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Further Studies of the Riboflavin-Binding Immunoglobulin IgG^{Gar}. Resolution into Fractions of Different Riboflavin Content and Aspects of Reassembly†

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ABSTRACT: A previously described human immunoglobulin with unusual flavin-binding activity, IgG^{Gar} [Farhangi, M., & Osserman, E. F. (1976) *N. Engl. J. Med. 294*, 177], is further characterized. The protein can be fractionated into two subpopulations, one of which is nearly completely saturated with riboflavin and one in which the binding sites are largely vacant. Possible differences between these fractions and/or their binding sites are explored. While electrophoretically distinct, the IgG^{Gar}–riboflavin complexes possess a basic similarity in the binding sites of both fractions as evidenced by spectroscopic examination. However, an important difference exists in that added riboflavin equilibrates reversibly with the vacant sites of native IgG^{Gar}, while the riboflavin in the occupied sites is essentially irreversibly bound. The tight association may be due to an in vivo combination of riboflavin with protein of different conformation than occurs in vitro, such as an incompletely assembled or folded tetramer. Accordingly, in vitro renaturation was examined. Studies of renaturation revealed that the reduced interchain disulfides within a tetramer reoxidize smoothly, although inter-heavy-chain bonds form less readily than inter-heavy–light-chain disulfides. Renaturation of IgG^{Gar}, unlike previously studied IgG molecules, does not proceed under conditions in which the protein structure had previously been significantly disrupted. The assembly defect is localized in the inability of the denatured heavy chain to refold into a stable species capable of combining with the light chain.

Several years ago, Farhangi & Osserman (1976) discovered and characterized an unusual human immunoglobulin. The protein IgG^{Gar} was isolated from a patient with multiple myeloma and shown to be an IgG2, with λ light chains. This immunoglobulin was remarkable because it occurred in the serum with bound hapten, riboflavin, which remained tightly associated throughout standard isolation and purification procedures. The average occupancy of antigen binding sites in the native protein was about 1.5 equiv of riboflavin/mol of IgG^{Gar} in the preparations Farhangi and Osserman studied.

Among both murine and human monoclonal antibodies described until then, IgG^{Gar} was unique in being the only documented example of an antibody–hapten system in which the hapten was associated with the protein in vivo as well as in the isolated, purified antibody. However, as the authors noted, the discovery was strongly favored by the fact that the hapten is a chromophore, with a bright color and a characteristic visible spectrum. Accordingly, they suggested the possibility of instances of colorless haptens bound to circulating myeloma and normal immunoglobulins.

In addition to identifying the chromphore as riboflavin, Farhangi and Osserman localized the binding site to the Fab fragment and demonstrated that additional riboflavin was bound to unoccupied sites up to a saturating level of 2 equiv/mol. Moreover, although riboflavin was the exclusive chromophore on the native protein, the vacant sites bound

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flavin mononucleotide (FMN) equally well, as determined in competition experiments. Flavin adenine dinucleotide (FAD) competed somewhat less effectively, and a variety of other aromatic heterocyclics as well as ε-dinitrophenyl-L-lysine bound still less tightly.

A final striking result of the investigation was that while a variety of agents could be found for dissociating riboflavin, including acid, urea, dimethyl sulfoxide, and high concentrations of certain salts, dissociation was invariably accompanied by irreversible denaturation.

In the past few years, we have continued and extended the study of this important antibody–hapten system as part of a larger investigation of the in vitro assembly of immunoglobulins (Sears et al., 1975, 1977a,b; Sears & Beychok, 1977; Friedman et al., 1978; Kazin & Beychok, 1978). In particular, we have been interested in the relationship between riboflavin binding and the stability of the four-chain structure.

In this paper, we show that IgG^{Gar} can be separated into two fractions of differing degrees of saturation with riboflavin and that there appear to be two classes of binding sites for riboflavin. In an accompanying paper, we report results on thermodynamic and kinetic features of riboflavin binding.

Materials and Methods

Purification of IgG^{Gar}. Serum containing IgG^{Gar} was very viscous. It was diluted initially with 2 volumes of 0.15 M NaCl, and the resultant solution was mixed with an equal volume of 0.024 M $CaCl_2$. Any clot which formed was removed by filtering the solution through cheesecloth. The protein was then precipitated by dialysis against 1.64 M ammonium sulfate (after adjustment of the pH to 7.2 with ammonium hydroxide) with three changes of 6 L each. The suspension was centrifuged at 10000 rpm for 20 min and the precipitate washed 3 times with a freshly made 1.64 M ammonium sulfate solution. The protein was then redissolved with a minimum amount of water and extensively dialyzed for several days against 0.01 M Tris-HCl (Trizma, Sigma) and 0.02 M NaCl (pH 7.8). Finally, the protein was applied to DEAE-cellulose (DE-23, Whatman) which was precycled and equilibrated with the same buffer used for dialysis. After being loaded, the protein was eluted with a 0.02–0.5 M salt gradient generated by the continuous mixing of 2 L of 0.01 M Tris-HCl and 0.02 M NaCl (pH 7.8) with 2 L of 0.01 M Tris-HCl and 0.5 M NaCl (pH 7.8). A flow rate of 50 mL/h was maintained by a Gilson pump. The eluate was collected in 13-mL fractions on an LKB fraction collector.

Figure 1 shows the elution profile, which is composed of two protein peaks. The proteins were pooled into A and B fractions as indicated, and the region of overlap was pooled as an additional fraction. These were then exhaustively dialyzed against water, frozen, and lyophilized. After each purification step, the purity of the protein was checked by sodium dodecyl sulfate ($NaDodSO_4$)[1]–polyacrylamide gel electrophoresis. If any high molecular weight impurities appeared in the sample after the ion-exchange step, they were removed by molecular sieve chromatography on Ultrogel AcA 34 (LKB Instruments).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. All samples were first dissolved in a buffer with neutral pH. Iodoacetamide (IAAm; Sigma) was then added at a final concentration of 0.1 M to prevent disulfide exchange. The sample solution was made 1% in $NaDodSO_4$ (Bio-Rad) by the addition of an appropriate volume of a 10% $NaDodSO_4$ solution. For achievement of complete denaturation, the sample solutions were placed in a boiling water bath for 1–2 min (Kazin, 1977). Gel samples also contained 0.1% (v/v) brom phenol blue as the tracking dye and sucrose to increase the solution density. Generally, 10–20 μg of protein of each sample was loaded in each well of a slab gel which was made up of 0.05 M sodium phosphate (pH 7.2), 0.1% $NaDodSO_4$, 0.13% *N,N,N′,N′*-tetramethylethylenediamine, 0.075% ammonium persulfate, and either 5% or 7.5% acrylamide [acrylamide/bis(acrylamide) = 37:1]. The reservoir buffer was 0.05 M sodium phosphate and 0.1% $NaDodSO_4$ (pH 7.2). $NaDodSO_4$ gel electrophoresis was performed according to standard methods (Weber & Osborn, 1975) on an Ortec Model 4200 slab gel system.

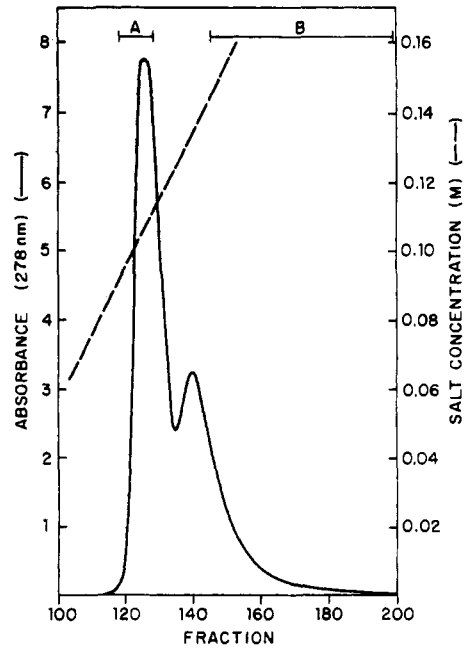

FIGURE 1: DEAE-cellulose chromatography of IgG^{Gar}. The ammonium sulfate precipitate of *Gar* serum was redissolved in 0.01 M Tris-HCl and 0.02 M NaCl buffer (pH 7.8) and loaded on a 2.5 × 100 cm DEAE-cellulose column equilibrated with the same buffer. The flow rate was 50 mL/h. Fractions of 13 mL were collected. The absorbance at 278 nm (—) and the salt concentration (---) were measured. The protein solution was pooled into fractions A and B, as indicated.

Isoelectric Focusing. Isoelectric focusing gel electrophoresis was carried out on an LKB Model 2117 Multiphor apparatus. An ampholine polyacrylamide gel plate (LKB) with pH range 5.5–8.5 was used. Generally, the sample was first dissolved or dialyzed against a low-salt buffer (or water) and transferred to the gel with a piece of filter paper (Whatman 3MM, Paratex) which had been soaked in sample solution. Two or three pieces were used for each sample to yield a final protein content of approximately 30 μg. Electrophoresis usually proceeded for 2 h and was stopped when the voltage reached a constant value. Several pieces of the gel were cut from its edge and soaked in water overnight to allow diffusion of ampholines out of the gel and into the solutions, which were used to identify the pH gradient on the gel. The rest of the gel was first soaked in a fixing solution—34.5 g of sulfosalicyclic acid and 115.0 g of trichloroacetic acid dissolved in a mixture of 300 mL of methanol and 70 mL of distilled water—for 40 min to precipitate the proteins and allow the ampholine to diffuse out of the gel prior to staining.

Nitrogen and Phosphate Determination. Analyses for total nitrogen (Jaenicke, 1974) and protein (Lowry et al., 1951) were carried out in conjunction with spectral measurements

[1] Abbreviations used: DTT, dithiothreitol; H chain, heavy chain of IgG; HOAc, acetic acid; L chain, light chain of IgG; $NaDodSO_4$, sodium dodecyl sulfate; TS buffer, 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.5.

to determine extinction coefficients. The flavin moiety was dissociated from the protein by following a previously described procedure (Koziok, 1971), and a phosphate-determination method was used (Jaenicke, 1974) to detect FAD or FMN.

Analytical Ultracentrifugation. Sedimentation velocity experiments were performed on a Spinco Model E analytical ultracentrifuge equipped with an automatic photoelectric scanner and an RTIC temperature control unit. Samples were run at 20 °C and scanned at 278 nm. The speed was 40 000 rpm for whole IgG^{Gar} protein and heavy-chain samples, and 44 000 rpm was used in the case of light-chain samples.

Circular Dichroism. Circular dichroism (CD) spectra were obtained on a Cary Model 60 spectropolarimeter equipped with a 6001 CD attachment. All measurements were done in a cell with 1-cm path length at room temperature. Molecular ellipticity is used to present data in the visible region and is expressed as degrees·centimeters^2 per mole of riboflavin.

Reduction and Reoxidation. Reduction and reoxidation experiments were based on previously published procedures (Sears et al., 1975; Sears, 1974; Sears & Beychok, 1977) with some modifications. For reduction experiments, protein in 0.15 M Tris-HCl, 0.15 M NaCl, and 2 mM EDTA (pH 8.0) was filtered through a 0.4 μM polycarbonate membrane. Dithiothreitol (DTT) was then added to these solutions at a DTT/protein molar ratio of 300:1. All procedures were performed in a sealed plastic working box which was purged with nitrogen in order to prevent air oxidation of the samples. At different time intervals, an aliquot was transferred to a vial, and the reduction reaction was stopped by the addition of concentrated iodoacetamide solution to give a final concentration of 0.1 M. Reduction was usually complete after 3 h. All alkylated samples were then subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis analysis.

In reoxidation experiments, the protein solution, after reduction with DTT in a 1:300 molar ratio, was passed through a 2.5 × 50 cm Bio-Rad P-2 column equilibrated with N_2-aerated 10 mM HOAc. The eluted protein solution was collected and kept under N_2 until the pH was raised. Reoxidation was initiated by adding $^1/_9$ volume of 0.5 M Tris-HCl and 1 M NaCl (pH 8.06) buffer. The final concentration was 0.05 M Tris-HCl, 0.1 M NaCl, and 9 mM acetate, and the ionic strength was 0.14 M. At appropriate times, aliquots of the reaction mixture were removed, reacted with 0.1 M iodoacetamide to stop further reoxidation, and analyzed for sulfhydryl content.

Separation of Heavy and Light Chains. Reduced protein was passed through a Sephadex G-100 column, equilibrated with a solution of 1 M propionic acid and 1 mM EDTA that had been bubbled with N_2. Separated H and L chains were collected around the peak position to achieve minimum contamination of the other component. For the L_2 reoxidation experiment, the L chain eluted from the G-100 column was passed through a P-2 column equilibrated with 10 mM HOAc and reoxidized by the procedure described above.

Sulfhydryl Determination. Free sulfhydryl in the reoxidation mixture was assayed with Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The experimental procedure and the method of calculating SH titers used in our laboratory have been described by Sears et al. (1977a).

Quantitative Densitometry. All the intermediates in reoxidation and reduction experiments were characterized by their respective migrations on the $NaDodSO_4$–polyacrylamide gel. The six bands on the gel were identified as H, L, HL, H_2, H_2L, and H_2L_2 by using the intermediates of a reference human IgGκ protein, *Fro*, that has been extensively studied in our laboratories (Sears et al., 1975). The slab gels were cut into 8 × 80 mm strips and scanned on a Gilford Model 250 spectrophotometer equipped with linear gel transport and a chart recorder. The method used for quantitative analysis of densitometer scans has been described (Sears et al., 1977a).

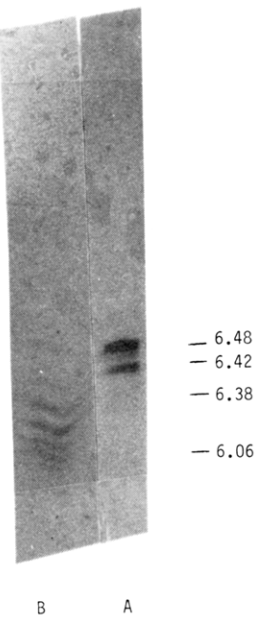

FIGURE 2: Isoelectric focusing gels of A and B proteins which were dissolved in distilled water. Total protein concentration in each gel was ~30 μg. The pH values of the gel in the region of the protein bands are indicated at the right-hand side.

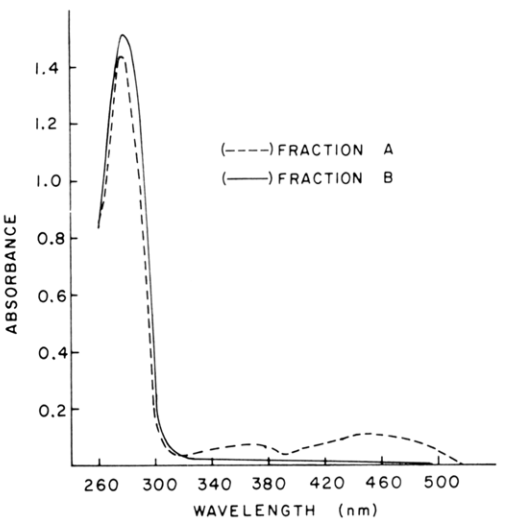

FIGURE 3: Absorption spectra of IgG^{Gar} protein. Absorption spectra of 3.24 μM A protein (---) and 3.87 μM B protein (—) in TS buffer.

Results

Gel Electrophoresis. Fractions A and B (see Figure 1) and *Fro* have similar $NaDodSO_4$ gel patterns, and their major bands possess the same mobility. This result indicates that the molecular weights of the *Fro* and *Gar* proteins are similar and is consistent with the value of 160 000 which was obtained for *Gar* by Farhangi & Osserman (1976).

As seen in Figure 2, the isoelectric focusing gel patterns of the A and B proteins are distinctively different. Fraction A shows two major components with isoelectric points of 6.4 and 6.5, while fraction B has five discernible bands with none watching those of A. The isoelectric points of these fall in the range 6.0–6.4.

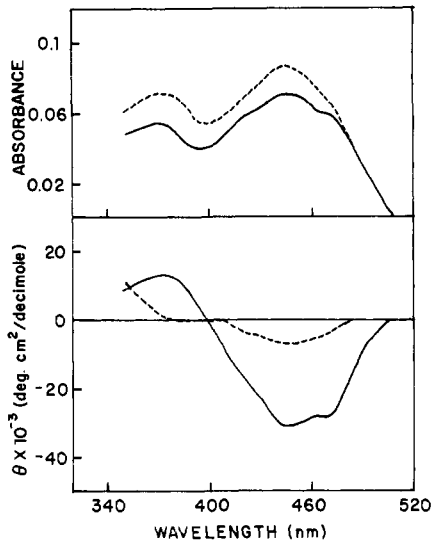

FIGURE 4: Absorption and CD spectra of *Gar* protein and riboflavin in the visible region. Upper panel: absorbance spectra of 2.4 μM A protein (—) and 6.88 μM riboflavin (---). Lower panel: CD spectra of 23.41 μM A protein (—) and 41.4 μM riboflavin (---).

Absorption Spectra. Native IgG^{Gar} absorbs both UV and visible radiation. Its absorption spectrum is shown in Figure 3. There is a major peak between 270 and 280 nm and peaks at 373 and 445 nm due to riboflavin. The characteristic protein UV band is at 275 nm in the case of fraction A and at 277 nm for fraction B. A shoulder which appears between 450 and 500 nm, seen in Figure 4, is evident in most flavin–protein complexes and has been attributed to vibronic structure (Strickland, 1974; Shiga et al., 1979).

The protein concentration can be calculated from the absorbance of the peak near 275 nm, after subtracting the contribution from riboflavin, and using a value of 22.9 for $\epsilon_{1\%}$. The value was based on nitrogen and Lowry (Lowry et al., 1951) analyses, and while anomalously high, its use is justified in that it yields self-consistent binding data (Chang et al., 1981). Riboflavin concentration (free or bound) was computed from the absorbance at 445 nm, using a molar extinction coefficient of 12.5 × 10^3 (Koziok, 1971). According to the absorption spectra of the A and B fractions, the molar ratio of riboflavin to protein was found to be close to 2 for A and 0.2 for B.

Circular Dichroism. The CD spectra of riboflavin in the visible region, shown in Figure 4, have a small negative band at 445 nm and a positive one at 373 nm, corresponding to absorption peaks at the same wavelengths. The riboflavin–protein complex of fraction A has a distinctively different spectrum from pure riboflavin. Its CD spectrum, shown in Figure 4, reveals that the negative ellipticity of free riboflavin at 445 nm in the bound form is increased by approximately 5-fold. A shoulder emerges at 470 nm, corresponding to one which appears in the absorption spectrum of fraction A protein, and is a common characteristic of most flavin–protein complexes (Shiga et al., 1979; Edmondson & Tollin, 1971; Nishikimi & Kyogoku, 1973). In addition, the positive ellipticity maximum at 373 nm increases, probably as a result of the interaction between aromatic side chains of the protein and the isoalloxazine ring (Edmondson & Tollin, 1971).

The protein of fraction B exhibits only weak CD bands in the visible region. However, after addition of riboflavin to the levels in fraction A, its CD spectrum is the same as the latter. This identical induced optical activity of bound riboflavin in both fractions implies a comparable environment for riboflavin irrespective of whether it was initially bound to the isolated protein available or bound to available sites after isolation.

Table I: $s_{20,w}$ Values of IgG^{Gar} Proteins

sample		buffer	$s_{20,w}$ (S)
IgG		0.15 M Tris-HCl, 0.15 M NaCl (pH 8.0)	6.64
L chain (alkylated):	5 μM	0.05 M Tris-HCl, 0.1 M NaCl (pH 7.5)	2.47
	7.7 μM		2.89
	15 μM		3.38
H chain (alkylated) (reduced)		0.05 M NaOAc, 0.1 M NaCl (pH 5.0)	11.51
			13.39
			15.55

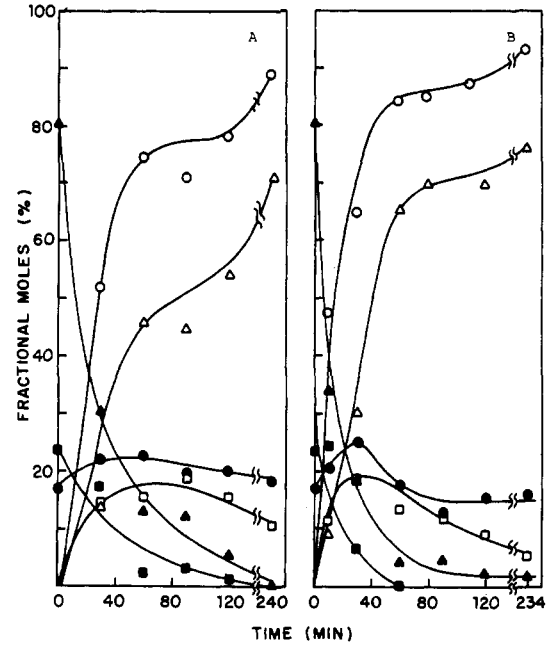

FIGURE 5: Reduction kinetics of IgG^{Gar} by DTT. Protein (8.64 μM) was reduced by DTT at a DTT/protein ratio of 50 (A) or 100 (B). Aliquots were removed from the reduction mixture at the times indicated in the figure, alkylated, and subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis. The fractional moles of each component were calculated as described in the text: L (○); H (△); HL (□); H_2 (●); H_2L (▲); H_2L_2 (■).

Sedimentation Velocity. $s_{20,w}$ values of native IgG^{Gar}, and of the separated heavy (H) and light (L) chains, are tabulated in Table I. The value for native IgG^{Gar} agrees with that of Farhangi and Osserman and is characteristic of four-chain IgG proteins. The light-chain value corresponds to a mixture of monomer and dimer, with dimer mainly at the highest concentration. The heavy chain, even at pH 5, is a very high molecular weight aggregate.

Reduction and Reoxidation. Reduction proceeds at neutral pH under conditions in which the protein molecular remains tetrameric, and only interchain disulfide bonds are reduced (Sears et al., 1975). Sulfhydryl analysis yielded a value of 12.0 ± 1.0 equiv of SH/mol at the completion of reduction with a 100-fold excess of DTT.

The time course of reduction is shown in Figure 5, at DTT excesses of 50- and 100-fold. There are several features to note in the figure. At the start of the reaction, a great deal of the protein is present as H_2L, although this is difficult to quantify since the bands for H_2L_2 and H_2L overlap on gels in unreduced samples. Reduction proceeds virtually to completion in about 3 h insofar as light-chain appearance is concerned, but only about three-fourths of the expected amount of heavy chain appears, the remainder persisting as H_2, even at a 100-fold excess of DTT. Since *Gar* protein has four

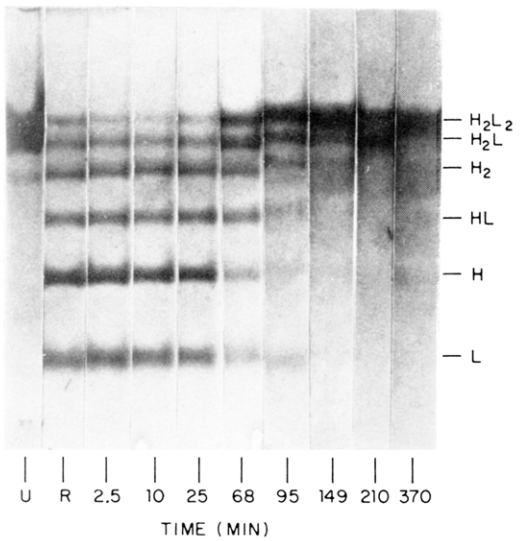

FIGURE 6: NaDodSO$_4$–polyacrylamide gels for the reoxidation of reduced, undissociated H_2L_2. Reoxidation intermediates are identified at the right. U and R stand for the unreduced and reduced samples, respectively. The rest of the gels contain the samples alkylated at the times indicated at the bottom of the figure. Total concentration of reduced H_2L_2 in the reoxidation system was 4.90 μM. A 31-μg sample of protein was loaded in each well.

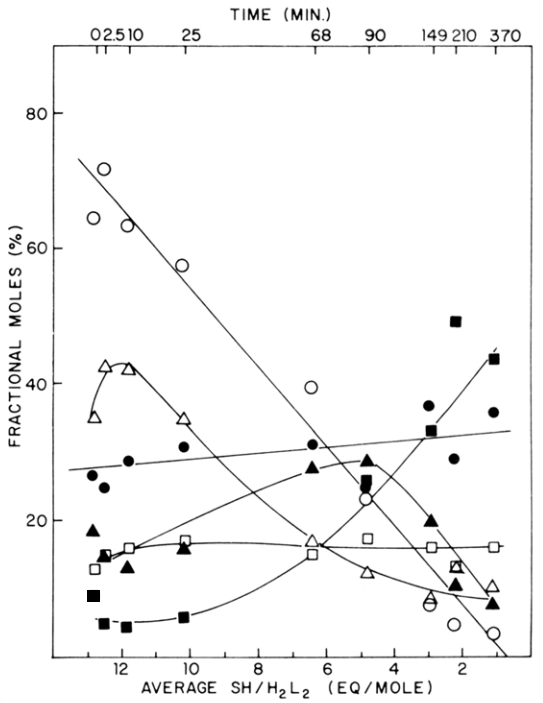

FIGURE 7: Quantitative analysis of the reoxidation gels of Figure 6. The fractional moles of each component are plotted as a function of SH equivalents/mole of total protein, which was measured at the times indicated at the top of the figure. The symbols are as defined in the legend of Figure 5.

inter-HH disulfide bonds, more H_2 than HL is expected to appear in the reduction mixture if the ease of reduction of an inter-HH bond is the same as that of an inter-HL bond. Were the reduction random, the maximum level of H_2 observed during reduction would be about 4 times that of HL (Sears & Beychok, 1977). Compared to the calculated differences, however, the experimentally observed difference between the fractional moles of the H_2 and HL is slight, suggesting that the inter-HH bonds are more sensitive to reduction by DTT than are the inter-HL bonds. Experiments were carried out at DTT/protein concentration ratios of 50 and 100, and the reduction patterns obtained under these two conditions were similar.

The covalent reassembly of reduced *Gar* protein is illustrated in Figures 6 and 7. The "zero" time sample, marked R, already shows significant amounts of H_2 and HL, but their concentrations remain relatively constant thereafter. This is similar to the behavior of these intermediates during reduction. During reoxidation, the excess of H_2 over HL is greater than in the reduction, but still well below the ratio expected for a reoxidation in which the disulfides form with equal facility.

All intermediates, H_2, HL, and H_2L, were present during reoxidation, showing that assembly of this protein proceeds by multiple pathways. Free L and H chains entirely reacted during the reoxidation, but H_2L_2 formation was incomplete, owing to the persistence of H_2 and HL even after many hours. The half-time of formation of H_2L_2, however, was only slightly longer (100 min) than that found for IgG *Fro* under the same conditions.

An investigation of the assembly from separated H and L chains was precluded by the irreversible denaturation of the separated H chain, under the experimental conditions used. Several common denaturing systems were used, including propionic and acetic acids, urea, and propylurea, but the denatured protein did not renature in typical renaturing systems. In one set of experiments, DTT was included in the renaturing system as a thiol–disulfide exchange catalyst to correct any incorrectly formed disulfide bonds. In another, chaotropic salts were added in an attempt to find a partially stable nonnative state. However, these attempts were unsuccessful.

It may be concluded from these results that once the reduced protein is denatured no recombination between H and L chains will occur. Isolated H chain has a strong tendency to form aggregates, even at low pH; isolated L chain can, however, be renatured after transfer to a nondenaturing environment. A covalent L_2 dimer forms at pH 7.5 with a half-time of 5 min; in contrast, L chain of *Fro* protein forms L_2 dimer at a much slower rate (Kazin & Beychok, 1978). By fluorescence methods described in the succeeding paper, it is shown that this L_2 dimer binds little or no riboflavin.

Discussion

In terms of net charge near neutral pH, and fractional saturation with riboflavin, IgG^{Gar} behaves as if it comprises two very similar, slightly heterogeneous subpopulations. One has an isoelectric point in the range 6.4–6.5 and is saturated, or nearly saturated, with riboflavin in its native form; the second is somewhat more acidic, with an isoelectric point in the range 6.0–6.4, and occurs with little or no riboflavin bound. Fraction A, which elutes from an anion-exchange column at pH 7.8, mainly contains the riboflavin-saturated form; fraction B contains the riboflavin-free form, which can be saturated with added riboflavin.

There is no direct evidence relating the net charge differences to the amount of riboflavin bound in the two fractions. Riboflavin is itself uncharged at pH 7.8 (Walsh, 1979; Massey et al., 1969). Moreover, analytical phosphate determinations showed no phosphorus on the dissociated chromophore, within the limits of experimental error, excluding a possible preference for charged FMN by the less acidic fraction. It is possible, of course, that the charge difference is generated by a different amide content in otherwise identical sequences, but why this should also be connected to sharply different occupation of the binding site by a neutral hapten is not at all evident.

A more plausible explanation, but again with no evidence at present, is that groups in or near the binding sites have altered pK_a values, depending on whether riboflavin is in the

binding site or not. Such pK_a changes could result from general conformational differences or different degrees of exposure of the prototropic groups to solvent. An acidic group bearing a negative charge when exposed to water might become protonated and neutral if riboflavin excludes solvent. Alternatively, a prototropic group might be a contact residue. For example, any side chain acting as a donor in a hydrogen bond would have an abnormally elevated pK_a value.

A still more perplexing facet of the nature of the binding interaction, already implicit in the work of Farhangi & Osserman (1976) and the basis of the studies in the following paper (Chang et al., 1981), is that the riboflavin binding sites of the two subpopulations differ with respect to reversibility. As noted above, riboflavin bound to the native protein cannot be removed except under conditions that denature the protein. Moreover, no attempts at reconstitution have thus far succeeded. In contrast, the binding of added riboflavin, although of very high affinity constant, is completely reversible.

There is some evidence, albeit inconclusive, that the distinction between irreversible and reversible riboflavin binding does not result from a difference in the sequence of residues and the conformation of the sites in the two cases. Sequencing studies of the variable regions of both heavy and light chains, being carried out by Dr. F. Garver (unpublished experiments), thus far reveal no instances of two residues occupying the same position. Furthermore, there is physicochemical evidence for the similarity in sites, as inferred from (1) the induced optical activity of added riboflavin, which is the same in the visible spectrum as that of riboflavin bound to the native protein, (2) the similarity in absorption spectra, (3) the complete quenching of riboflavin fluorescence by both kinds of sites, and (4) the noncovalent character of the irreversible binding.

This last point adds only little to the argument for similarity of sites but is clearly important in its own right since covalent bonding might explain the observed irreversibility. Examples of covalent attachment of flavin moieties to proteins are, indeed, well-known [for a recent review, see Singer et al. (1976)]. However, labilization of such bonds at neutral pH by denaturation of the protein with urea is unlikely, even if not absolutely ruled out.

If covalent bonding is excluded, then some posttranslational conformational event may occur that locks any already bound riboflavin into the site and makes its release from the native protein kinetically forbidden. It is possible that such irreversibly bound riboflavin must come into contact with the site at a stage when assembly is not complete. Such an obligatory sequence of events might prevent riboflavin dissociation, except under denaturing conditions.

Finally, as a possible clue to the failure of IgG^{Gar} to reassemble after reduction and chain separation, the stabilities of the interchain disulfide bonds were examined. Reduction at neutral pH with 50- and 100-fold excesses of DTT revealed that inter-HH (H_2) disulfides were more susceptible than inter-HL (HL) bonds, but aside from this feature, reduction proceeded smoothly. Similarly, reoxidation of the reduced protein, at neutral pH and without prior chain separation, led to reconstituted protein, although the yield was less than 100%.

These results suggest that the underlying reason for the failure of IgG^{Gar} to reassemble after chain separation is that recombination of chains does not yield a noncovalently assembled tetramer. It has been established that in vitro covalent assembly of human and mouse Ig molecules depends on the prior formation of that native noncovalently associated tetramer structure, and this does not occur with IgG^{Gar}. Instead, separated H chains, whether alkylated or not, rapidly form high molecular weight aggregates that do not go on to react with light chains.

In contrast, the separated light chains appear to be behaving normally, at least insofar as the ability to renature and to form L_2 dimers. The half-time for formation of the covalent L_2 dimer is much faster than occurs with a human κ chain (Kazin & Beychok, 1978), but this probably results from the lower pK_a of the sulfhydryl group in a penultimate residue as compared to a terminal residue (Pink & Milstein, 1967; Jocelyn, 1972). In addition, alkylated *Gar* light chains occur as a monomer–dimer equilibrium at neutral pH, and the presence of the noncovalent dimer may also speed formation of the disulfide bonds.

The successful in vitro renaturation of IgG^{Gar} then apparently depends on finding conditions for renaturation of isolated H chains without formation of irreversible aggregates.

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