$ and $P(HH)$ are not linear functions of r and that their ratio is not constant. The curves in this figure were plotted from the data of a typical unseparated chain reoxidation (Sears et al., 1975) using $P(HL)_{av} = 1 - fM_L$ (eq 1 and 9) and $P(HH)_{av} = 1 - (r/4) + fM_L$ (eq 13).[5] Although the ratio is not constant nor sharply discontinuous, as would be expected for independently reacting bonds (see footnote 3), it does approach a constant value of one after the first half of the reaction.

It is concluded that the interchain disulfides of this immunoglobulin do not reoxidize in vitro by entirely independent means. Rather, some degree of cooperativity is exhibited between reoxidizing disulfides.

Nonrandom Reoxidation: Cooperative Bond Formation. Cooperativity in this system implies that the state or reoxidation of one (or more) bond in a molecule influences the tendency of another bond in the molecule to form a disulfide. The essential elements to resolve in this process are the degree of cooperativity and the mechanism through which it is manifested in terms of the four individual disulfide bonds of the molecule. Although the degree and mechanism of cooperativity in the experimental system cannot yet be fully evaluated because of the limitations in the general theory discussed above, it is possible to arrive at a general idea of the limits of cooperative behavior by considering certain extreme cases. Specifi-

[4] Actually, the probability ratio is discontinuous in the nonrandom model. $P(HL)/P(HH)_{av}$ will be constant only until the SH titer drops to a value at which either $P(HL) = 1$ or $P(HH)_{av} = 1$ depending on whether the ratio is greater than or less than one. A probability of one occurs when all bonds of one type are formed so the probability cannot exceed this value. Thus, for SH titers beyond this point the ratio varies proportionally to r since one probability value is now constant at one while the other varies as r.

[5] The value for the probability of the inter-HL bond formation is designated as an average here because the parameter $1 - fm_L$ allows no distinction between the first and second inter-HL bonds made in the molecule. By contrast, the first and second inter-HH bonds formed in a molecule can be distinguished (F. Friedman, unpublished observation). The fractions of molecules containing one, two, or no HH bonds are found as follows: $P(1HH) = 2(fM_{H_2} + fM_{H_2L} + fM_{H_2L_2}) - (2 - (r/2) + 2fM_L)$; $P(2HH) = (2 - (r/2) + 2fM_L) - (fM_{H_2} + fM_{H_2L} + fM_{H_2L_2})$; $P(0HH) = 1 - (fM_{H_2} + fM_{H_2L} + fM_{H_2L_2}) = fM_H + fM_{HL}$. The data, however, show too much scatter at present to plot these separately and only $P(HH)_{av}$ is determined: $P(HH)_{av} = \frac{1}{2}[P(1HH) + 2P(2HH)]$.

cally, one can ask how the reoxidation would appear if the cooperativity were absolute in the sense that the bonds form sequentially with certain bonds unable to reoxidize until others are first formed.

The patterns on sodium dodecyl sulfate gels allow one to identify three pathways of absolute cooperativity:

$$H + L \rightarrow HL \xrightarrow{+HL} H_2L_2 \quad (I)$$

$$H + L \rightarrow HL \xrightarrow{+H} H_2L \xrightarrow{+L} H_2L_2 \quad (II)$$

$$H + H \rightarrow H_2 \xrightarrow{+L} H_2L \xrightarrow{+L} H_2L_2 \quad (III)$$

Reaction pathways I, II, and III were first proposed by Scharff and Laskov (1970) as possible intracellular assembly routes for immunoglobulins. Although these equations appear to suggest that noncovalent assembly coincides with covalent assembly, they are in fact equally valid for the reoxidations of preassembled noncovalent tetramers. For example, reaction I, which states that two inter-HL bonds must be formed prior to any inter-HH bonds, corresponds to the reoxidation depicted in Figure 4, disruption of the molecules by sodium dodecyl sulfate at any stage would leave only H, L, HL, and H_2L_2 to be identified by the gels. Comparable reaction schemes can be drawn for the remaining two equations. The interesting finding to emerge from these equations is that in each case one or more possible intermediates is absent from the reaction scheme: H_2 and H_2L are missing in I; H_2 in II; and HL in III. In the in vitro reoxidation experiments, however, all three intermediates are always present. We thus conclude that the cooperativity in the experimental system is not absolute at least in so far as reactions I, II, and III are concerned.

Discussion

It is concluded that the reoxidation in vitro of the four interchain disulfides of the IgG1κ described in preceding papers (Sears et al., 1974, 1977a) is a nonrandom and partially cooperative process. This conclusion stems from the failure of the theoretical random model case and all other models with independently interacting disulfides to account adequately for the prominent features of the experimental profiles. The cooperativity is judged to be partial rather than absolute because the data are also not consistent with any identifiable pathways for this system in which the formation of one type of bond would necessarily precede the formation of another. It should be noted that cooperativity customarily refers to the thermodynamic states in protein reactions whereas the system studied here is not in equilibrium with respect to disulfide bond formation and the cooperativity is kinetic in nature.

The exact degree and mechanism of cooperativity in the experimental system has not yet been elucidated because the experimental information is inadequate for a unique determination of the probabilities of the 12 possible microstates in this system. The additional information needed to solve this problem should be forthcoming from studies in which the reoxidative behavior of one set of bonds can be isolated and analyzed separately. One such study was described in the preceding paper where H chains reoxidized in the presence of alkylated L chains which were irreversibly blocked in their ability to form covalent inter-HL bonds.

In summary, the theoretical analysis suggests the following mechanism of disulfide bond formation in this molecule: The first disulfide bond formed may be any of the four possible bonds, but the probability that it is an HL bond is roughly twice the probability that it is an HH bond. Once the first inter-HL bond is formed, the reaction probability for the second such bond diminishes relative to that for one or both inter-HH disulfides. The first inter-HL disulfide appears to drive the molecule into a conformational state in which the second HL bond closes with significantly greater difficulty.

An alternative theoretical treatment based on second-order rate constants has been developed by Percy et al. (1975) who attempted to stimulate earlier IgG reoxidation experiments from the same laboratory (Petersen and Dorrington, 1974). The present analysis points up two possible sources of difficulty in the theory by Percy et al. (1975) which may account for their relatively poor simulation of IgG reoxidation data that are qualitatively very similar to our data for IgG1κ(*Fro*). First, Percy et al. assume that the ratio of bond forming probabilities for the HL and HH bonds is constant in contrast to the results illustrated in Figure 3 where the ratio is more biphasic rather than constant in nature. Second, the model by Percy et al. assumes freely interacting chains and intermediates rather than noncovalently associated tetramers. However, as earlier studies from the same laboratory clearly showed (Bigelow et al., 1974), the conditions of their reoxidation experiments strongly favor rapid noncovalent chain association into tetramers.

The finding that the interchain disulfides in IgG1κ(*Fro*) form via specific pathways rather than by a wholly random process suggests parallels between this work and recent studies on disulfide bond formation in single-chain proteins (Wetlaufer, 1973; Wetlaufer and Ristow, 1973; Creighton, 1975). In both the single chain and multichain cases, the crucial difficulty is to sort out the kinetic and thermodynamic determinants leading to defined pathways. There are, however, significant distinctions. In particular, for single-chain reoxidations it is important to maintain concentrations low enough that intermolecular contacts are minimized. The opposite is, of course, true in covalent assembly processes where the concentrations must be high enough to ensure rates of association that meet cellular synthetic requirements.

Even though the thrust of the theory developed here is discussed in terms of disulfide reoxidations, no assumptions have in fact been made as to the "direction" of the reactions and the theory equally applies to the opposing reactions of disulfide reduction. As discussed in the following paper (Sears et al., 1977b), the theoretical framework here provides a useful basis for the analyzing experiments in which the reductive susceptibilities of the interchain disulfides of immunoglobulins are examined with reducing agents.

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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.