Binding of Phosphorylcholine to an IgM Waldenström as Studied by Fluorescence Spectroscopy and Circular Dichroism†

Walter F. Riesen,* Joseph Schlessinger,‡ and Jean-Claude Jaton

ABSTRACT: The binding characteristics of an IgM Waldenström (FR) for the ligand phosphorylcholine has been studied by fluorescence spectroscopy. Upon phosphorylcholine addition, IgM FR exhibited 83% enhancement of the tryptophanyl fluorescence, which was associated with a red shift of the emission maximum (5 nm). The same properties were observed with the 7S IgM subunits. The association constant K_A for phosphorylcholine was 6×10^4 M^{-1} for IgM FR and the 7S subunit, as determined by fluorescence titration, a value in agreement with that obtained by equilibrium dialysis. No significant decrease in the K_A value was found in the presence of 3 M urea; in 6 M urea, the increase in fluorescence intensity was 36% of the value obtained in the absence of denaturing agent. In contrast, only 4% of fluorescence enhancement was noted upon binding in 3 M GuHCl and no enhancement could be seen when the concentration of GuHCl was increased to 5 M, thus suggesting complete unfolding of the protein and subsequent loss of binding activity. The pH dependence study of the phosphorylcholine binding to IgM FR indicated no significant differences in the fluorescence enhancement between pH 5 and 8, whereas at more acidic or alkaline pH values, the enhancement became smaller. At pH 3.0 and 10.0, no enhancement was seen, suggesting no binding of the ligand, a fact confirmed independently by equilibrium dialysis. When the spectroscopic properties of the IgM FR were compared with those of murine myeloma proteins that bind the same ligand, large differences were recorded in the amplitude of the phosphorylcholine induced enhancement of the fluorescence and in the shift of the emission maximum wavelength. This suggests that the human and murine proteins interact differently with the small ligand phosphorylcholine thus implying that the variable domains of these molecules are not identical.

Antibodies are endowed with two kinds of interconnected activities, which are mediated through distinct parts of their molecules: the specific binding of antigen by the Fab parts and effector functions, like the binding of complement, the triggering of lymphocytes and the activation of mast cells, due to the Fc moiety. These functions that are unique for molecules belonging to the same class or subclass are usually expressed only after antigen binding. The mechanisms by which they are triggered are still not understood in molecular terms. Investigations on the effect of antigen binding on the conformation of the antibody molecule should, therefore, contribute to the understanding of how antigens induce these events.

The effect of ligand binding on chromophoric groupings (such as tryptophan, tyrosine, and phenylalanine) in proteins is a very sensitive probe for conformational changes within these proteins. The specific binding of small haptenic molecules to homogeneous antibodies (Holowka et al., 1972; Jaton et al., 1975) and to some human (Ashman and Metzger, 1969) and mouse myeloma proteins (Pollet and Edelhoch, 1973, 1974; Jolley et al., 1973) has been successfully used to study ligand-induced conformational changes in antibody molecules. Whereas these studies were mainly centered around antibodies belonging to the IgG and IgA class of immunoglobulins, little is known as yet about the behavior of IgM antibodies.

In a previous report we described an IgM Waldenström that has specific binding activity for the hapten phosphorylcholine (Riesen et al., 1975). Since changes in optical parameters of mouse myeloma proteins upon binding of phosphorylcholine have recently been demonstrated (Pollet and Edelhoch, 1973, 1974), this human IgM protein should be useful for comparing the effects of ligand binding on antibodies of the same specificity but of different immunoglobulin class and species by optical methods.

Experimental Procedure

IgM FR with binding activity against phosphorylcholine has been described previously (Riesen et al., 1975). Phosphorylcholine–calcium chloride, acetyl choline (reagent grade), and choline chloride were purchased from Sigma (St. Louis, Mo.), dithiothreitol (DTT[1]) from Calbiochem (Los Angeles, Calif.) and iodoacetamide from Fluka (Buchs).

Isolation of IgM FR. IgM FR was purified from the serum of patient F.R., suffering from Waldenström's macroglobulinemia, by affinity chromatography using a phosphorylcholine–Sepharose immunoadsorbent as previously described (Chesebro and Metzger, 1972). Specific elution of the bound protein was effected by phosphorylcholine at a concentration of 5×10^{-3} M in borate buffered saline, pH 8.

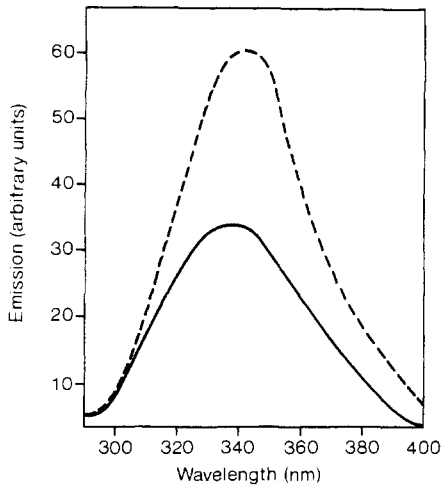
Fluorescence Measurements. Emission spectra were determined on a Hitachi Perkin-Elmer, Model MPF-3 spectrofluorometer equipped with a thermostated cell compartment at 25.0 ± 0.5 °C. Excitation was done at 280 and at 295 nm (bandwidth 8 nm). The emission spectra were recorded in the wavelength range of 290–400 nm (bandwidth 8 nm). Protein

† From the Basel Institute for Immunology, Basel 5, Switzerland, the Institute for Clinical and Experimental Cancer Research, University of Berne, Tiefenau Hospital, 3004, Berne, Switzerland, and the Department of Chemical Physics, The Weizmann Institute of Science, Rehovot, Israel. *Received November 20, 1975.* Supported in part by the Swiss National Foundation for Scientific Research.

* Address correspondence to Institute for Clinical and Experimental Cancer Research, Tiefenau Hospital, 3004 Berne, Switzerland.

‡ Present address: Department of Chemistry, Cornell University, Ithaca, N.Y. 14850.

[1] Abbreviations used are: CD, circular dichroism; CPL, circular polarization of luminescence; GuHCl, guanidine hydrochloride; DTT, dithiothreitol; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.



IGURE 1: Emission spectra of IgM FR without (—) and with excess (- - -) of phosphorylcholine in 0.01 M Tris–0.14 M NaCl, pH 7.5. Excitation was at 280 nm.

solutions were adjusted to an optical density of 0.1 at 280 nm when the excitation was done at 280 nm or 0.1 at 295 nm when the excitation was at 295 nm. The concentrations of phosphorylcholine and acetylcholine, which were determined on a weight basis, ranged between 10^{-7} M and 10^{-3} M.

Preparation of 7S Subunits (IgMs). The subunits were produced by reduction with 0.01 M DTT in 0.1 M Tris–NaCl buffer, pH 8.6, for 60 min at 37 °C, followed by alkylation with a 10% molar excess of iodoacetamide. IgMs was then purified by Sephadex G-200 gel filtration.

The ligands were added in small increments of 10 μl to 2.0 ml of the IgM solution with gentle stirring. The total volume of the added ligand did not exceed 100 μl. The maximum enhancement of emission, which is obtained when essentially all binding sites are occupied by the ligand, was determined by adding excess phosphorylcholine (dry) until no further increase in emission occurred. In order to calculate the association constant, the ratio of the observed fluorescence enhancement to maximum enhancement was taken as the fraction of antibody sites occupied. The concentration of free ligand was calculated as the difference between the concentration of total ligand and that of bound ligand. Corrections were made for dilution. Unless otherwise stated, all experiments were performed in 0.01 M Tris–0.14 M NaCl, pH 7.5.

A control experiment done with an IgM Waldenström that exhibits no binding activity for phosphorylcholine (as determined by equilibrium dialysis) indicated that no correction had to be made for nonspecific enhancement or quenching of fluorescence.

Circular Dichroism (CD). Circular dichroism measurements were performed with a Jasco J-20 recording spectropolarimeter at 22 °C. All experiments were done in triplicate. The mean residue ellipticity, (θ), in units of degree cm^2/dmol, was calculated from the relation $(\theta) = \theta M/10 \times lc$, where θ is the measured ellipticity in degrees, M is the mean residue molecular weight (110), c is the concentration of antibodies in g/ml, and l is the path length of the cell (l = 1 cm). The concentration of IgM was 0.7 mg/ml based on OD_{280} = 0.9 and an extinction coefficient of 12.5 (Metzger, 1970). IgM–phosphorylcholine complexes were formed by adding a tenfold molar excess of hapten over IgM binding-site concentration. Phosphorylcholine at the highest concentration used showed neither absorbancy nor transition bands in the wavelength range studied (260–310 nm). The average experimental error was estimated to be ±2.0° cm^2 dmol^{-1}.

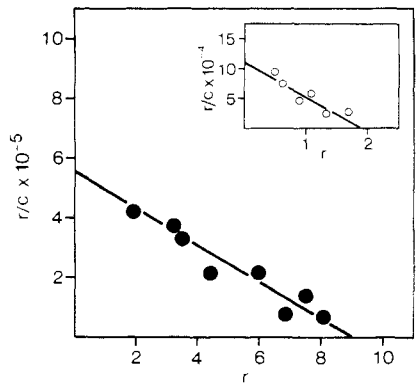


FIGURE 2: Scatchard plot of the binding of phosphorylcholine to pentameric (19S) IgM FR (●) and monomeric (7S) IgM FR (○), as studied by fluorescence enhancement. r is defined as mol of hapten bound per mol of each molecular species; c is the concentration of unbound ligand.

Circular Polarization of Luminescence (CPL) Measurements. Full details about the instrument for measuring CPL spectra of proteins were given elsewhere (Schlessinger et al., 1975). The CPL spectrum is expressed by the emission anisotropy factor g_{em}, defined as $g_{em} = 2\Delta f/f$, where $\Delta f/f$ is the extent of circular polarization of the fluorescence (f) defined as positive for left handed circular polarization (Steinberg, 1975). The excitation wavelength was 275 nm with a resolution of 30 nm. The bandwidth of the analyzing monochromator was 15 nm. The spectrum was recorded between 300 and 380 nm. All measurements were taken at 22 °C. Sample cells of 2-mm light path were used.

Results

Fluorescence Studies with IgM FR. IgM FR, upon binding of phosphorylcholine, exhibited a percentage increase of the tryptophanyl fluorescence of 83% when excitation was done at 280 nm (Figure 1). This fluorescence enhancement was associated with a red shift of the emission maximum wavelength from 337 to 342 nm. With excitation at 295 nm, a percentage increase of 92% was recorded. The fluorescence enhancement upon phosphorylcholine binding was similar for the 19S IgM (83%) as for the 7S IgMs (80%), and the maximum emission wavelength was virtually the same.

The ligand-induced enhancement of the fluorescence was used to determine the binding affinity, the stoichiometry, and the homogeneity of the reaction of IgM FR with phosphorylcholine. The Scatchard plot of the binding data is given in Figure 2. The association constant was found to be 6×10^4 M^{-1}, at 25 °C; at saturation, 9.0 binding sites/molecule of IgM (mol wt 900 000) were occupied by the phosphorylcholine hapten. For the 7S IgMs the same K_A value and 1.9 binding sites/molecule (mol wt 180 000) were calculated. Homogeneity of binding is indicated by the straight line, obtained by plotting r/c vs. r. Since IgM FR has previously been found to cross-react with other choline derivatives, such as acetylcholine and choline chloride, the effect of these ligands on the fluorescence of this protein was also studied. The comparison of the emission maxima, the percentage increase of the fluorescence enhancement, and the association constants are given in Table I. In spite of the 15-fold difference in affinities measured with these ligands, the emission maximum wavelength and the fluorescence enhancement were similar.

Dependence on pH. The pH dependence of the binding of phosphorylcholine to IgM FR is illustrated in Figure 3. In order to prevent the possible stabilization of the protein con-

TABLE I: Fluorescence Changes in IgM FR upon Binding of Phosphorylcholine and Choline Derivatives.

Ligand	Emission Maximum (nm)	% Enhancement of Tryptophanyl Fluorescence (Excitation at 280 nm)	Association Constant, K_A (M^{-1})
Phosphoryl-choline	342	83%	6×10^4
Acetylcholine	342	80%	4×10^3
Choline chloride	342	81%	4×10^3

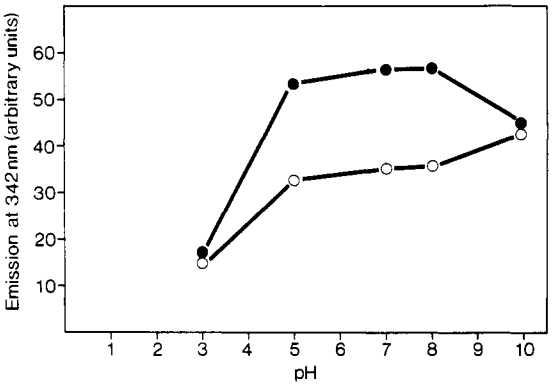


FIGURE 3: pH dependence of emission at 342 nm for IgM FR. (○) IgM alone; (●) with excess PC.

formation by hapten binding, no titrations with free and phosphorylcholine bound IgM FR were performed; instead, IgM FR was dissolved in 0.01 M acetate–0.01 M Tris buffer adjusted to pH values between 3.0 and 10.0. In order to avoid the precipitation of IgM FR below pH 6, 1 M NaCl had to be added to all buffer solutions. Under these conditions, a percentage increase in emission of 50% was noted upon saturation with phosphorylcholine, as opposed to 83% in 0.14 M NaCl. The pH range of 5–8 yielded a constant difference in fluorescence emission intensity, suggesting no significant alterations in binding affinity of the protein for phosphorylcholine. However, at more acidic or alkaline pH values the percentage increase in fluorescence enhancement became smaller, and at pH 3.0 and 10.0 no fluorescence enhancement was noted, thus, suggesting the absence of hapten binding at these pH values. Lack of phosphorylcholine binding at pH 3.0 and 10.0, respectively, was independently confirmed by equilibrium dialysis.

Denaturation Studies. The effect of denaturing agents on the fluorescence of IgM FR in the absence and the presence of phosphorylcholine is shown in Figure 4. In the absence of phosphorylcholine, a steady increase in emission intensity was observed in solutions of IgM FR containing increasing concentration of urea (3–10 M), indicating a change in the microenvironment of the chromophores, which probably reflects the unfolding of the polypeptide chains of the protein. Similarly, an increase in fluorescence intensity was observed in the presence of GuHCl (3–5 M). However, no further increase was noted when the concentration of GuHCl was raised to 7 M. This suggests that unfolding of the protein was essentially complete beyond a concentration of 5 M in GuHCl. In addition, GuHCl significantly quenched the intensity of the protein fluorescence. Further evidence for the denaturation of IgM FR was provided by a red shift of the emission maximum wavelength from 337 to 342 nm in 10 M urea and to 346 nm in 5–7 M GuHCl.

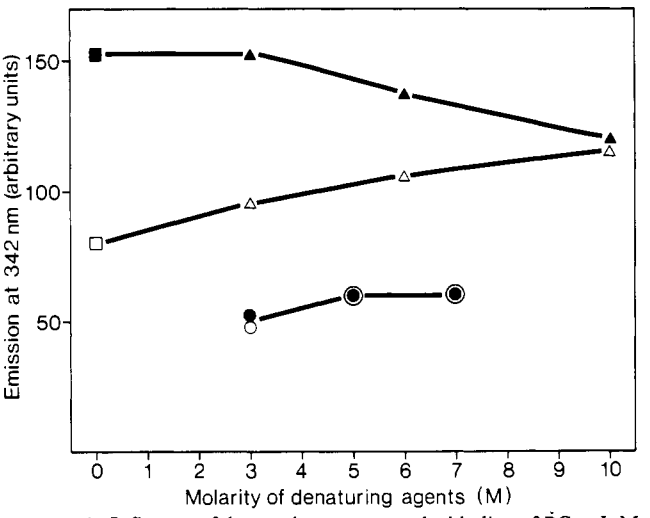


FIGURE 4: Influence of denaturing agents on the binding of PC to IgM FR. (△) IgM alone in urea; (▲) in urea + excess PC; (○) IgM alone in GuHCl; (●) in GuHCl + excess PC; (□) IgM alone in 0.01 M Tris–0.14 M NaCl, pH 7.5; (■) in 0.01 M Tris–0.14 M NaCl, pH 7.5 + excess PC.

Upon addition of phosphorylcholine, no fluorescence enhancement was noted in the presence of 5 and 7 M GuHCl and only 4% in 3 M GuHCl. In contrast, a substantial fluorescence enhancement was found upon phosphorylcholine addition even at concentrations of urea as high as 6 M, where the percent increase in fluorescence intensity was still 36% of that recorded after addition of phosphorylcholine to the native antibody. In the presence of 3 M urea, the association constant for the binding of phosphorylcholine was 2×10^4 M^{-1}, as compared with a value of 6×10^4 M^{-1} in the absence of urea.

After reduction of the IgM with 0.01 M DTT in the presence of 6 M urea, no enhancement of the fluorescence was observed upon addition of phosphorylcholine, suggesting that the dissociated heavy and light polypeptide chains (which are produced under these conditions) lack binding activity.

Circular Dichroism (CD). The CD spectrum of IgM FR was measured in the wavelength range of 260–310 nm in the absence and the presence of excess of phosphorylcholine ligand (Figure 5A). The shape of the spectrum in this region is rather uniform with a slightly negative ellipticity. The major positive bands between 260 and 310 nm, which are commonly observed with antibodies of the IgG class (Cathou et al., 1968), could not be noted in the CD spectrum of IgM FR. Two different IgM Waldenström proteins and IgM isolated from a normal human serum pool exhibited CD spectra similar to that of IgM FR.

Since the CD spectra of the proteolytic fragments of immunoglobulins appear to be additive (Cathou et al., 1968), the isolated Fab and Fc fragments of IgM might exhibit transition bands of similar amplitude but of opposite sign. In fact, Fab and Fc fragments of IgM FR exhibited essentially the same CD spectra as that of the native IgM. The 7S monomer obtained after reduction and alkylation of protein FR showed a CD spectrum similar to that of the pentameric IgM FR. Upon addition of phosphorylcholine, small changes in ellipticity were observed in the 270–300 nm range, which were, however, only slightly above the limits of experimental error. With the 7S subunit or the Fab fragment from IgM FR the same small changes in ellipticity were recorded as a result of ligand binding.

TABLE II: Fluorescence Characteristics of PC Binding Monoclonal Immunoglobulins.

Protein	Emission Maxima (nm)	Enhancement of Tryptophanyl Fluorescence (%)		Reference
		Excitation at 280 nm	Excitation at 295 nm	
IgM FR	342	83	92	This paper
IgMs FR	342	80	nd	This paper
TEPC-15 IgA	332	25	18	Pollet and Edelhoch, 1974
HOPC-8 IgA	332	25	18	Pollet and Edelhoch, 1974
McPc-603 IgA	333	10	0	Pollet and Edelhoch, 1974
MOPC-167 IgA	333	12	12	Pollet and Edelhoch, 1974
MOPC-511 IgA	334	13	13	Pollet and Edelhoch, 1974
ALPC-43 IgG	334	10	7	Pollet and Edelhoch, 1974

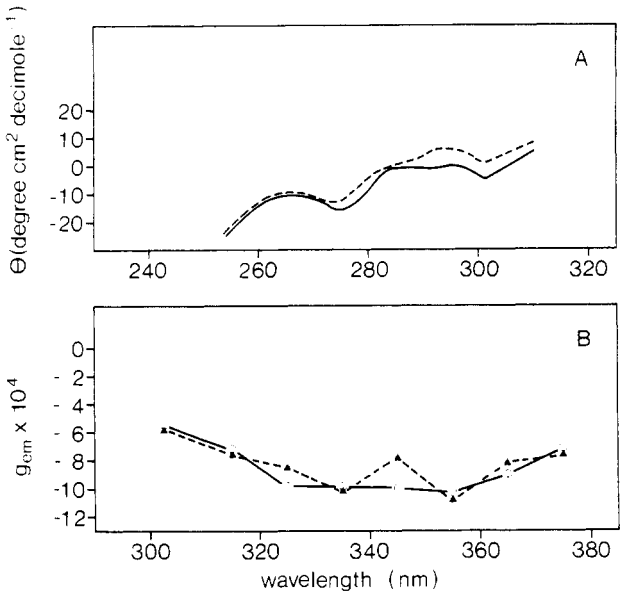


FIGURE 5: (A) Near uv CD spectrum of IgM FR in the presence (- - -) or the absence (—) of PC. (B) CPL spectrum of IgM FR in the presence (- ▲ -) or the absence (- O -) of PC.

Circular Polarization of Luminescence (CPL). The CPL spectrum of IgM FR in the range of 300–380 nm (Figure 5B) is similar in shape but more negative in the amplitude than that recorded for rabbit antibodies and mouse myeloma proteins (Givol et al., 1974). Upon addition of phosphorylcholine, no significant change in the anisotropy throughout the spectrum was observed.

Discussion

In the present report the characteristic features of the binding site of a monoclonal human IgM with phosphorylcholine activity have been analyzed by fluorescence spectroscopy and CD. The comparison with the data obtained by Pollet and Edelhoch (1973, 1974) on phosphorylcholine binding of mouse myeloma proteins is given in Table II. The murine proteins, which were shown to exhibit considerable structural homology (Barstad et al., 1974), and the human IgM FR show an enhancement of the tryptophanyl fluorescence after binding of the ligand. This might suggest structural similarities between these phosphorylcholine binding murine and human immunoglobulins, even though they do not share idiotypic specificities (Cosenza and Riesen, unpublished). However, there are several spectroscopic differences between IgM FR and the murine myeloma proteins (Table II). (1) The amplitude of the phosphorylcholine induced enhancement of the fluorescence is markedly different for the human IgM FR (83%) than for the murine proteins (10–25%). (2) While the mouse myeloma proteins show a small blue shift (2 nm) of the emission maximum wavelength, IgM FR exhibits a red shift of 5 nm that suggests that the tryptophanyl residues, residing in nonpolar microenvironment, became more exposed to solvent. (3) The phosphorylcholine-induced fluorescence enhancement of IgM FR is greater with excitation at 295 nm than when the excitation was done at 280 nm. The reverse was found for three murine proteins, while for two the same fluorescence enhancement was observed at both excitation wavelengths (Table II). It is noteworthy that at 295 nm there is very little absorption by tyrosyl residues.

The association constant of IgM FR for phosphorylcholine, as determined by fluorescence enhancement, was 6×10^4 M^{-1} and 9.0 binding sites/molecules were calculated. These values correspond to previous findings obtained by equilibrium dialysis (Riesen et al., 1975). Homogeneity of the binding reaction is indicated by the linearity of the Scatchard plot. The phosphorylcholine induced fluorescence enhancement of the pentameric IgM and of the monomeric IgM subunit were virtually the same with respect to percentage increase (80%) and maximum wavelength (342 nm), and the same affinity was calculated for both molecular species. This indicates the absence of interaction between the 5 IgM subunits.

It is noteworthy that the cross-reacting ligand, acetylcholine, with an association constant 15-fold lower than that for phosphorylcholine induced virtually the same percentage increase of fluorescence enhancement as phosphorylcholine and the same red shift of the maximum emission wavelength; this suggests that the cross-reacting ligand has the same effect on the conformation of the binding site of the protein as phosphorylcholine, provided the ligand concentration is adequate.[2] A similar observation was made by Morris et al. (1974) using CD for studies on the interaction of mouse myeloma proteins with phosphorylcholine and choline derivatives. This may be relevant for considerations on the stimulation of cells by cross-reacting antigens, for which these cells have a low affinity (Cramer and Braun, 1973; Varga et al., 1973).

The studies on the effect of denaturing agents on the fluorescence of IgM (FR) showed no further change in the protein fluorescence intensity above 5 M GuHCl, suggesting that the protein was completely unfolded. This observation is in agreement with the results of others (Noelken and Tanford, 1964; Cathou et al., 1967), whereas the complete denaturation of the protein in the presence of urea requires concentrations above 10 M. In 3 M urea, the affinity of the protein for phosphorylcholine was of the same order of magnitude as that of

[2] Since the effect of acetylcholine is very similar to that induced by phosphorylcholine, it appears likely that the choline moiety is responsible for the fluorescence change.

the native antibody, and in 6 M urea there was still substantial binding of phosphorylcholine. This would imply that the conformation in the binding site was only partially affected by 6 M urea.

The significance of the observed changes in the tryptophanyl fluorescence in relation to changes in the conformation of the protein remains unclear, since it is difficult to distinguish between effects produced on chromophores located in the combining site that interact directly with the ligand and effects of chromophores residing in parts of the molecule not participating in the combining region. It is possible that the observed fluorescence changes merely reflect the interaction of tryptophanyl residue(s) located in the combining site with phosphorylcholine. Alternatively, these spectral changes could be due to tryptophanyl residue(s) outside the antigen binding region; they would then be the expression of ligand-induced modifications in the antibody conformation. Amino acid sequence analysis of IgM FR indicate that there are no tryptophanyl residues within the hypervariable regions of the light chain (Riesen and Jaton, 1976b); the sequence of the heavy chain is not yet established.

Circular dichroism has proven to be a valuable probe for the assessment of ligand-induced conformational changes in immunoglobulin molecules (Holowka et al., 1972; Pollet et al., 1974; Morris et al., 1974; Jaton et al., 1975). Small CD changes were indeed observed upon addition of phosphorylcholine to IgM FR. However, the quantitation of these changes is unreliable due to the small ellipticity values observed with IgM FR. For comparison, the phosphorylcholine binding TEPC-15 mouse myeloma IgA exhibited a 15% CD change upon ligand binding (Morris et al., 1974; Pollet and Edelhoch, 1974). Ashman et al. (1971) found no major conformational changes in a nitrophenyl-binding human γM macroglobulin upon ligand binding.

Recently circular polarization of luminescence, CPL, was introduced as a sensitive probe for the analysis of structural changes in proteins (Steinberg, 1975). The studies on the CPL of IgM (FR) in the absence and presence of phosphorylcholine indicated no evidence for a spectral change upon ligand binding, an observation in agreement with CPL studies of phosphorylcholine binding mouse myeloma proteins (Givol et al., 1974). It thus seems that in contrast to CD and fluorescence spectroscopy, in this particular hapten–antibody system, the CPL method does not reveal spectral changes.

Whatever the significance of the ligand-induced spectral changes observed, the human and murine PC binding proteins do markedly differ from each other in the mode of interaction with the simple antigenic determinant PC. This would imply that the variable domains of these proteins are not identical. Amino acid sequence studies currently undertaken to establish the primary structure of the Fv domains of the human Waldenström's macroglobulin FR (Riesen et al., to be published) and the PC binding murine myeloma proteins (Barstad et al., 1974; Rudikoff and Potter, 1974) are expected to reveal the degree of homology in antibody variable regions specific for the same antigenic determinant. The primary structure together with crystallographic and optical data should help delineate the common conformational features controlling their specificity.

Note Added in Proof

It is remarkable that the sequence of heavy chain FR in the first hypervariable region, which is known to be involved in hapten binding, is identical with that of five heavy chains derived from phosphorylcholine binding mouse myeloma proteins, except for an Asp/Glu interchange at position 35 (Riesen et al., 1976a).

Acknowledgments

We thank Dr. P. Moser (Ciba-Geigy, Basel) and Dr. H. Steffen (F. Hoffmann-La Roche, Basel) for giving us the opportunity to use their instruments and for their help, and Dr. D. G. Braun, for helpful comments and criticisms of the manuscript.

References

Ashman, R. F., Kaplan, A. P., and Metzger, H. (1971), *Immunochemistry 8,* 627.

Ashman, R. F., and Metzger, H. (1969), *J. Biol. Chem. 244,* 3405.

Barstad, P., Rudikoff, S., Potter, M., Cohn, M., Konigsberg, W., and Hood, L. (1974), *Science 183,* 962.

Cathou, R. E., and Haber, E. (1967), *Biochemistry 6,* 513.

Cathou, R. E., Kulczyski, A., Jr., and Haber, E. (1968), *Biochemistry 7,* 3958.

Chesebro, B., and Metzger, H. (1972), *Biochemistry 11,* 766.

Cramer, M., and Braun, D. G. (1973), *J. Exp. Med. 138,* 1533.

Givol, D., Pecht, I., Hochmann, J., Schlessinger, J., and Steinberg, I. Z. (1974), *Proc. Int. Congr. Immunol., 2nd 1,* 39–48.

Holowka, D. A., Strosberg, A. D., Kimball, J. W., Haber, E., and Cathou, R. E. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3399.

Jaton, J.-C., Huser, H., Braun, D. G., Givol, D., and Schlessinger, J. (1975), *Biochemistry 14,* 5312.

Jolley, M. E., Rudikoff, S., Potter, M., and Glaudemans, C. P. J. (1973), *Biochemistry 12,* 3039.

Metzger, H. (1970), *Adv. Immunol. 12,* 57.

Morris, D. H., Williams, R. E., and Young, N. M. (1974), *Biochem. Biophys. Res. Commun. 61,* 1167.

Noelken, M. E., and Tanford, C. (1964), *J. Biol. Chem. 239,* 1828.

Pollet, R., and Edelhoch, H. (1973), *J. Biol. Chem. 248,* 5443.

Pollet, R., Edelhoch, H., Rudikoff, S., and Potter, M. (1974), *J. Biol. Chem. 249,* 5188.

Riesen, W., Braun, D. G., and Jaton, J.-C. (1976a), *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Riesen W., and Jaton, J.-C. (1976b), *Biochemistry* (in press).

Riesen, W., Rudikoff, S., Oriol, R., and Potter, M. (1975), *Biochemistry 14,* 1052.

Rudikoff, S., and Potter, M. (1974), *Biochemistry 13,* 4033.

Schlessinger, J., Roche, R. A., and Steinberg, I. Z. (1975), *Biochemistry 14,* 255.

Steinberg, I. Z. (1975), in Concepts in Biochemical Fluorescence, Chen, R., and Edelhoch, A., Ed., Marcel Dekker, New York, N.Y. (in press).

Varga, J. M., Konigsberg, W. H., and Richards, F. F. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3269.