Hapten Stabilization of Antibody Conformation*

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ABSTRACT: The effect of ε-DNP-lysine on the tertiary structure of the Fab portion of anti-DNP antibodies in solutions of 4 M guanidine hydrochloride (Gd·HCl) was studied as a function of time by measuring the circular dichroism and intrinsic tryptophan fluorescence of Fab. The circular dichroism spectrum of native anti-DNP Fab shows a number of bands, particularly those at 232 nm and in the 250–300-nm region, which are sensitive to the conformational state of the protein; after 5 hr in 4 M Gd·HCl these bands disappear. The presence of hapten markedly retarded loss of these bands. Significant optical activity in the 250–300-nm regions was retained even after 24 hr in 4 M Gd·HCl. Quenching of intrinsic Fab tryptophan fluorescence by bound ε-DNP-lysine was found to be higher when the hapten was present throughout the time of exposure of protein to denaturant than when it was absent. The binding of hapten to antibody, therefore, stabilizes the tertiary structure of the Fab portion in such a way as to at least partially maintain compactness of the molecule and rigidity and environmental asymmetry of the optically active chromophores. These results are consistent with the interpretation that the amino acid residues in the binding site are located in at least several nonconsecutive portions of the polypeptide chains which are brought into a close spatial relationship by the native molecular conformation.

The effect of Gd·HCl on the β-structure component of Fab was also studied. The intensity of the 217-nm band was essentially unchanged after 24 hr in 6 M Gd·HCl. There thus appears to be considerable retention of native β structure under strong denaturing conditions.

Recent optical rotatory dispersion studies on the denaturation of high-affinity rabbit anti-DNP[1] antibody and its Fab fragment in solutions of 4 M Gd·HCl have shown that the presence of the hapten, ε-DNP-lysine, in the binding site substantially decreases the extent of denaturation (Cathou and Haber, 1967). However, because of the difficulty of interpreting complex optical rotatory dispersion spectra, the stabilization process could not be described in any detail.

The circular dichroism of high-affinity rabbit anti-DNP, particularly the hapten binding Fab fragment, has recently been studied (Cathou *et al.*, 1968). Since the circular dichroism spectrum showed far more detail than the corresponding optical rotatory dispersion spectrum, and since some of the bands have been tentatively identified, we reinvestigated the haptenic stabilization of antibody structure on denaturation by Gd·HCl. In particular, we studied the effects of bound hapten on the circular dichroism bands as a function of time of exposure to denaturing agent.

As an independent measure of molecular conformation and compactness we investigated the effect of hapten on the average distance of tryptophan residues from the hapten binding site, a parameter which can be measured by the technique of fluorescence quenching (Velick *et al.*, 1960).

We report here that by all criteria examined, the binding of hapten to antibody stabilizes the tertiary structure, in agreement with our earlier results (Cathou and Haber, 1967). In addition, it is probable that some aromatic amino acid residues, most likely tryptophan and/or tyrosine are located near or at the binding site, and that these are firmly held in an asymmetric environment by the presence of hapten, even after extensive exposure of the protein molecule to Gd·HCl.

Haptenic stabilization of antibody tertiary structure suggests that the amino acid residues involved in the binding site, in analogy with those in enzyme active sites (Blake *et al.*, 1965; Kartha *et al.*, 1967), are located in at least several nonconsecutive portions of the polypeptide chains of the Fab fragment and are brought into a close spatial relationship by the molecular conformation.

Methods and Materials

High-affinity rabbit anti-DNP and its papain fragments were prepared as previously described (Cathou and Haber, 1967). Antibody was purified from serum collected at least 6 weeks after immunization; this results in preparations with average association constants, K_0, of $\geq 10^9$ moles/l. for ε-DNP-lysine. Ultra Pure Gd·HCl from Mann Research Laboratories was dried for several days in a vacuum desiccator before use. ε-DNP-lysine was obtained from Mann Research Laboratories. All solutions were prepared in PBS buffer.

Concentration Determination. Concentration of the anti-DNP Fab fragments and ε-DNP-lysine were determined spectrophotometrically using extinction coefficients previously reported (Steiner and Lowey, 1966; Eisen and Siskind, 1964).

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[1] The abbreviations used are: anti-DNP, antibody directed against the ε-DNP-lysine determinant; Gd·HCl, guanidine hydrochloride; PBS, phosphate-buffered saline (0.01 M potassium phosphate buffer–0.15 M NaCl, pH 7.4).

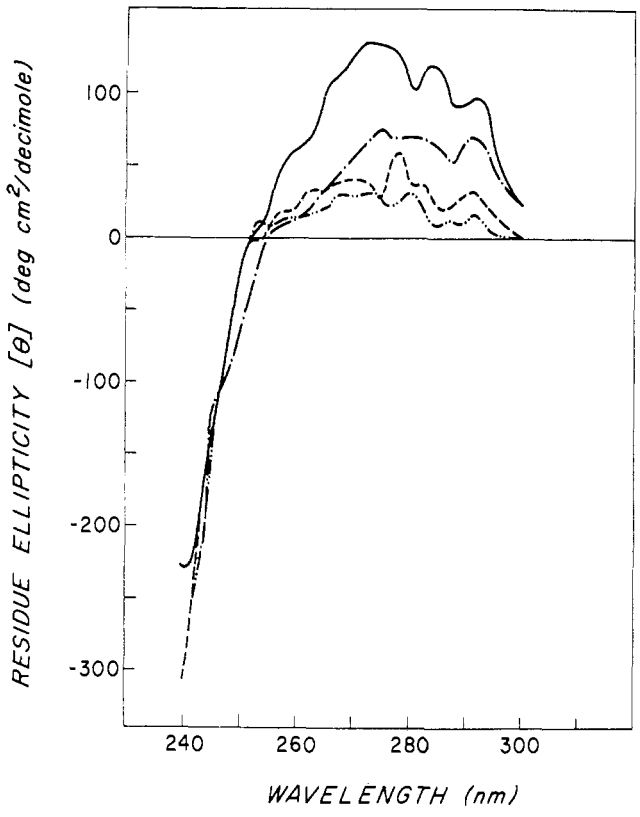

FIGURE 1: Circular dichroism spectra of rabbit anti-DNP Fab in 4 M Gd·HCl (pH 7.4) at 30°, at several time intervals. (——) Fab in 0.01 M phosphate buffer–0.15 M NaCl (pH 7.4), no Gd·HCl present; (–·–) Fab in 4 M Gd·HCl after 20 min; (– – –) Fab in 4 M Gd·HCl after 2 hr, 15 min; (– · · –) Fab in 4 M Gd·HCl after 4 hr.

Circular dichroism measurements were made using a Cary Model 6001 recording spectropolarimeter with circular dichroism attachment. Spectra were obtained at 30°, in cells with path length ranging from 5 cm to 1 mm. Samples generally contained 0.1–0.4 mg/ml of protein and, in experiments with hapten, the ε-DNP-lysine was in threefold molar excess over that of protein. Since these concentrations of protein are at least 10^3 greater than the average dissociation constant, this is a sufficient quantity of hapten to saturate the binding sites. Solution absorbances were less than 1.5. Ellipticity is expressed in residue ellipticity, $[\theta]$, which is defined as

$$[\theta] = \frac{M\theta}{10cl} \quad (1)$$

where M is the mean residue weight, θ is the observed ellipticity in degrees, c is the concentration of optically active solute in grams per cubic centimeters, and l is the path length in centimeters. A mean residue weight of 108 was used (Crumpton and Wilkinson, 1963). Correction of ellipticity for the solvent effect on the rotational strength of optically active transitions was not included. Knowledge of the effective refractive index of the environment surrounding any optically active group in a protein is limited, thus making such a correction of dubious value.

Calculation of Ellipticity Retention between 300 and 250 nm. Quantitative estimation of ellipticity was determined by measuring the area under the positive branch of the spectrum between 300 and 250 nm. The per cent ellipticity retention was calculated by comparing the area under a given curve (area x) to the area under the curve of native anti-DNP Fab (area y). The results are expressed as

$$\% \text{ ellipticity } (E) = \frac{(\text{area } x)}{(\text{area } y)}(100) \quad (2)$$

Area was estimated by cutting and weighing a tracing of the desired section of the curve. Error is estimated to be within ±5%.

Fluorescence quenching measurements were made on an Aminco-Bowman spectrofluorometer equipped with a Hanovia 150-W xenon lamp. The concentration of Fab was 0.12 mg/ml (2.4×10^{-6} M) and the solvents were PBS or 4 M Gd·HCl as indicated. An excitation wavelength of 282 nm and an emission wavelength of 348 nm were employed (uncorrected). All measurements were made with a solution volume of 1.5 ml in a thermostated cell at 30°.

The quenching of Fab fluorescence was performed by titrating a solution of Fab with a solution of ε-DNP-lysine (Velick *et al.*, 1960). To compare efficiencies of fluorescence quenching for various solutions, the initial fluorescence intensity of Fab, F_i, and a final fluorescence intensity, F_f, obtained after a given addition of hapten (1.7×10^{-6} M) were recorded and used to calculate Q_{final}

$$Q_{final} = 1 - \frac{F_f}{F_i} \quad (3)$$

Values of Q_{final} were determined for Fab in 4 M Gd·HCl as a function of time both with and without the initial presence of hapten. For the experiments in which hapten was not initially present, solutions of Fab in 4 M Gd·HCl were made up and allowed to stand for a given time. F_i was then measured and hapten was added. F_f was then measured again and Q_{final} for this time interval calculated.

With hapten initially present, the fluorescence measured after a given time is F_f. To calculate Q_{final} for these cases, F_i was assumed to be the value of F_i obtained at the same time interval for Fab in 4 M Gd·HCl in the absence of hapten.

Results

All data presented are for the papain fragment Fab I (terminology of Porter, 1959) of anti-DNP. The results obtained for the Fab II fragment were qualitatively similar.

Circular dichroism spectra from 300 to 250 nm of Fab in PBS and in 4 M Gd·HCl for time intervals up to 4 hr after solution mixing are shown in Figure 1. The spectrum of native Fab in PBS shows at least four bands. Spectra of the Fab–ε-DNP-lysine complex for time intervals in 4 M Gd·HCl up to 3.5 hr are shown in Figure 2. The circular dichroism spectra of *native* Fab and of the Fab–ε-DNP-lysine complex in PBS are similar within experimental error from 300 to 210 nm (Cathou *et al.*, 1968). The changes occurring in this region of the spectrum on denaturation are complex and appear to affect all of the ellipticity bands.

Figure 3 shows the spectra obtained after 6 hr in 4 M Gd·HCl. There is a striking difference observable between the

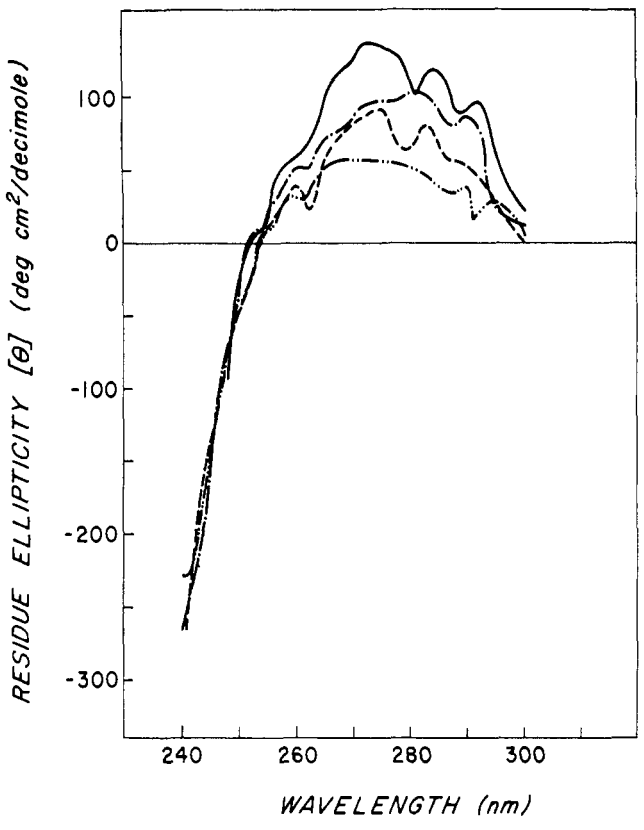

FIGURE 2: Circular dichroism spectra of the rabbit anti-DNP Fab–ε-DNP-lysine complex in 4 M Gd·HCl (pH 7.4) at 30°, at several time intervals. (——) Complex in 0.01 M phosphate buffer–0.15 M NaCl (pH 7.4) no Gd·HCl present; (–·–) complex after 15 min in 4 M Gd·HCl; (– – –) complex after 1 hr, 25 min in 4 M Gd·HCl (– · · –) complex after 3 hr, 30 min in 4 M Gd·HCl.

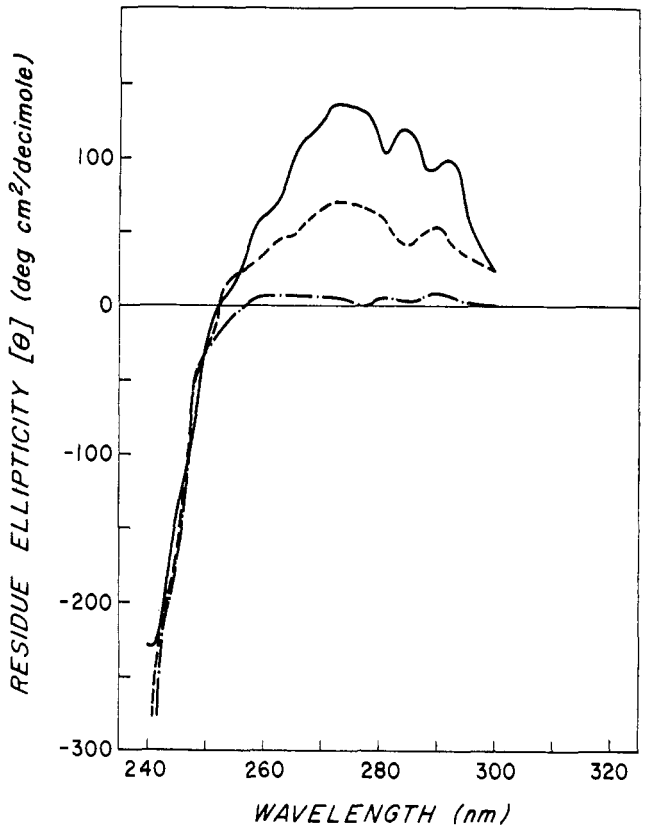

FIGURE 3: Circular dichroism spectra of rabbit anti-DNP Fab and the Fab–ε-DNP-lysine complex in neutral buffer and 4 M Gd·HCl. (——) Fab and the Fab–ε-DNP-lysine in 0.01 M phosphate buffer–0.15 M NaCl (pH 7.4) no Gd·HCl present; (– – –) Fab–ε-DNP-lysine complex after 6 hr in 4 M Gd·HCl; (–·–) Fab after 6 hr in 4 M Gd·HCl.

ellipticity curves of Fab and of the Fab–hapten complex. Approximately half of the intensity of the bands of the complex is retained, while the bands of Fab have essentially disappeared.

The per cent of native Fab ellipticity from 300 to 250 nm retained after a given time in the presence and absence of hapten is shown in Table I. At 15 min, the rate of denaturation as measured by the loss of ellipticity, was twice as fast for Fab as for the Fab–hapten complex. In the absence of hapten about 80% of the native ellipticity in the 300–250-nm region was lost after 4 hr while in the presence of hapten only 50% was lost. Between 4 and 6 hr in 4 M Gd·HCl, there was essentially no further change in ellipticity in the presence of hapten, whereas in its absence almost all of the ellipticity had disappeared (Figure 3 and Table I). After 24 hr about 40% of the native ellipticity was retained with hapten while only 5% was retained without hapten.

Figure 4 shows the circular dichroism spectra from 225 to 250 nm of Fab in PBS and in 4 M Gd·HCl with and without hapten after 6.5 hr. The circular dichroism spectrum has a negative ellipticity band at 240–242 nm and a positive one at 232–234 nm in this region (Cathou *et al.*, 1968). After 6.5 hr, *ca.* 30–40% of the ellipticity of the 232–234-nm band was retained (Figure 4) in the presence of hapten while in its absence the band had diminished to a shoulder after as short a time as 1–2 hr. By 24 hr, in the presence of hapten the 232–234-nm band was also reduced to a shoulder.

There is thus significant hapten-dependent preservation of native Fab ellipticity at 232 and 250–300 nm on exposure of Fab to 4 M Gd·HCl.

The 217-nm band of Fab was essentially unaffected after 24 hr in 4 M Gd·HCl. On exposure of Fab to 6 M Gd·HCl for 24 hr, more than 80% of the ellipticity at 217 nm was retained. This figure is somewhat higher than that (*ca.* 50%)

TABLE 1: Per Cent Ellipticity, *E*, Retained from 250 to 300 nm of Anti-DNP Fab in the Presence and Absence of the Hapten, ε-DNP-lysine, As a Function of Time of Fab in 4 M Gd·HCl (pH 7.4) at 30°.[a]

Time	% *E* (+Hapten)	% *E* (−Hapten)
15 min	77	52
4 hr	46	20
6 hr	51	7
24 hr	37	5

[a] Please see eq 2 in the text for the definition of per cent ellipticity.

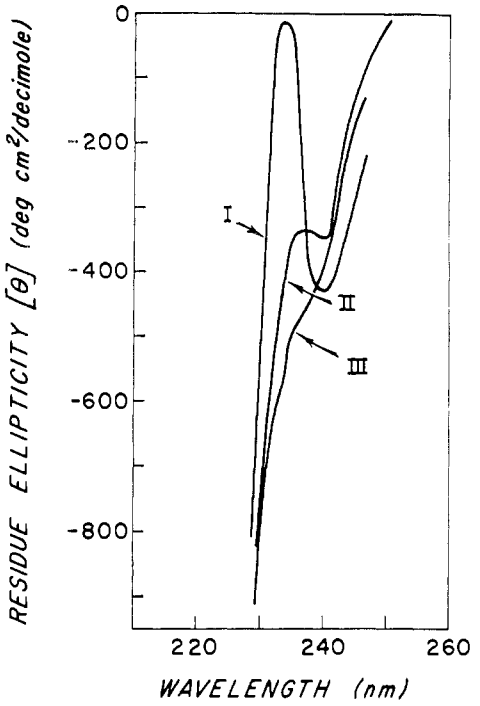

FIGURE 4: Circular dichroism spectra of rabbit anti-DNP Fab and the Fab–ε-DNP-lysine complex in neutral buffer and 4 M Gd·HCl. Curve I: Fab and the Fab–ε-DNP-lysine complex in 0.01 M phosphate buffer–0.15 M NaCl (pH 7.4) no Gd·HCl present; curve II: Fab–ε-DNP-lysine complex after 6.5 hr in 4 M Gd·HCl; curve III: Fab after 6.5 hr in 4 M Gd·HCl.

reported previously (Cathou *et al.*, 1968); the difference may be due to differences in stability among antibody preparations.

Fluorescence quenching data for Fab in 4 M Gd·HCl both with and without hapten initially present are given in Table II. In PBS, Q_{final} was found to be 0.72, a value similar to Q_{max} values previously reported for anti-DNP (Velick *et al.*, 1960). With hapten initially present, the value of Q_{final} of Fab in 4 M Gd·HCl was reasonably constant over the first *60* min. If, instead of being present initially, hapten was added to Fab in 4 M Gd·HCl after the time intervals given in Table II, values of Q_{final} were about the same for the first *30* min and then decreased with time. After 60 min, significantly more of the activity measured by hapten fluorescence quenching was retained when hapten was initially present.

TABLE II: Fluorescence Quenching of Fab in 4 M Gd·HCl As a Function of Time.[a]

Time (min)	Q_{final} (+ε-DNP-Lys Initially Present)	Q_{final} (−ε-DNP-Lys Initially Present)
1	0.52	0.57
5	0.60	0.56
15	0.54	0.45
30	0.56	0.48
60	0.50	0.35
360	0.29	0.13

[a] Please see text for experimental details. Fab concentration = 2.4×10^{-6} M; ε-DNP-lysine concentration = 1.7×10^{-6} M; Q_{final} in PBS (no Gd·HCl) for the same Fab and ε-DNP-lysine concentrations was 0.72.

Discussion

Bands Sensitive to Conformational Change. As was reported in a previous paper (Cathou *et al.*, 1968), the bands at 232 and in the 300–250-nm region can be used as sensitive conformational probes. The present data reveal significant hapten-dependent retention of all of these bands after 6 hr in 4 M Gd·HCl and even after 24 hr in the case of the 300–250-nm bands. In the absence of hapten, no significant retention of ellipticity is observed after 6 hr (Figure 3).

The positive circular dichroism bands from 300 to 250 nm arise from aromatic and possibly disulfide containing residues which are held in asymmetric environments in the native Fab structure (Beychok, 1967). The sign of the band is not necessarily an intrinsic property of the residue but rather is dependent on the relative orientation of the peptide linkage and the residue (Horwitz *et al.*, 1969). Comparison of the circular dichroism spectrum of native Fab in the 300–250-nm region with a recently published spectrum of tryptophan (Strickland *et al.*, 1969) reveals some similarity of the spectra and suggests that perhaps tryptophan residues make a large contribution to this part of the Fab circular dichroism spectrum.

Based on a comparison of ellipticity values and on band position as a function of pH, the positive 232–234-nm band has been attributed to tyrosine (Cathou *et al.*, 1968). This band is more sensitive to conformational change than the bands from 300 to 250 nm. Although the presence of hapten retards the loss of ellipticity at 232–234 nm in 4 M Gd·HCl, hapten stabilization is no longer observed after 24 hr, whereas there is still significant retention of the bands from 300 to 250 nm.

It is possible that the 232-nm band may reflect the particular asymmetric environment imposed by association of the heavy and light chains. The optical rotatory dispersion spectra of intact IgG immunoglobulins exhibit a Cotton effect around 240 nm (Steiner and Lowey, 1966; Cathou and Haber, 1967; Ross and Jirgensons, 1968; Tanford, 1968). Tanford has shown that on disassociation of the heavy and light chains of nonspecific rabbit IgG, and subsequent recombination under conditions in which random reassociation can occur, the 240-nm Cotton effect is not regenerated (Tanford, 1968). However, on recombination of separated heavy and light chains of human myeloma IgG, this Cotton effect can be fully restored. He has therefore suggested that recombination of unique heavy and light chains is necessary for complete restoration of native conformation and the 240-nm Cotton effect. The circular dichroism spectra of rabbit nonspecific IgG, anti-DNP, and human myeloma IgG (Cathou *et al.*, 1968; Ross and Jirgensons, 1968) exhibit two circular dichroism bands in this region at 235 and 240 nm. For nonspecific IgG and anti-DNP these bands have been shown to arise primarily from the 232 and 242-nm bands of the Fab fragment (Cathou *et al.*, 1968)

Thus the Cotton effect centered at about 240 nm in the intact IgG probably contains contributions from the circular dichroism band at 232 nm due to tyrosine.

Inspection of the amino acid sequences of several human and mouse light chains (Kabat, 1968), rabbit γG heavy chain (Wilkinson, 1969; Fruchter *et al.*, 1970), and several human γG heavy chains (Press and Hogg, 1969; Edelman *et al.*, 1969) reveals the presence of a number of tryptophan and tyrosine residues in the loops formed by the intrachain disulfide bonds and in section 105–109 of DAW. Although complete amino acid sequences of rabbit light and heavy chains are presently unavailable, it is probable that tryptophan and tyrosine residues will be found in positions similar to those in other species. Ellipticity at 232 and 250–300 nm therefore probably arises from the specific folding of these regions.

Preservation in the presence of hapten of the various circular dichroism bands must reflect stabilization of the native tertiary structure responsible for the asymmetric environments of the aromatic amino acid residues. Such stabilization suggests interaction of the hapten with at least several nonconsecutive regions of one or both polypeptide chains rather than with an isolated residue or a few vicinal residues on a single chain (Cathou and Haber, 1967). This concept is depicted schematically in Figure 5. This would be the case if the antibody active site, like an enzyme active site, were composed of contact amino acids separated in the linear sequence of the polypeptide chains which are brought into a close spatial relationship by the molecular conformation. Recently reported amino acid sequence data of the variable region of several γG heavy chains and a μ chain reveal alternating segments of constant and variable regions (Edelman *et al.*, 1969; Press and Hogg, 1969; Wikler *et al.*, 1969). Assuming the binding site involves nonconsecutive residues, it is possible that contact amino acids which form the site may come from several of these variable positions.

Alternatively, the presence of hapten in the binding site could stabilize native structure by maintaining the interaction of the heavy and light chains, since it is as been shown that it is harder to disassociate reduced and alkylated chains of anti-DNP in 1 M propionic acid in the presence of hapten (Metzger and Singer, 1963). If this were the case, the site could contain a consecutive sequence of amino acids in the heavy chain *and* a similar sequence in the light chain. Such a structure would still be consistent with our results. Experiments are now underway in our laboratory to establish whether one or both chains are necessary for hapten stabilization.

Flourescence Quenching. In the interaction of ε-DNP-lysine with an anti-DNP combining site, much of the antibody fluorescence is quenched. The mechanism for this quenching involves single–singlet energy transfer from tryptophan residues to bound ε-DNP-lysine (Velick *et al.*, 1960). Such energy transfer is sensitive to the distance between the donor and acceptor molecules (Stryer and Haughland, 1967). For a given antibody preparation, relative changes in Q_{final}, the amount of hapten quenching, will thus reflect changes in the antibody structure near the active site if the hapten is still bound to the same extent.

As seen in Table II, after 60 min values of Q_{final} were significantly higher when hapten was initially present. This suggests a hapten-dependent preservation of antibody activity in 4 M Gd·HCl and is consistent with the hapten stabilization revealed in the circular dichroism data.

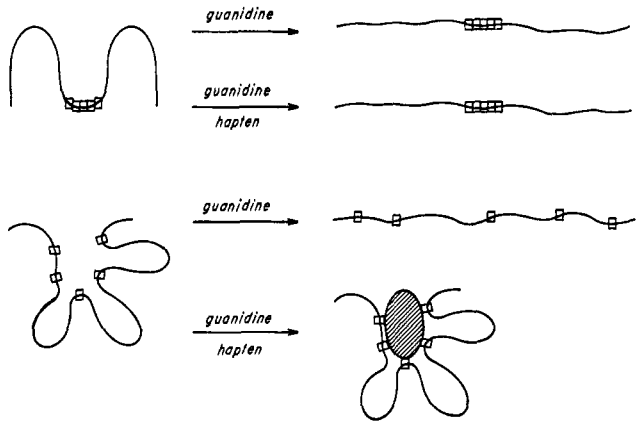

FIGURE 5: Schematic models of two possible configurations of the antibody combining site and the effects of Gd·HCl denaturation on each. The small blocks represent contact amino acids. The number and size of these are not to be taken literally. The large oval in the lower figure represents hapten. In the upper set of figures, the site is composed of several vicinal amino acids along a single polypeptide chain. The presence of hapten has no effect on the course of denaturation. In the lower set of figures, the amino acids comprising the binding site are sequentially separated along the length of the polypeptide chain(s). One or both chains could contribute contact amino acids. The presence of hapten in the site stabilizes this conformation.

The value of Q_{final} with hapten initially present at 360 min given in Table II may be slightly high. This is a result of uncertainty in the estimation of F_i for solutions initially containing hapten. The value of F_i, for a given time interval, was assumed to be the same as that obtained from a solution of Fab in 4 M Gd·HCl in the absence of hapten for a comparable time interval. We have found that F_i for Fab under these conditions (4 M Gd·HCl, no hapten) increases 25–30% over a period of 30 min. Denaturation of the Fab molecule produces changes in the environment of tryptophan residues, thereby affecting changes in the total fluorescence intensity. The fluorescence of tryptophan-containing proteins has previously been shown to have variable behavior in the presence of a denaturant (Steiner *et al.*, 1964). In the presence of increasing concentrations of urea, the fluorescence of lysozyme and human serum albumin is quenched while that of thyroglobulin and pepsin is enhanced. This is presumably not due to the effect of urea or Gd·HCl on the intrinsic fluorescence of tryptophan as the fluorescence energy yield of free tryptophan at neutral pH is essentially the same in phosphate buffer and in phosphate buffer plus 4 M Gd·HCl (Weinryb and Steiner, 1970). Hapten stabilization of native Fab structure (considered independently of hapten quenching) would tend to decrease the rate of increase of F_i thus leading to lower values of Q_{final}. If one assumes that the increase of F_i in the presence of hapten parallels the decrease in optical activity (as shown in Table I), then after 6 hr Q_{final} = 0.20 rather than 0.29 The other values of Q_{final} are essentially unaffected. Q_{max} = 0.20 is probably a lower limit. *However, the assumption necessary to give this lower value of Q_{final} implies hapten stabilization of the Fab native structure.* Consequently, the differences in Q_{final} with and without hapten for $\geq$60 min are probably real.

The time-dependent decrease in hapten fluorescence quenching activity of Fab in 4 M Gd·HCl could result from either of two processes. The first is a decrease in K_0, the association constant, as denaturation proceeds, thereby yielding fewer hapten–Fab complexes for a given hapten concentration. Up to 30 min after solution mixing, especially when hapten was present initially, little change in Q_{final} was observed (Table II). This is consistent with previous results which indicated that, at least initially, association constants of anti-DNP and *p*-nitrophenol were similar in 4 M Gd·HCl and in neutral buffer. Since this is a high-affinity antibody, determination of precise values of K_0 for the hapten, ε-DNP-lysine, by means of equilibrium dialysis is difficult. However, in both 4 M Gd·HCl and in PBS after an equilibration time of 6 hr, K_0 was found to be of the order of 10^8. Also, at 6 hr in 4 M Gd·HCl, in the presence of a threefold molar excess of ε-DNP-lysine, both antibody binding sites were found to fully bind hapten; at the concentrations of antibody and hapten used, K_0 must have been at least 10^8 (Cathou and Haber, 1967). Therefore, at the concentrations of Fab and hapten used in the present fluorescence-quenching experiments, essentially all of the hapten should have been bound.

A second more likely mechanism which would lead to decreased values of Q_{final} involves molecular expansion or "loosening" on denaturation. Tryptophans can move out of the quenching sphere of the hapten as the Fab expands and uncoils. The initial presence of hapten could inhibit the molecular expansion by stabilizing native tertiary structure.

A comparison of the circular dichroism and fluorescence-quenching data in Tables I and II indicates that the former technique is more sensitive in detecting changes in conformation. However, one must bear in mind that the two techniques measure different physical parameters. Circular dichroism spectra reflect changes in *three-dimensional* structure and are sensitive indicators of the interactions of optically active chromophores with other residues in their immediate environments, while fluorescence quenching is primarily a *linear* distance-dependent phenomenon which reflects compactness of the molecule. Fluorescence quenching involves one type of residue (tryptophan) while circular dichroism may arise from several types of residues. In addition, it has been shown that when the donor and acceptor are separated by only short distance, such that the efficiency of transfer lies in the range 100–90%, the energy transfer is relatively insensitive to distance (Stryer and Haugland, 1967; Eisenger *et al.*, 1969). For the tryptophan–ε-DNP-lysine system, we have calculated that 90% efficiency of transfer is achieved at an approximate separation of 10–15 Å. Thus, quenching of tryptophan fluorescence will not be substantially changed until the average distance of the tryptophan residues from the site exceeds this value.

It is thus difficult to make a rigorous quantitative comparison of the two sets of data. Nonetheless, when the data for time intervals of 15 min and 6 hr are compared, it appears that the presence of hapten has a greater retarding effect on the rate of optical activity loss than on the rate of fluorescence quenching activity loss. This suggests that on denaturation a particular residue can probably attain a degree of flexibility sufficient to destroy its asymmetric orientation before molecular expansion causes significant linear displacement of the residues. Hapten stabilization of the molecule against such a mechanism would be more readily reflected by optical activity results than by the distance dependent energy-transfer data.

β Structure. The 217-nm circular dichroism band of Fab has been previously assigned to the n–π* transition of polypeptides in the β conformation (Pysh, 1966; Townend *et al.*, 1966; Cathou *et al.*, 1968). The intensity of this band is essentially unchanged after 24 hr in 6 M Gd·HCl. For poly-*l*-lysine in the β form, the 217-nm band is strongly solvent dependent. Values of the molar ellipticity at 217 nm for poly-*l*-lysine increase from 9000 deg cm²/dmole in sodium dodecyl sulfate to 23,000 deg cm²/dmole in alkali (Sarkar and Doty, 1966). An increase of similar magnitude of the molar ellipticity at 217 nm of Fab on change of solvent from PBS to 6 M Gd·HCl (no data available) would still indicate more than 40% retention of the native β structure.

Therefore, it is reasonable to conclude that substantial preservation of native β structure occurs for Fab in 6 M Gd·HCl even after 24 hr. Retention of native structure under conditions normally assumed to completely denature protein has been reported. Most recently, Doi and Jirgensons (1970) reported that the 217-nm band of γ-immunoglobulin and its tryptic fragments were only slightly affected by acid denaturation. Portions of the β structure of β-lactoglobulin were shown to be quite resistent to alkaline denaturation (Townend *et al.*, 1967). It has been demonstrated that 7 M Gd·HCl at pH 7 is not sufficient to complete the dissociation of lactate dehydrogenase subunits (Millar *et al.*, 1969). More generally, Miller and Goebel (1968) found that hydrodynamic parameters (commonly used criteria) are quite insensitive to large regions of associated residues, and that proteins in 6 M Gd·HCl could therefore have sizeable amounts of highly associated, though not regularly ordered, regions.

Acknowledgment

The authors gratefully thank Dr. Hermann Kalckar and Dr. Donald Wallach for the use of their Cary 6001 spectropolarimeter.

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The Covalent Structure of a Human γG-Immunoglobulin. VI. Amino Acid Sequence of the Light Chain*

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ABSTRACT: The amino acid sequence of the constant region of the κ chain of the human γG-immunoglobulin, Eu, has been determined. This and previous studies establish the complete sequence of a light chain isolated from an intact serum immunoglobulin. With the exception of two residues, the sequence of the constant region is the same as that of constant regions of type K urinary Bence-Jones proteins which are Inv (3+). At position 108 the Eu light chain contains glycine instead of arginine. In addition, the Eu light chain contains aspartic acid at position 122 as has been found in two Bence-Jones proteins; in a third Bence-Jones protein, asparagine has been found at this position. The variable region of the Eu κ chain resembles variable regions of Bence-Jones proteins belonging to subgroup I. In view of the fact that it shows a larger amount of variation than other light chains of this subgroup, it may belong to a new subgroup.

Much of our present knowledge of antibody structure has been obtained through the study of immunoglobulin light chains which are readily obtained in the form of urinary Bence-Jones proteins (Edelman and Gally, 1962; for a recent review, see Edelman and Gall, 1969). Light chains contain a region of variable sequence and a region of relatively constant sequence (Hilschmann and Craig, 1965). The variable region has been implicated in the antigen binding function of antibody molecules (Singer and Thorpe, 1968). The constant (C_L) region of the light chain is presumably required to allow it to associate with the heavy chain so that V regions of both chains can form an antigen-combining site.

In the previous paper in this series (Cunningham *et al.*, 1968) we have presented the amino acid sequence of the variable region of the light chain of the human γGl myeloma protein Eu. We now report the complete amino acid sequence of the Eu light chain.

Materials and Methods

The techniques employed for isolation of light chains and CNBr fragments, and for gel filtration, ion-exchange chromatography, and amino acid analysis have been described previously (Edelman *et al.*, 1968; Waxdal *et al.*, 1968a,b; Cunningham *et al.*, 1968). Conditions for digestion with carboxypeptidase A, carboxypeptidase B, and leucine aminopeptidase have also been described (Gottlieb *et al.*, 1968). High-voltage paper electrophoresis was performed at pH 4.7 and 2.0 (Schwartz and Edelman, 1963).

Sequence Determinations Using the Dansyl-Edman[1] *Method.*

* From The Rockefeller University, New York, New York 10021. *Received December 8, 1969.* This work was supported in part by grants from the National Science Foundation (GB 8371) and the U. S. Public Health Service (AM 04256 and AI 09273).

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: DNS and dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; Asx, aspartic acid or asparagine; Glx, glutamic acid or glutamine.