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*RECOVERY OF ANTIGENIC SPECIFICITY AFTER DENATURATION AND COMPLETE REDUCTION OF DISULFIDES IN A PAPAIN FRAGMENT OF ANTIBODY**

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It would now appear reasonably certain that the secondary and tertiary structure, and in some instances quaternary structure, of a number of proteins is dependent only on the information contained in the amino acid sequence.[1-8] This fits well with current concepts of protein synthesis which provide only for the colinear transfer of sequential information from the base triplets of the genetic template to the amino acids of the protein molecule without providing any mechanism for the direct transfer of conformational information.[9, 10] Antibodies are a unique group of proteins characterized by a great diversity with respect to specificity of their binding properties, but also, within any single class of antibodies, such as γ_{1A}, γ_{1M}, or γ_2, by a great similarity in charge, amino acid composition, and over-all three-dimensional structure. It has been difficult to envision a specific amino acid sequence and consequently a different genetic template determining each of the multitude of binding site conformations possible.[11] An alternative view holds that specificity is generated by three-dimensional folding more or less independent of the amino acid sequence. This is said to occur at the time of protein synthesis through the mediation of antigen. The stabilization of this structure has been postulated to be related to noncovalent interactions[12] or to a specific arrangement of disulfide bonds.[13]

A direct test of the relationship between amino acid sequence and antibody specificity has not been possible because of the chemical heterogeneity of antibody preparations available to date. Indirect tests of the role of noncovalent bonds in stabilization of the conformation of the binding site have been carried out by Karush[13] and by Buckley, Whitney, and Tanford.[14] However, in neither of these investigations could antibody specificity be regenerated after both cleavage of disulfide bonds and unfolding of secondary structure.

In the present investigation a papain-derived antibody fragment[15] containing the specific binding site is subject to full reduction of its disulfide bridges and unfolding

of its secondary structure as evidenced by optical rotatory dispersion. Upon removal of the denaturant and oxidation of the disulfide bridges, a large part of the original binding specificity is regained.

Materials and Methods.—Preparation of univalent antibody: Gamma globulin was isolated by alcohol fractionation of serum[16] from rabbits immunized with ribonuclease (RNase). Anti-RNase was precipitated with RNase at equivalence. The precipitate was dissolved in 0.5 *M* formic acid, and filtered through a 2 × 60-cm column of Sephadex G-100, developed with the same solution. Anti-RNase appears in the void volume of this column.

Anti-RNase was reacted with papain, the course of the reaction followed by a pH stat, and terminated when three peptide bonds per mole of antibody had been cleaved. Fraction I was separated by carboxymethyl-cellulose chromatography.[15]

In order to facilitate determination of protein concentration in dilute solution, a portion of the material from anti-RNase F-I was acetylated with acetic anhydride C^{14} (New England Nuclear Co.—specific activity 10 mc/mmole) to the extent of 1 mole of acetate per mole of protein. This limited acetylation did not result in any decrease in binding capacity of the univalent antibody.

Antibody assay: A method for determining antigen binding by univalent antibody was devised and proved suitable for assay at extremely low protein concentration. RNase was iodinated with I^{125}. [17] Samples of anti-RNase F-I and RNase I^{125} were mixed in a buffer containing 0.1 *M* tris-acetate pH 7.5 and 1 mg/ml egg white lysozyme (Worthington, lot #LY629A). The presence of lysozyme in the buffer, a protein similar in charge and molecular weight to RNase, prevented nonspecific adsorption of RNase I^{125} to glass, gel, and antibody protein. After 24 hr incubation at 4°, the mixture was applied to a 1.5 × 55-cm column of Sephadex G-100 and the effluent allowed to pass through a 0.5-ml coil of no. 20 Teflon tubing contained within the well of a scintillation counter. Constant flow rates were maintained with a metering pump, and the radioactivity of the effluent was periodically integrated by a scaler and printer.

Reduction: Samples of anti-RNase F-I were reduced for 2 hr in the presence of 8 *M* urea and 0.1 *M* mercaptoethanol. Urea was freed of cyanate ion by passage through a mixed bed ion exchange resin. Guanidinium chloride was made from recrystallized guanidinium carbonate (Eastman) by neutralization with HCl and was then twice recrystallized from ethanol. In order to determine the degree of reduction, a portion of each of the samples was carboxymethyllated[2] and then subjected to amino acid analysis according to Spackman, Stein, and Moore.[18]

The half-cysteine content of anti-RNase F-I was determined by cysteic acid analysis[19] employing the automatic amino acid analyzer.

Optical rotatory dispersion: Rotatory dispersion studies were performed with a Cary model 50 spectropolarimeter employing anti-RNase F-I in 0.1 *M* sodium chloride solution, in 8 *M* urea solution, and in 8 *M* urea and 6 *M* guanidinium chloride after reduction and carboxymethylation. Protein concentrations were determined by absorbance at 280 mμ, employing an extinction coefficient of 1.38. Rotations were corrected for refractive index of water, 8 *M* urea,[20] and 6 *M* guanidinium chloride.[21] Because of the considerable absorbance of guanidinium chloride below 210 mμ, sufficient dispersion data could not be obtained to define a trough of the negative Cotton effect. Consequently, an analysis of the dispersion data between 450 and 300 mμ was made using the two-term Drude equation derived by Shechter and Blout.[22–24]

$$[R'] = A_{(\alpha,\rho)\,(193)} \frac{\lambda^2_{193}}{\lambda^2 - \lambda^2_{193}} + A_{(\alpha,\rho)\,(225)} \frac{\lambda^2_{225}}{\lambda^2 - \lambda^2_{225}},$$

where $[R']$ = mean residue rotation corrected for refractive index, λ = wavelength in mμ, $A_{(\alpha,\rho)(193)}$ and $A_{(\alpha,\rho)(225)}$ constants related to the crossover points of the Cotton effect related to λ. From a graphical representation of this equation, $A_{(\alpha,\rho)(193)}$ and $A_{(\alpha,\rho)(225)}$ can be calculated and compared to previously determined values for known fully helical and fully random polypeptides.

Oxidations: After 2 hr of reduction at an appropriate protein concentration, samples were dialyzed against 1 mmolar mercaptoethanol in tris-acetate buffer pH 8.0 at 23° for 24 hr. At the end of this time the dialysis fluid was changed to 0.1 *M* tris-acetate pH 8.0 and the dialysis terminated 24 hr later.

Density gradient centrifugation: Acetyl C^{14} anti-RNase and the reoxidized protein were centrifuged 18 hr at 100,000 × *g* in a linear sucrose gradient (20–5% w/v) employing hemoglobin as a marker.

Results.—Isolation of anti-RNase: The lack of appreciable contamination of the anti-RNase peak with RNase was demonstrated in experiments employing fluorescein-labeled RNase.[25] No fluorescence (employing an Aminco-Bowman fluorometer) could be demonstrated in the void volume of the column. After neutralization and removal of a small amount of precipitate formed, the remaining protein solution can be 95 per cent precipitated with RNase at equivalence.

Determination of antigen binding: The binding of RNase-I^{125} to antibody proved a sensitive and reproducible assay for univalent antibody binding activity. It is apparent from Figure 1*A* that when RNase I^{125} is run with Fraction I from normal

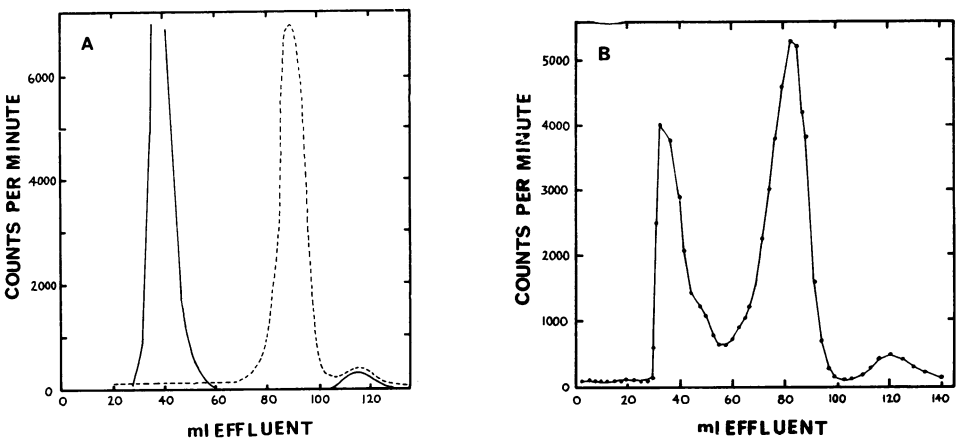

FIG. 1.—(*A*) Separation of RNase-I^{125} from anti-RNase F-I on a 1.5 × 55-cm column of Sephadex G-100 in 0.1 *M* tris acetate pH 7.5 containing 1 mg/ml lysozyme.——, 30 μg antiRNase F-I incubated with RNase I^{125}; - - - - , 500 μg papain Fraction I from normal rabbit gamma globulin with RNase I^{125}. (*B*) Ten μg of anti-RNase F-I with RNase I^{125}.

gamma globulin on Sephadex G-100, all the radioactivity is found within the included volume of the gel without any evidence for nonspecific binding of RNase-I^{125}. A minor second peak is observed which probably represents contaminating free iodide but is well resolved from the RNase I^{125} peak. When an excess of anti-RNase F-I is added, nearly all the radioactivity is found in the excluded volume of the column with the exception of the small trailing iodide peak which does not change in size. This indicates that all the labeled RNase is capable of being bound to antibody and that there has been no destruction of antigenic determinants during the iodination process. A typical assay is illustrated in Figure 1*B*. Counts under the first and second peak are integrated and compared to values obtained from various standards. In this way it is possible to quantify as little as 0.1 μg of antibody.

Reduction: It is apparent from the cysteic acid analyses in Table 1 that there are 12 half cysteines or 6 disulfide bonds in anti-RNase F-I. This agrees with the analyses of Crumpton and Wilkinson[26] for rabbit papain Fragment I. The degree of reduction obtained under various conditions can be deduced from S-carboxymethyl cysteine analyses. Reduction is incomplete in 8 *M* urea at pH 7.5. However, in 10 *M* urea or in 6 *M* guanidinium chloride, essentially complete reduction is obtained.

TABLE 1

CYSTEIN CONTENT AND DEGREE OF REDUCTION OF ANTI-RNASE F-I*

Reaction	Cysteic acid† (moles per mole protein)	S-Carboxymethylcystein‡ (moles per mole protein)
Performic acid oxidation	12.4	—
Reduction, 8 *M* urea, pH 7.5, and carboxymethylation	—	9.3
Reduction, 10 *M* urea, pH 7.5, and carboxymethylation	—	11.1
Reduction, 10 *M* urea, pH 8.5, and carboxymethylation	—	11.7
Reduction, 6 *M* guanidinium chloride, pH 7.5, and carboxymethylation	—	11.2
Reduction, 6 *M* guanidinium chloride, pH 8.5, and carboxymethylation	—	12.4

* Calculated on the basis of a molecular weight of 49,000 as determined by long-column equilibrium centrifugation[35] and a specific extinction coefficient of 1.38.
† Corrected for 6% loss in hydrolysis.[19]
‡ Corrected for 9% loss in hydrolysis.[36]

Unfolding of secondary structure: Ultraviolet rotatory dispersion studies of anti-RNase F-I under various conditions are illustrated in Figure 2. In 0.1 *M* NaCl a small trough in the dispersion diagram at 245 mμ is evident, with a larger trough at 225 mμ, a crossover point at 220 mμ, and a large positive peak at shorter wavelengths. Specific interpretation of this unconventional pattern is difficult, but it certainly represents an ordered secondary structure. There is no suggestion of the trough at 233 mμ generated by the negative Cotton effect of an α helix.[22] In 8 *M* urea, while negative rotation is increased and the small trough at 245 mμ is abolished, the over-all shape of the first portion of the negative Cotton effect is the same. Similarly, after reduction in 8 *M* urea of 4 to 5 of 6 disulfides, no additional change except increased negative rotation is observed. However, after reduction in 6 *M* guanidinium chloride, the minimum at 225 mμ is abolished and the curve takes on the form of a random coil. A minimum at 210 mμ could not be observed because of solution absorbance, but analysis by the two-term Drude equation of the visible dispersion pattern is consistent with a random coil. A plot of $[R']\ (\lambda^2 - \lambda^2_{193})/\lambda^2_{193}$ versus $\lambda^2/(\lambda^2 - \lambda^2_{225})$ yielded the values $A_{(\alpha,\rho)(193)} = -267$ and $A_{(\alpha,\rho)(225)} = -240$. Figure 3 shows these two constants plotted with a line defined by $A_{(\alpha,\rho)(193)}$ and $A_{(\alpha,\rho)(225)}$ of polyglutamic acid at pH 4 (fully helical) and at pH 7 (fully random). Fully reduced anti-RNase F-I in 6 *M* guanidinium chloride falls nearly on this line and very close to the position of fully random polyglutamic acid. When these data are used to calculate helix content,

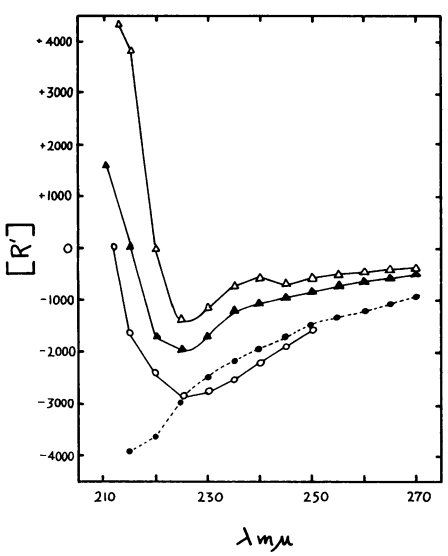

FIG. 2.—Ultraviolet optical rotatory dispersions. △——, Anti-RNase F-I in 0.1 *M* NaCl; ▲——, anti-RNase F-I in 8 *M* urea; ○——, anti-RNase F-I reduced and carboxymethylated in 8 *M* urea; ●- - -, anti-RNase F-I reduced and carboxymethylated in 6 *M* guanidinium chloride.

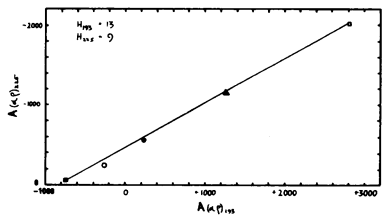

FIG. 3.—Plots of $A_{(\alpha,\rho)}$ (193) versus $A_{(\alpha,\rho)}$ (225). □, Polyglutamic acid, pH 4 in water solution; ■, polyglutamic acid pH 7 in water solution; ●, RNase; ▲, Bovine serum albumin; O, anti-RNase F-I reduced and carboxymethylated in 6 *M* guanidinium chloride.

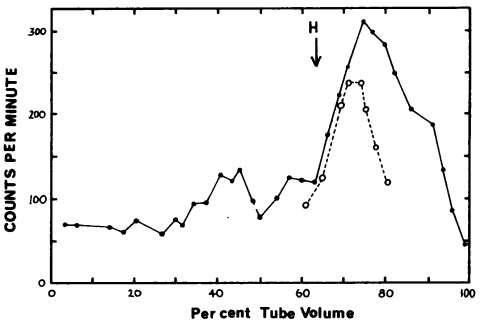

FIG. 4.—Sucrose density gradient centrifugation in 20% to 5% (w/v) linear gradient. ●——, Reoxidized acetyl C^{14} anti-RNase F-I; O- - -, acetyl C^{14} anti-RNase F-I; *H*, hemoglobin.

H_{193} = 13 per cent and H_{225} = 9 per cent. These values are among the lowest reported either for proteins or synthetic polypeptides. RNase, a protein of low helical content (25%), and bovine serum albumin, a protein of moderately high helical content (55%), are shown for comparison.

Reoxidations: Table 2 shows the per cent regain of specific binding activity of

TABLE 2

ANTIGEN BINDING OF ANTI-RNASE F-I REDUCED AND THEN OXIDIZED WITH AIR

Conditions of reduction*	Conditions of oxidation†	Protein remaining in solution‡ %	Binding activity after oxidation§ %
8 *M* Urea, pH 7.5	1 mmolar Mercaptoethanol, pH 8.0	30	56
8 *M* Urea, pH 7.5	Carboxymethylated	45	0
8 *M* Urea, pH 7.5	0.1 *M* Acetic acid	52	0
10 *M* Urea, pH 7.5	1 mmolar Mercaptoethanol, pH 8.0	62	22
10 *M* Urea, pH 7.5	Carboxymethylated	65	0
10 *M* Urea, pH 7.5	0.1 *M* Acetic acid	46	0
6 *M* Guanidinium chloride, pH 8.0	1 mmolar Mercaptoethanol, pH 8.0	53	27
6 *M* Guanidinium chloride, pH 8.5	1 mmolar Mercaptoethanol, pH 8.0	58	20

* All reductions were done in 0.1 *M* mercaptoethanol.
† All oxidations were done at a protein concentration of 10 μg/ml.
‡ Determined by radioactive counting.
§ Expressed as a percentage of activity of an equal concentration of acetyl C^{14} anti-RNase F-I.

antibody reduced under different conditions. Protein losses of unreduced controls were approximately the same and are probably related to adsorption to glass vessels and dialysis tubing. There was no evidence for the formation of insoluble products of oxidation.

Partially (5–6/8 disulfides) or fully reduced materials, after carboxymethylation, are completely inactive. Oxidation at pH 3.5 also results in an inactive product. This would suggest that, as in the instance of RNase,[27] oxidation at this pH results in random arrangement of disulfide bridges with consequent failure of formation of the specific conformation necessary for binding antigen.

The data in Table 3 indicates that recovery of activity is very dependent on protein concentration during oxidation. Concentrations which are consistent with

TABLE 3

CONCENTRATION DEPENDENCE OF ANTI-RNASE F-I OXIDATION*

Protein concentration, μg/ml	Protein remaining† in solution, %	Binding activity‡ after oxidation, %
90	31	8
45	31	26
9	30	56

* Reduction done in 0.1 *M* mercaptoethanol, 8 *M* urea, pH 7.5.
† Determined by radioactive counting.
‡ Expressed as a percentage of activity of an equal concentration of acetyl C^{14} anti-RNase F-I.

successful oxidations of other proteins[2] yield very little activity with anti-RNase F-I.

Prolonged dialyses, first against mercaptoethanol solutions and then against buffer at pH 8, seemed essential since simple dilution of the denatured and reduced material into pH 8 buffer or rapid separation by Sephadex G-25 resulted in the recovery of very little activity.[2]

Figure 4 shows that by density gradient ultracentrifugation, the sedimentation velocity of the major component of the reoxidized material is similar to that of the starting material, 3.2S. However, in the reoxidized material a heavier aggregate (7.8S) is apparent as well as some lighter material.

Discussion.—Gamma globulin is a compact globular protein which seems to have very little evidence for α helix content by analysis of optical rotatory dispersion by the equation of Moffitt[28] or by deuterium exchange.[37] Consequently, a change in optical rotation at a single wavelength[14] cannot be interpreted as evidence for unfolding and may simply reflect alterations in the effective dielectric constant of the environment of various portions of the molecule.[22-24] In the present study, the far ultraviolet rotatory dispersion pattern of anti-RNase F-I confirms that α helix is not present in this part of the molecule. The nature of the dispersion curve strongly suggests an ordered or repeating structure although its identity cannot now be defined. Eight *M* urea or partial reduction in 8 *M* urea abolishes the small trough at 245 mμ but does not alter the over-all shape of the dispersion curve. This suggests that the partially reduced protein in 8 *M* urea retains a considerable part of its original conformation. However, reduction in 6 *M* guanidinium chloride clearly abolishes the trough at 225 mμ and gives a dispersion curve identical to that of a random coil with respect to the region which can be observed above 215 mμ. This impression is confirmed by the results of the two-term Drude analysis of visible dispersion data. Unlike dispersion analyses by the Moffitt equation which are useful in defining the content of α helix in proteins comprised only of helices and random coils and which may not be applied to the study of nonhelical proteins,[24] the present analysis allows specific identification of a random coil as opposed to other more ordered structures.

It is evident that disulfide bridges between various parts of a peptide chain or between two peptide chains limit the number of conformations which an otherwise random coil can assume. In order to define the role of the amino acid sequence alone in determining conformation, cleavage of all disulfide bridges prior to renaturation is clearly essential. In the present investigation, evidence of complete reduction of disulfide bridges is given.

The striking relationship between activity regained and protein concentration during oxidation may simply relate to aggregation as demonstrated in RNase

oxidations at much higher concentrations.[2] However, unlike RNase, antibody papain Fraction I contains two peptide chains held together by a single disulfide bridge. The formation of an interchain disulfide bridge in an incorrect position prior to complete refolding and reformation of the intrachain bridges may result in an altered and inactive conformation. Dilution, of course, favors intramolecular over intermolecular reactions. This allows each chain to assume its correct conformation prior to assembly of the two-chain complex. The role of the amino acid sequence in determining conformation may be related directly only to intramolecular folding of the individual chains. It has been demonstrated previously that separated, undenatured A and B chains recombine readily to form an active product, even when the interchain disulfide bond is not permitted to reform.[29-31]

The identity of the reoxidized material as an A-piece B chain complex is supported by the sedimentation velocity obtained. It is not surprising that a portion of the product has polymerized to a heavier material while another part remains in the form of single chains. The principal 3.2S peak may well, of course, contain some A-chain and B-chain dimers which would be expected to have a similar sedimentation velocity.

A possible criticism of interpretation of these data is that optical rotatory dispersion studies may fail to detect localized regions of preserved conformation in an otherwise random structure. Yet it is evident from these experiments that a unique arrangement of the disulfide bridges in antibody is essential for preservation of the specific binding activity. This is supported by the demonstration that oxidation at pH 3.5, which is known to cause random arrangement of disulfides in RNase results in an inactive product. Since the effect of oxidation at low pH is a change in the charge of certain amino acid side chains and consequently the nature of the information contained in the sequence, the unique arrangement of disulfide must be determined by sequential information.

It is of considerable interest that the material subject to partial reduction and partial denaturation in 8 *M* urea at pH 7.5 does not regain specific activity after oxidation at pH 3.5. This may be taken to indicate that though 1–2 of the 6 disulfide bonds are resistant to reduction, a specific arrangement of the others is essential for preserving the conformation of the binding site.

It is now highly probable that both chains participate in formation of the binding site.[29-31] Consequently, the pairing of disulfides in both chains must be considered. Assuming that only one disulfide connects the two chains,[32] and as suggested in previous data, there are 3 disulfide bonds within the B chain and 2 within the A chain,[26] the probability of any single arrangement forming by random chance is $1/(35)(6!/3!2^3)\ (4!/2!2^2)$, or 0.000635.[33, 34] The activity regained in these experiments exceeds this by a factor of over 400. These findings make it highly likely that the amino acid sequence alone determines the conformation of the binding site, and that the specificity of an antibody must be determined at some time prior to protein synthesis.

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