Investigations of the Molecular Basis for the Temperature-Dependent Insolubility of Cryoglobulins. II. Spectroscopic Studies of the IgM Monoclonal Cryoglobulin McE†

C. R. Middaugh, G. J. Thomas, Jr., B. Prescott, M. E. Aberlin, and G. W. Litman*

ABSTRACT: The secondary and tertiary structures of the monoclonal, IgM-κ cryoglobulin, McE, and four noncryoglobulin IgM-κ immunoglobulins have been compared. The far-ultraviolet circular dichroism spectrum of the cryoimmunoglobulin (at 37 °C) exhibits a 38% reduction in the 218-nm ellipticity minimum relative to the other proteins and the fluorescence emission maximum is slightly blue shifted and decreased in intensity (~40%). These differences have been at least partially localized to the Fabμ region of the cryoglobulin by a variety of spectroscopic techniques and do not appear to be a consequence of differential light scattering or to be directly attributable to known differences in amino acid composition between the proteins. Temperature studies at noncryoprecipitating concentrations of the cryoglobulin reveal atypical changes at low temperatures in both far-ultraviolet circular dichroism and fluorescence spectra that are consistent with changes in environment of some aromatic residues. Both far-ultraviolet absorbance at 189 nm and laser Raman spectroscopy indicate little or no change in the secondary structure of the cold precipitated cryoglobulin. The concentration dependence of the low temperature induced changes in McE suggests that they are of intramolecular origin. It has not yet been possible to positively localize these changes to a particular region of the cryoglobulin molecule. It is hypothesized that a low-temperature-induced conformation change in cryoimmunoglobulin McE is either a prerequisite for or an integral component of the cryoprecipitation process.

Cryoimmunoglobulins are immunoglobulins which exhibit temperature-dependent, reversible insolubility as the temperature of certain sera is reduced from 37 to 0 °C (Lerner et al., 1947). Frequently, 10^3-10^4-fold changes in solubility can occur over a temperature range of less than 10 °C. Monoclonal cryoimmunoglobulins consisting of only a single immunoglobulin type have been described for the IgM, IgG, and IgA classes. The most frequently encountered monoclonal cryoimmunoglobulins are of the μ heavy chain and κ light chain classes. Mixed cryoglobulins consist of a mixture of immunoglobulins which usually include a monoclonal IgM component. Polyclonal cryoimmunoglobulins without a principal monoclonal component have also been described (Grey and Kohler, 1973; Brouet et al., 1974). Monoclonal cryoimmunoglobulins are usually found in association with lymphoproliferative disorders, while mixed and polyclonal cryoglobulins are often associated with nonneoplastic disease including collagen-vascular disorders and both acute and chronic infections. Depending on the relative concentration and temperature sensitivity of the cryoglobulins, their presence can result in increased blood viscosity and intravascular precipitation. The occurrence and clinical significance of cryoimmunoglobulins are well documented (Meltzer and Franklin, 1966; Grey and Kohler, 1973; Brouet et al., 1974); however, the molecular basis for cryoglobulin behavior is not understood. It is felt that cryoimmunoglobulins bear a general structural resemblance to their noncryoglobulin counterparts. Furthermore, those structural properties which have been associated with the cryotendencies of particular monoclonal cryoimmunoglobulins do not occur necessarily in other cryoglobulins of the same class, although they may exhibit similar temperature-dependent insolubility characteristics.

In order to examine possible conformational differences between cryoglobulin and noncryoglobulin IgM proteins, we have analyzed the secondary structure of the IgM cryoglobulin McE and four noncryoglobulin IgM proteins using far-UV CD,[1] far-UV absorption, infrared, and laser Raman spectroscopy. Tertiary structure has been studied employing near-UV CD, near-UV absorption, fluorescence, and laser Raman spectroscopy. In addition, the possible role of a temperature-induced conformational change(s), as suggested by the sharp temperature and concentration dependence of cryoprecipitation in the cold-induced insolubility of McE, has been assessed.

Materials and Methods

Isolation of Cryoglobulin and Noncryoglobulin IgM. An enriched cryoprecipitate obtained during plasmapheresis of patient McE was generously provided by Dr. L. Crombie. The cryoprecipitate was stored either at −20 or at 0 °C and prior to use was warmed to 37 °C and centrifuged at 3000*g* for 30 min at 37 °C to remove any residual debris. The plasma was transferred from the centrifuge to glass bottles contained in an ice bath. Upon contacting the prechilled glass, a white precipitate formed and continued to intensify until the contents

† From the Sloan-Kettering Institute for Cancer Research (C.R.M., M.E.A., and G.W.L.), New York, New York 10021, and the Department of Chemistry, Southeastern Massachusetts University (G.J.T. and B.P.), North Dartmouth, Massachusetts 02747. *Received September 21, 1976.* These studies were supported in part by NIH AI-13528, NIH CA-08748, and NIH CA-17404 (G.W.L.); NIH AI-11855 (G.J.T.). This work will be submitted to Cornell University by C.R.M. in partial fulfillment of the requirements for the Ph.D. degree. Portions of this work have been presented previously (Middaugh, C. R., Aberlin, M. E., and Litman, G. W. (1976), *Biophys. J. 16*, 60a). A preliminary report describing the clinical history of patient McE and characterizing protein McE as a monoclonal IgM cryoglobulin has been made previously (Zinneman, H. H., Fromke, V. L., and Seal, U. S. (1973), *Clin. Chim. Acta 43*, 91).

[1] Abbreviations used: CD, circular dichroism; UV, ultraviolet; $\tilde{\epsilon}$, mean residue extinction coefficient; $(Fc)_{5\mu}$ and Fabμ are defined in Putnam et al. (1973).

of the bottle was in a semisolid form. After 2 h, the bottles were centrifuged at 0 °C at 7500*g* for 30 min. The supernate was discarded and 0.15 M NaCl was added to equal the original plasma volume. The cryoprecipitate was warmed to 37 °C and redissolved by gentle agitation with a glass rod. This procedure was repeated 7–10 times and was sufficient to remove contaminating plasma proteins. The final precipitate was dissolved in either 0.15 M NaCl or in a minimal volume of 0.15 M NaCl–0.1 M Tris-HCl–0.02% NaN_3 (pH 8.0) and chromatographed at 37 °C on a 2.4 × 120 cm column of Bio-Gel A-1.5m, 200–400 mesh (Bio-Rad, Richmond, Calif.), in the NaCl–Tris buffer. This chromatography step was necessary to separate aggregated from nonaggregated forms of IgM. All cryoglobulin preparations were either used immediately or stored at −20 °C.

The following noncryoglobulin, monoclonal IgM proteins from patients with Waldenström's macroglobulinemia or chronic lymphocytic leukemia have been included in these studies: ESm, Hof, Sch, and WSm (IgM, κ); Cog and Wil (IgM, λ). The proteins were isolated from serum or from recalcified plasma by a combination of euglobulin precipitation and gel filtration techniques. Plasma or serum was dialyzed for 24 h at 4 °C against a 150-fold volume excess of 1 mM NaH_2PO_4 (pH 7.35). The flocculant precipitate which formed was isolated by centrifugation at 7500*g* for 30 min at 4 °C. The supernate was discarded and the precipitate dissolved in a volume of 0.15 M NaCl equivalent to one-half the original plasma volume followed by dialysis against a 50-fold volume excess of 1 mM NaH_2PO_4–2 mM EDTA (pH 7.35) (repeated 4–5 times) and subjected to chromatography on a 2.4 × 120 cm column of Bio-Gel A-1.5m, 200–400 mesh, developed in the same buffer. The chromatographic fractions corresponding to ~19S IgM were pooled and concentrated at 4 °C by ultrafiltration employing an Amicon pressure concentrator fitted with a PM 30 membrane.

Proteins were exchanged into appropriate buffer solutions by prolonged dialysis (48–72 h, 0 °C) against 10 000 × the original sample volume. Prior to spectroscopic analysis, samples were centrifuged (37 °C) at 20 000*g* for 30 min and the supernatants passed through a 0.45-μm Millipore filter. The concentrations of the protein solutions were determined from their absorbance at 280 nm employing the following experimentally determined dry weight extinction coefficients ($E_{1cm}^{1\%}$ 280 nm) in 0.15 M NaCl (pH 7.0): McE = 12.2 ± 0.2, McE Fabμ = 12.7 ± 0.3, McE $(Fc)_5\mu$ = 11.2 ± 0.2, ESm = 12.1 ± 0.2, ESm Fabμ = 13.2 ± 0.3, ESm $(Fc)_5\mu$ = 11.0 ± 0.2, Hof = 11.8 ± 0.3, Hof Fabμ = 13.4 ± 0.3, Hof $(Fc)_5\mu$ = 10.8 ± 0.1, Sch = 12.4 ± 0.2, Sch Fabμ = 13.5 ± 0.2, Sch $(Fc)_5\mu$ = 11.1 ± 0.2, Wil = 12.4 ± 0.3, Wil Fabμ = 13.2 ± 0.2, Wil $(Fc)_5\mu$ = 11.3 ± 0.2, WSm = 12.2 ± 0.1, WSm Fabμ = 12.8 ± 0.2, WSm $(Fc)_5\mu$ = 11.2 ± 0.2.

$(Fc)_5\mu$ and Fabμ fragments were prepared by proteolysis at 56 °C with trypsin as described by Plaut and Tomasi (1970).

Each immunoglobulin and its fragments were judged to be homogeneous by the criterion of double diffusion analysis (employing antisera to whole human serum and human immunoglobulin heavy and light chains), analytical ultracentrifugation and gel filtration at 37 °C, and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate in 15-cm gels of fully reduced and alkylated protein.

Amino Acid Compositions. Amino acid compositions were determined employing a Beckman Model 121 amino acid analyzer equipped with a system AA computing integrator. A single column methodology was used. Samples for amino acid analysis were hydrolyzed in 6 N HCl at 110 ± 0.3 °C for 20–72 h in evacuated (<40 μmHg) ampules. Tyrosine determinations were extrapolated to zero time. Tryptophan was estimated spectroscopically following reaction of protein dissolved in 10 M urea (Heico) with *N*-bromosuccinimide (Spande and Witkop, 1967).

Circular Dichroism. CD spectra were determined in the near (320–250 nm) and far (250–205 nm) UV regions employing a Cary Model 60 recording spectropolarimeter equipped with a 6002 CD attachment. The temperature of the sample cell was controlled by circulating water from a thermostated water bath through a jacketed cell holder. Solution temperatures were monitored by a thermistor probe inserted directly into the equilibrated sample solution. For near-UV spectroscopy, protein concentrations of 0.5–1.0 mg/mL were measured in cylindrical quartz cells of optical paths 0.5–2.0 cm. Determinations in the far-UV were made using protein concentrations of 0.03–0.8 mg/mL and cells of optical paths 0.01–2.0 cm. Molar ellipticities were calculated as described previously (Litman et al., 1973) employing a mean residue molecular weight of 108 (Crumpton and Wilkinson, 1963). Possible light-scattering artifacts were investigated by varying the amount of scattered light that was detected by a movable phototube in conjunction with an adjustable sample cell diaphragm that allowed the acceptance half-angle of the collected radiation to be varied between 0.6 and 45° (Schneider and Harmatz, 1976). Unless stated otherwise, all spectra were determined at the instrument standard acceptance half-angle of 8°. Possible absorption flattening artifacts were investigated by measurement of the far-UV absorption peak (190 nm, see below) under conditions identical with those employed in the CD determinations employing aqueous 0.15 M NaF, pH 7.5, as a solvent. This region of the spectrum has been shown to be sensitive to absorption flattening (Urry et al., 1970). The NaF was found to have no detectable effect on the structure of the proteins. All spectra were determined in triplicate and on at least three samples prepared independently by identical isolation procedures.

Fluorescence. Intrinsic fluorescence emission spectra were measured with a Cary 50-026-900 differential recording spectrophotometer equipped with a 450-W short-arc xenon lamp, as described previously (Sonenberg, 1971). All measurements were made employing front surface illumination with an angle between the exciting and emitting beams of 23°. Sample preparations and handling, as well as variable temperature measurements, were performed essentially as described above.

Absorption Spectroscopy. Far-UV, near-UV, and visible absorbance measurements were made with a Cary 1605 recording spectrophotometer equipped with temperature jacketed cuvettes. Special precautions were taken for the far-UV measurements pertaining to the cleaning and handling of all cuvettes and solutions (Gratzer, 1967), and the instrument was continuously flushed with nitrogen prior to and during all experimental determinations. Stray light levels were judged to be within acceptable limits as indicated by the applicability of Beer's law within the range of protein concentrations investigated (0.01–0.2 mg/mL). The slit widths employed throughout the wavelength range (250–185 nm) did not exceed 0.75 mm at any time. Partial correction for the temperature-dependent reduction of the end absorption of water in the far-UV (Woods and O'Bar, 1970) was made by connecting both sample and reference cells to a common temperature-controlled circulator system. Heat loss through connecting lines, however, produced a detectable temperature effect (see

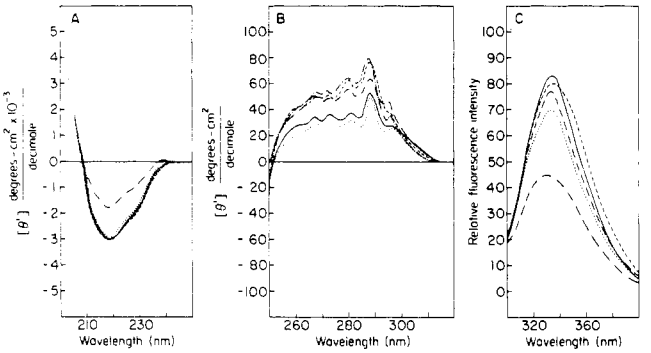

FIGURE 1: CD and fluorescence emission spectra of cryoimmunoglobulin McE and four noncryoglobulin human IgM proteins. All spectra were recorded at 37 °C in 0.01 M sodium hydrogen phosphate–0.15 M NaCl (pH 7.0). (A) Far-UV CD spectra, protein concentration of 0.03 mg/mL; (B) near-UV CD spectra, protein concentration 1.0 mg/mL; (C) fluorescence emission spectra produced by excitation at 275 nm, protein concentration 0.03 mg/mL. Symbols: (—) Esm; (- - -) Sch; (• • •) WSm; (- • - •) Hof; and (- • • • • •) cryoglobulin McE.

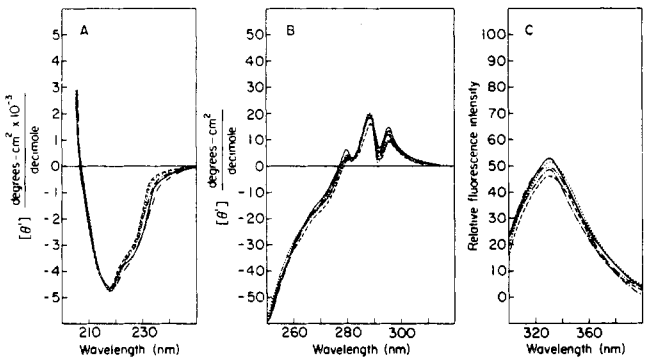

FIGURE 2: CD and fluorescence emission spectra of cryoimmunoglobulin McE $(Fc)_5\mu$ fragment and four comparison IgM $(Fc)_5\mu$ fragments. Experimental conditions and symbols are the same as in Figure 1.

Results). Error produced by expansion and contraction of the sample volume was compensated by normalization to changes in absorbance of standard copper sulfate solutions. For far-UV spectroscopy, samples were contained in 1.0-mm cells of fused silica (Suprasil).

Near-Ultraviolet and Visible Absorption Spectroscopy. Measurements in the near-UV and visible region were performed on samples held in 1.0 or 10.0 mm matched cells of fused silica and were filtered immediately prior to examination. Possible light-scattering contributions at the various experimental conditions investigated were examined by plotting the log of the wavelength of observation against the log of absorbance and extrapolating these data into absorbing regions (Leach and Scheraga, 1960). Unless otherwise indicated, these corrections were negligible at the given experimental conditions.

Raman Spectroscopy. Concentrated solutions of proteins (>50 mg/mL) are required for Raman spectroscopy. Samples of immunoglobulins were prepared at 50–150 mg/mL, according to availability, in 0.15 M NaCl (pH 7.0). Aliquots of 25 μL were drawn into glass capillary cells of 1.0 mm internal diameter (Kimax, No. 34507). The cells were sealed and centrifuged prior to mounting in the sample illuminator of the Raman spectrometer where the cells were thermostated to maintain the solution temperature constant to ±0.5 °C.

Spectra were excited with the 488.0-nm line of an argon-ion laser (Coherent Radiation, Model CR-2) and were recorded on a Spex Ramalog spectrometer. Further details of the

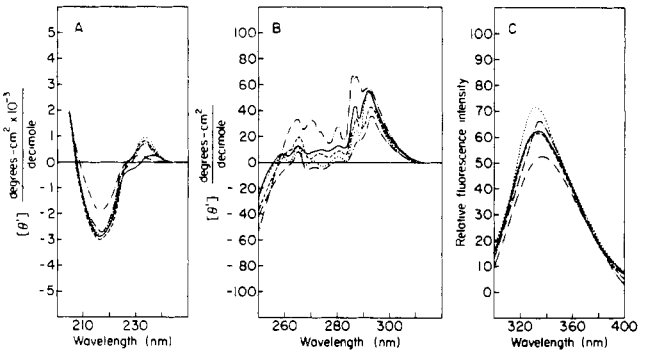

FIGURE 3: CD and fluorescence emission spectra of cryoimmunoglobulin McE Fabμ fragment and four comparison IgM Fabμ fragments. Experimental conditions and symbols are the same as in Figure 1.

Raman instrumentation and sample thermostat are given elsewhere (Thomas, 1971; Thomas and Barylski, 1970). Raman frequencies are reported to a precision of ±2 cm^{-1} for either strong or sharp lines and ±4 cm^{-1} for weak or broad lines.

Results

Far-Ultraviolet Circular Dichroism. All CD spectra in the far-UV of the IgM proteins and their fragments at 37 °C are characterized by the presence of a well-defined ellipticity minimum at 217–218 nm, which has been attributed to the $n\rightarrow\pi^*$ transition of the peptide group of polypeptides in the β conformation (Figures 1A, 2A, and 3A) (Cathou et al., 1968; Ghose, 1971; Greenfield and Fasman, 1969; Litman et al., 1971; Pysch, 1966), although other compensatory contributions may be present in this region of the spectrum (Cathou and Dorrington, 1975; Stevens et al., 1968). Spectra of the intact proteins are also characterized by a broad band between 225 and 235 nm (Figure 1A) that has previously been assigned to a transition occurring in the C-terminal half of the Fc region (Dorrington et al., 1972; Litman et al., 1973). This is confirmed both by the absence of such a band in the Fabμ fragments (Figure 3A) and by its presence in the $(Fc)_5\mu$ portion (Figure 2A) of the molecules investigated in the present study. It is possible, however, that the loss of this band in the Fabμ fragment may be partially attributable to the enhanced 230–240-nm positive band (Figure 3A). A small peak of positive ellipticity is often resolvable between 240 and 235 nm, apparently due to tyrosine residue(s) in the Fabμ portion of the molecule (see below). It is evident from Figure 1A that the spectrum of cryoimmunoglobulin McE differs significantly from those of the comparison IgM proteins. The ellipticity minimum at 218 nm in McE is only 62% as intense as that of the other IgM proteins. The broad band at 235–225 nm is resolvable in McE, although it is reduced in intensity compared with the other proteins. The small positive peak at 239 nm likewise shows detectable differences relative to the other two molecules in which such a peak is resolvable.

These differences can be partially localized to a particular region of the cryoglobulin by examination of the far-UV CD spectra of the Fabμ and $(Fc)_5\mu$ fragments of each of the proteins (Figures 2A and 3A). The spectra of the five $(Fc)_5\mu$ fragments are essentially identical, each with an ellipticity minimum at 218 ± 1 nm of amplitude, −4700 ± 100 deg cm^2 $dmol^{-1}$. The weak band at 235–225 nm (in the intact proteins, Figure 1) appears slightly broadened (235–222 nm) and significantly enhanced (in the fragments, Figure 2) with some minor variations noted among the individual fragments. More

significant differences are apparent, however, in the spectra of the Fabμ fragments. For example, the minimum in ellipticity at 217 ± 1 nm is 36 ± 3% lower in amplitude in the cryoimmunoglobulin Fabμ fragment than in the other four fragments (Figure 2). Marked differences are also resolvable in the 240–225-nm region. Three of the proteins display strong positive peaks (900 ± 150 deg cm^2 dmol^{-1}) at 234 ± 1 nm. Protein ESm displays a weaker peak (200 ± 20) at 237 (± 1) nm, while cryoimmunoglobulin McE displays a peak of similar intensity (280 ± 20) shifted to 232 ± 1 nm. In studies of the Fab fragments of IgG molecules, a band in this region has been assigned to tyrosine residues (Cathou et al., 1968; Dorrington and Smith, 1972).

Near-Ultraviolet Circular Dichroism. Examination of CD spectra in the near-UV (320–250 nm) of protein McE and the four comparison proteins and their respective fragments demonstrates additional differences between these proteins and the cryoglobulin. The CD spectra in the near-UV of the intact macroglobulins (Figure 1B) reveal a complex series of positive peaks. The two well-resolved bands at 288 ± 1 nm and 281 ± 1 nm and the well-resolved shoulder at 296 ± 1 nm can probably be attributed to tryptophan side chains (Strickland, 1974), although some contributions from tyrosine and cystine residues also are possible in this region. Below 277 nm are a pair of weak positive bands at 273 ± 1 nm and 268 ± 1 nm and a broad shoulder beginning at approximately 265 nm. Assignments in this region are highly speculative, with contributions from tryptophan, phenylalanine, tyrosine, and cystine all possible (Goodman and Toniolo, 1968; Strickland, 1974). The spectra of the cryoimmunoglobulin over the entire near-UV region are intermediate in appearance between those of the other proteins.

The spectra of the (Fc)$_{5\mu}$ fragments (Figure 2B) are somewhat less complex, and can be resolved into well-defined peaks at 296 ± 1 nm, 288 ± 1 nm, and 280 ± 1 nm with a broad shoulder beginning at approximately 270 nm. By reference to spectra of model compounds, the three prominent bands may be due to tryptophan transitions as noted above.

Differences become evident, however, upon examination of the near-UV CD spectra of the various Fabμ fragments (Figure 3B). For the four "normal" IgM molecules, well-resolved peaks occur at 292 ± 1 nm, 287 ± 1 nm, 272 ± 2 nm, and 265 ± 1 nm while a peak which is somewhat variable from one fragment to another occurs between 263 and 255 nm. However, strikingly different features are observed for the McE Fabμ fragment. The ratio of the relative intensity of the 292- and 287-nm peaks is reversed, with the 287-nm band predominant in the cryoimmunoglobulin spectra. Furthermore, this peak appears to consist of at least two components, while the analogous peak that is observed in the spectra of the noncryoimmunoglobulin Fabμ fragments cannot be resolved into more than a single band.

A number of other differences are also evident below 285 nm: The 281-nm peak is more pronounced than the analogous band in the other proteins. Below 275 nm, the 272-nm and 265-nm peaks are replaced by a series of three peaks, a well-resolved pair of bands of almost equal intensity at 264 ± 1 nm and 270 ± 1 nm, and a shoulder at 274 ± 1 nm. There is also no longer any evidence for a transition in the region below 263 nm.

Scattering Artifacts. The possibility that the differences in the CD spectra between the cryoimmunoglobulin and the other IgM proteins arise from differences in light scattering has been examined by two independent methods. Plots of log λ vs. log a in the visible region of the spectrum extrapolated into the UV suggest no significant contribution to the observed spectra. The second method employed to monitor possible scattering artifacts involved varying the acceptance angle of the light detection system. A variation of less than 2% in both signal frequency and strength between sample positions of extreme acceptance angle geometry argues strongly that differential light scattering is not responsible for any of the major features of the observed spectra between 40 and 2 °C.

Fluorescence. To further investigate possible differences between the cryoimmunoglobulin and the cold soluble homologues, the intrinsic fluorescence emission spectra of the individual proteins and their Fabμ and (Fc)$_{5\mu}$ fragments at 37 °C were obtained by excitation at 275 nm of protein solutions at concentrations of 0.03 mg/mL. Figure 1C presents the spectra of the intact cryoimmunoglobulin and four comparison spectra. Each of the spectra (as well as those derived from the individual fragments) are characteristic of emission predominantly from tryptophanyl residues (Kronman and Holmes, 1971). The emission maximum of protein McE appears slightly blue shifted (330 ± 1 nm) relative to the emission peak of the other proteins (334 ± 1 nm) and is significantly (59 ± 5%) less intense. In Figure 2C, the intrinsic fluorescence spectra of the (Fc)$_{5\mu}$ portions of the proteins are compared. The relative fluorescence intensity of the (Fc)$_{5\mu}$ fragments is similar (50 ± 4), but the cryoglobulin peak displays a small blue shift (329 ± 2 nm) in relation to the other proteins (332 ± 1 nm).

In Figure 3C it can be observed that the intensity of emission from the McE Fabμ fragment is only 81 ± 6% that of the other proteins. Furthermore, this emission maximum is apparently red shifted (337 ± 1 nm) in relation to the other peaks (333 ± 1 nm). Comparable to results described above for the CD measurements, the reduction in intensity of the fluorescence emission peak in the native cryoimmunoglobulin molecule may be at least partially attributed to differences in the spectral properties of the Fabμ portion of the molecule (but see below). It should be noted that the differences in fluorescence emission properties described above cannot be attributed simply to differences in amino acid composition between the proteins. The cryoimmunoglobulin actually contains an excess of tryptophan residues in its heavy chain (12–13 vs. 8–10) when compared with other IgM molecules. Analysis of the Fabμ fragment assigns this tryptophan excess to the Fd region of the heavy chain. The possibility of energy transfer from tyrosine to tryptophan residues obscures a more definitive interpretation, however, since the cryoglobulin heavy chain is deficient in tyrosine side chains (11 vs. 14–17 for noncryoglobulin IgM).

Near-Ultraviolet Absorption. As further evidence for a spectral difference residing in the Fabμ fragment of protein McE, the near-UV absorption spectra of McE Fabμ and the Fabμ fragments of the other proteins were determined. An increased intensity of the shoulder at 291 and 284 nm for the cryoimmunoglobulin is consistent with the postulated difference in environment of some aromatic residues. These differences, however, can be at least in part understood as a consequence of the abnormal aromatic amino acid composition of the cryoimmunoglobulin.

Effect of Temperature on Circular Dichroism and Far-UV Absorption. The effect of temperature upon the CD spectrum of the cryoimmunoglobulin at a nonprecipitating concentration of 0.03 mg/mL is illustrated in Figure 4. Between 40 and 25 °C, the spectrum remains essentially unaltered. However, at temperatures below 25 °C, a complex series of changes occurs. The ellipticity minimum at 218 nm decreases and a series of bands of negative amplitudes and low intensities can be re-

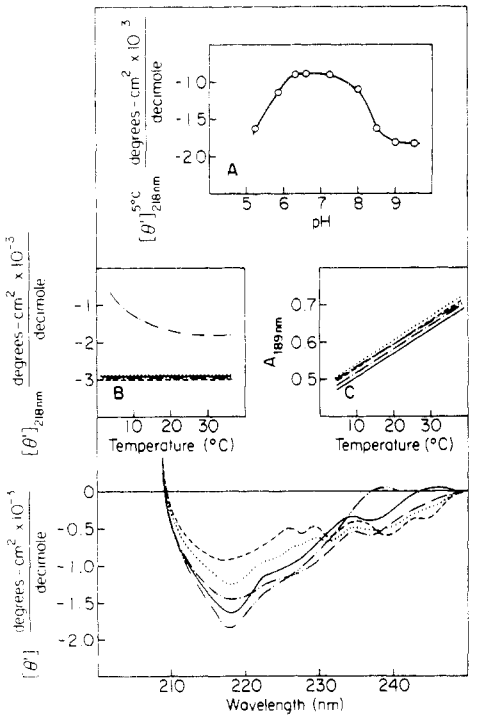

FIGURE 4: Effect of temperature on the far-UV CD spectrum of cryoimmunoglobulin McE in 0.01 M sodium hydrogen phosphate–0.15 M NaCl (pH 7.0). Protein concentration, 0.03 mg/mL. For clarity, spectra at only five temperatures are illustrated. Symbols: (- · · · · ·) 37.8 °C; (—) 19.0 °C; (- · · ·) 13.1 °C; (· · ·) 9.7 °C; and (- - -) 5.5 °C. (A) Effect of pH on the 218-nm CD ellipticity minimum of McE and four comparison IgM proteins; (B) effect of temperature on the 218-nm ellipticity minimum of McE and the same four IgM proteins. The symbols are the same as in Figure 1. Measurements were made at approximately 2 °C intervals. (C) Effect of temperature on the far-UV absorbance at 189 nm of cryoglobulin McE and the four reference molecules. Measurements were made at approximately 2 °C intervals in 0.15 M NaF (pH 7.0). Symbols again as in Figure 1.

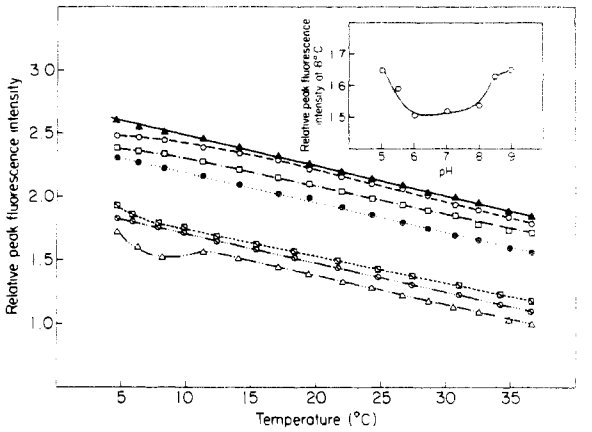

FIGURE 5: Effect of temperature on the relative peak fluorescence intensity of cryoimmunoglobulin McE and its Fabμ and (Fc)$_{5\mu}$ fragments and on the four comparison IgM proteins. Protein concentration, 0.03 mg/mL. Symbols: (Δ- · · · · · · Δ) McE; (■- - - -■) McE Fab; (○- · · · · · · · · ○) McE (Fc)$_{5\mu}$; (● · · · ●) WSm; (□- · · · · · · · □) Hof; (○- - -○) Sch; and (▲—▲) Esm. Inset shows the effect of pH on the relative peak fluorescence intensity of cryoglobulin McE at 8.0 °C.

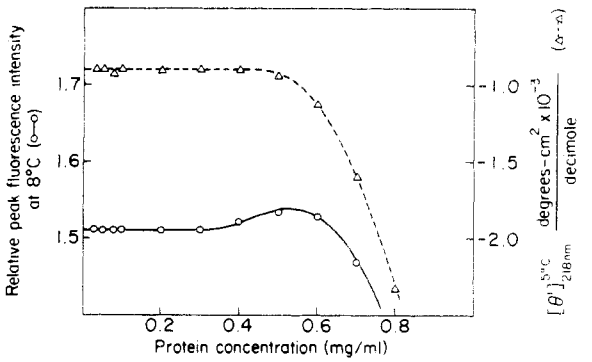

FIGURE 6: Effect of cryoimmunoglobulin McE concentration on the relative peak fluorescence intensity of the protein at 8.0 °C and on the 218-nm CD ellipticity minimum at 5.0 °C.

solved between 250 and 225 nm. No evidence of increased light scattering or absorption flattening was present at low temperatures. In order to partially quantify these changes, the ellipticity at 218 nm is plotted vs. incubation temperature for McE and the comparison molecules (inset B, Figure 4). The ellipticity at this wavelength is seen to change very gradually between 25 and 5 °C. The absence of a more abrupt temperature-dependent change in these data suggests a lack of cooperativity in the generative process. The far-UV CD spectra of the four other IgM proteins remain essentially unchanged over this temperature range. Within a time period of minutes it has been found that the spectral changes induced by lowering the temperature are completely reversible within the limits of detectability of these measurements (~2%). These changes are also pH dependent (inset A, Figure 4), being greatest between pH 6 and pH 8. At pHs less than 5 or greater than 12, alterations consistent with pH-induced conformational change occur. Attempts to demonstrate temperature-induced spectral changes, similar to those described above, in isolated (Fc)$_{5\mu}$ and Fabμ fragments of the cryoimmunoglobulin molecule have not been successful.

The changes in the far-UV CD spectra of the cryoimmunoglobulin suggest that changes in the secondary structure of the protein may be occurring as the temperature is lowered. To investigate such a hypothesis, the far-UV absorption spectra of protein McE and the other proteins were examined as a function of temperature using the same protein concentrations employed in the CD studies. Each protein displayed a single broad (peptide) peak at approximately 189 nm with a weak shoulder at 225 ± 2 nm. The mean residue extinction coefficients of the peptide-bond chromophore were very similar for each of the five proteins and were found to be between 5560 and 5900 after correction for side-chain contributions (Gratzer, 1967). For the cryoimmunoglobulin, $\bar{\epsilon}$ was calculated to be 5580, which is within the range of $\bar{\epsilon}$ for the other proteins. These $\bar{\epsilon}$ values can be compared with values of 4100, 6900, and 5800 at 190 nm for poly(L-lysine) in the α-helical, random, and β conformations, respectively (Rosenheck and Doty, 1961), and with estimates of 5800 and 5600 for myeloma and normal human IgG (Ross and Jirgensons, 1968). The effects of temperature upon the peptide peaks are shown in Figure 4, inset C. All five proteins including the cryoimmunoglobulin display a linear decrease in absorbance with temperature (due to incomplete compensation of the temperature-dependent end absorption of water; see Materials and Methods) over the range 38 to 4 °C, with no evidence of thermal anomalies.

Temperature Dependence of Intrinsic Fluorescence. In an attempt to confirm the temperature-dependent spectral changes in CD described above, the intrinsic fluorescence of each of the five proteins and their respective fragments was examined over the temperature range 38 to 5 °C (Figure 5). It has been demonstrated that a monotonic decline in quantum

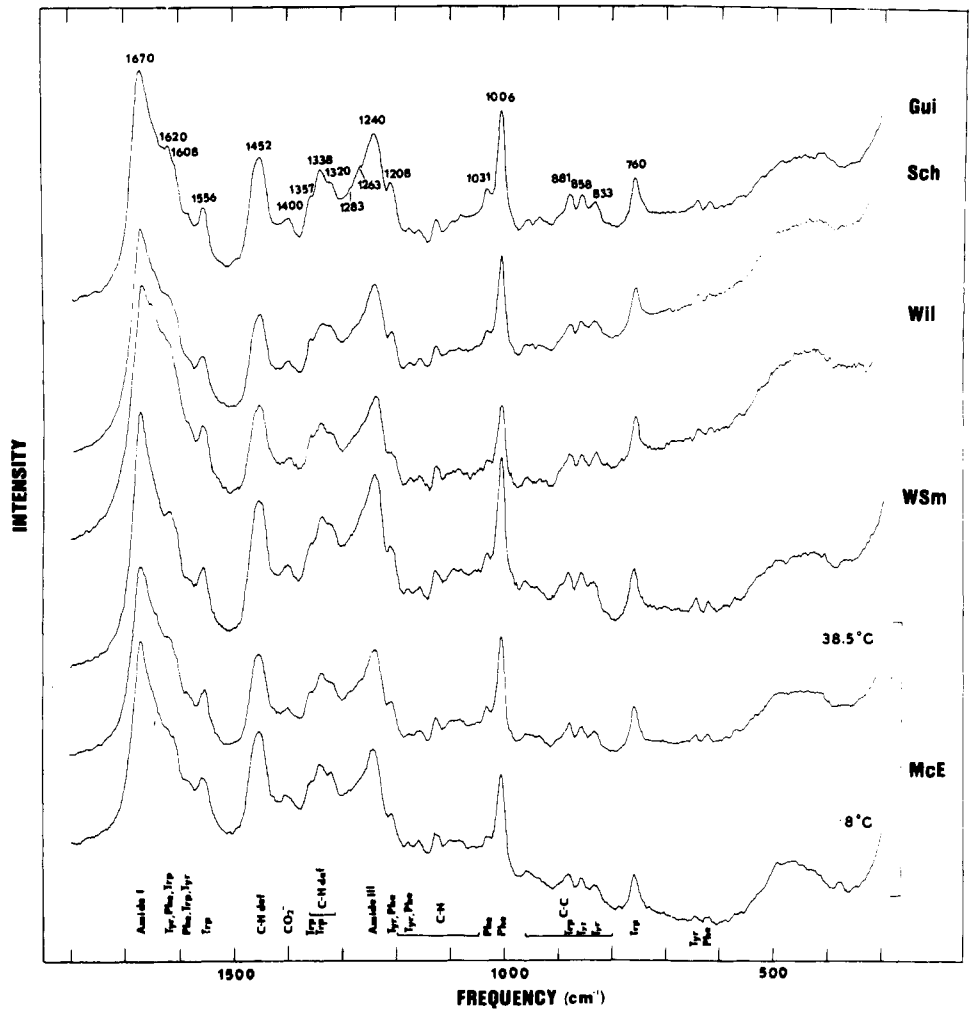

FIGURE 7: Raman spectra of cryoimmunoglobulin McE at −8.0 and 38.5 °C (150 mg/mL) and of IgM proteins WSm (90 mg/mL), Wil (50 mg/mL), Sch (90 mg/mL), and IgG Gui (80 mg/mL) at 38.5 °C in 0.15 M NaCl, pH 7.0. Conditions: excitation wavelength, 488.0 nm; radiant power, ~300 mW; spectral slit width, 10 cm^{-1}; scan speed, 25 cm^{-1}/min; rise time, 10 s. The small peak at 380 cm^{-1} in the −8 °C spectrum of McE is not a Raman line but a grating ghost, which often occurs with strongly scattering samples.

yield occurs with increasing temperature for tryptophan and tyrosine side chains in aqueous solution (Gally and Edelman, 1964). Any variation in emission intensity of either chromophore that does not fall monotonically with temperature is usually taken to be indicative of structural alterations. All proteins and their fragments show the expected positive linear dependence of fluorescence intensity with decreasing temperature between 37 and 12 °C. The Fabμ and $(Fc)_{5}\mu$ fragments of the four cold soluble IgM molecules are not shown in Figure 6, but all eight plots are approximately linear between 38 and 5 °C. On the other hand, below 12 °C, a number of proteins display deviations from this linearity. The most dramatic change appears for the cryoimmunoglobulin and consists of a well-resolved depression between 12 and 5 °C. Two of the other proteins (Sch and Hof) display slight downward inflections below 12 °C. A slight dip is also evident for the Fabμ fragment of protein McE, although it is not comparable in magnitude to that shown by the intact protein. Using the intensity of the fluorescence emission peak at 8 °C as a measure of the magnitude of this "anomaly" in the fluorescence vs. temperature plot of McE, the dip is at a maximum between pH 6 and 8 (inset, Figure 5), but rapidly disappears between pH 5 and 6 and between pH 8 and 9. As was found in the CD measurements, pH extremes (less than 4, greater than 12) produce relatively large changes in fluorescence intensity, possibly as a result of major structural alterations of the proteins. At neutral pH (pH 6 to 8) none of the changes in intensity described above appears to be accompanied by detectable shifts in the position of the emission maxima. The intensity of Rayleigh scattered light at 275 nm was used to monitor the possibility of changes in light scattering. No differences were found to accompany any of the atypical low-temperature-induced spectral changes shown in Figure 5.

Concentration Dependence of Temperature-Induced Changes in Fluorescence and CD Spectra. The effect of protein concentration upon the unusual CD and fluorescence spectral characteristics of the cryoimmunoglobulin at low temperature is shown in Figure 6. Both the relative peak fluorescence intensity and the ellipticity minimum at 218 nm appear independent of protein concentration between 0.03 and 0.35 mg/mL at lower temperatures, despite the fact that light scattering becomes detectable at protein concentrations greater than 0.2 mg/mL. At concentrations exceeding 0.4 mg/mL, both parameters display large changes in intensity that are accompanied by visible precipitation of the protein, which renders meaningful interpretation of the data impossible.

Near-Ultraviolet Absorption Spectroscopy. Attempts to detect thermal anomalies in UV spectra of McE and its fragments at low noncryoprecipitating concentrations (approximately 0.03–0.10 mg/mL) have been unsuccessful. At higher

protein concentrations scattering produced by precipitation of the cryoimmunoglobulin prohibits reliable measurement of temperature-dependent spectra. Examination of the temperature-dependent absorption spectra of intact proteins Sch, Hof, WSm, and ESm reveals no evidence for unusual changes as the temperature is lowered.

Raman Spectroscopy. The applicability of laser-Raman spectroscopy to both aqueous and solid proteins makes this technique especially useful for investigating questions concerning changes in secondary structure of cryoimmunoglobulins upon precipitation.

Figure 7 shows the Raman spectra obtained from aqueous solutions of cryoimmunoglobulin McE at −8 and 38.5 °C of IgM proteins WSm, Wil, Sch at 38.5 °C, and of the IgG_1-λ myeloma protein Gui, at 38.5 °C. Each spectrum of Figure 7 exhibits the same frequencies and intensities for conformationally sensitive Raman lines of the peptidyl backbone (the amide I and amide III modes, Frushour and Koenig, 1975). These are as follows: amide I appears as a fairly sharp and intense Raman line centered between 1668 and 1670 cm^{-1} in each spectrum, while amide III appears as a broad and moderately intense Raman line centered between 1238 and 1242 cm^{-1} in each spectrum. These features are characteristic of the antiparallel β-pleated sheet structure of proteins (Chen and Lord, 1974) and indicate that the predominant secondary structure in each case is of the β type. The present results are also consistent with those of Painter and Koenig (1975) and Pezolet et al. (1976), who reported a predominance of β structure in the protein chains of heterogeneous populations of rabbit and human immunoglobulins on the basis of their Raman spectra. The other prominent lines in the Raman spectra of Figure 7 are assignable to vibrations of aromatic amino acid residues and other chemical groups in the side chains, as indicated along the abscissa.

The changes produced in the Raman spectrum of cryoimmunoglobulin McE by lowering the temperature from 38.5 to −8 °C are very small. There are no significant changes in the amide I and amide III lines which would suggest a significant difference in structure between the dissolved and cryoprecipitated states of McE. We do note, however, some minor and reproducible changes in the intensities of Raman lines of the aromatic amino acid residues, possibly suggesting minor differences in their environments in the two states. An example is the 1320 cm^{-1} line of tryptophan which is somewhat sharper at −8 °C than that at 38.5 °C in the McE spectrum (Figure 7). It should be noted that the physical state of the McE sample at −8 °C was that of a translucent gel, which we presume corresponds to that of the cryoprecipitate formed in more dilute preparations. No freezing of the solvent was detectable.

Each of the other IgM preparations as well as the IgG protein was also examined at −8 °C, with no precipitation and no apparent changes in the Raman spectra.

The Raman spectrum of the Fabμ portion of McE is nearly identical with that of the parent molecule. A greater background in the spectrum of the $(Fc)_5\mu$ fragment made quantitative comparison of Raman intensities more difficult. The conclusions about secondary structure reached above regarding McE are directly applicable to both the Fabμ and $(Fc)_5\mu$ fragments. No changes were detected in the spectra of either fragment at −8 °C and no gelation or precipitation was detected.

The search for changes in the Raman spectrum of cryoimmunoglobulin McE was repeated with sample preparations more concentrated (to 200 mg/mL) and more dilute (to 80 mg/mL) than those shown in Figure 7. In every case, no change in the protein's secondary structure could be detected upon cryoprecipitation. Examination of the amide I band (Litman et al., 1973; Timasheff et al., 1967) in infrared spectra of immunoglobulin films of each protein at both precipitating and nonprecipitating temperatures was also consistent with no change in secondary structure at low temperatures.

Discussion

In summary, there exist significant differences between cryoimmunoglobulin McE and a number of cold-soluble IgM molecules with regard to certain spectral features of the proteins. While a reduction in the 217–218-nm CD ellipticity minima of protein McE is suggestive of a major difference in secondary structure between the cryoimmunoglobulin and the other IgM proteins, this is not supported by several other critera. Far-UV absorption, infrared, and Raman spectroscopic results are all consistent with the existence of very little difference in secondary structure between the proteins. We presume, therefore, that the observed differences in the far-UV CD spectra reflect positive differential contributions of other optically active chromophores in the far-UV region. The fluorescence emission spectra also are consistent with this interpretation; the reduction of intensity and small blue shift of the fluorescence emission maximum of McE relative to the other IgM's cannot be directly correlated with differences in amino acid composition and, therefore, probably reflect a difference in the environment of some of the aromatic residues of McE.

Examination of the Fabμ and $(Fc)_5\mu$ fragments of McE and the other IgM proteins allows the proposed conformational difference to be partially localized to the Fabμ portion of the cryoimmunoglobulin molecule. Reductions in both the intensity of the 217-nm CD ellipticity and the fluorescence emission maxima are manifested in this part of the molecule. Interestingly, the fluorescence emission maximum of this fragment appears to be very slightly red shifted relative to the other Fabμ fragments, while the $(Fc)_5\mu$ portion displays a small blue shift, similar to that observed with the native cryoimmunoglobulin. Thus, while the localization of the spectral differences seen between the intact cryoglobulin molecule and its "normal" counterparts is not absolute nor easily quantifiable (resulting from the degradation of the Cμ2 domain during trypsin proteolysis at 56 °C), we feel justified in concluding that these observations suggest an abnormality in cryoimmunoglobulin tertiary structure that at least partially involves the Fabμ region. Gel filtration and electrophoretic studies of both the intact cryoglobulin and its Fabμ fragment under both native and denaturing conditions suggest a slightly greater Stokes radius relative to their homologues, consistent with this hypothesis (Middaugh and Litman, manuscript in preparation).

It is possible that an intrinsic structural difference between the cryoimmunoglobulin and the comparison proteins might result in the production of an McE Fabμ fragment that differs spectroscopically because of the proteolytic cleavage process itself, rather than as a result of any intrinsic difference between the fragment and homologous regions in the reference proteins. For a number of reasons we feel that this is unlikely. If the protease produced internal disruption of peptide bonds in the McE Fabμ fragment, such breaks should be detectable in the amino acid sequence analysis of the whole fragment. However, McE Fabμ yields a single sequence that is completely homologous with the sequence of the isolated cryoimmunoglobulin κ light chain (J. M. Kehoe, personal communication), as predicted for an intact Fabμ fragment with a pyrrolidonecarboxylic acid blocked heavy chain. Furthermore, the chroma-

tographic distribution (under denaturing conditions) of the Fabμ fragment as a single, ~50 000 molecular weight species is inconsistent with secondary, internal proteolytic cleavage. A unique alteration in the tertiary structure of the cryoimmunoglobulin as a result of the proteolysis is also unlikely upon consideration of the localized domain structure of immunoglobulin molecules (Davies et al., 1975).

The effect of temperature on certain spectral features of the cryoimmunoglobulin at noncryoprecipitating concentrations reveals a further difference between McE and the comparison proteins. Both CD and fluorescence measurements as a function of temperature reveal irregularities that are not found upon similar analysis of the other IgM proteins. Such changes suggest that a conformational alteration occurs in cryoglobulin McE at low temperatures. These changes appear to be quite distinct from those appearing for immunoglobulins as a result of high-temperature denaturation, being both lesser in magnitude and in the case of the CD measurements, in the opposite direction to that usually observed for such disorder transitions (Gally and Edelman, 1964; Kincaid and Jirgensons, 1972; Pollet et al., 1972). As suggested above, we interpret these changes as arising primarily from changes in the environment of certain aromatic chromophores rather than from changes in cryoglobulin secondary structure. Measurements as a function of temperature of secondary structure-sensitive vibrational modes by infrared and Raman spectroscopy and of the far-UV absorption peak near 190 nm are all consistent with this hypothesis.

It has so far not been possible to localize the temperature-dependent conformational sensitivity to any particular region of the molecule. This inability of the isolated fragments to undergo the conformational change could reflect the fact that spatially distant parts of the cryoimmunoglobulin pentamer are involved in the process and explain our inability to localize the blue shift of the intact cryoglobulin to the Fabμ region. The significance of the apparent lack of cooperativity in the conformational transition is unknown, but one possibility is a change in segmental flexibility of the cryoglobulin, as opposed to a more localized alteration (Holowka and Cathou, 1976). Evidence from x-ray crystallographic analysis of immunoglobulin fragments suggests the occurrence of invariant aromatic amino acid residues in cavities between structural domains (Davies et al., 1975; Poljak, 1975). Recent fluorescence studies of Bence-Jones proteins and immunoglobulin light chains are consistent with this observation (Longworth et al., 1976). If aromatic residues are involved in interdomain stability interactions, their perturbation in the cryoglobulin might be reflected in some of the atypical spectral properties described in this work.

Although aggregation itself can induce major conformational changes in polypeptides (Hammes and Schullery, 1968; Pearce, 1975), the lack of concentration dependence (see Figure 6) under the experimentally accessible conditions employed in this study argues strongly that an intramolecular phenomenon is responsible for the atypical thermal effects observed. Furthermore, under many of the conditions employed in these experiments, it is clear that the spectral changes may actually precede cryoprecipitation of protein McE. Additional data have shown that the effects of a wide variety of solutes which both inhibit and enhance cryoprecipitation can be correlated with their effect upon the temperature-dependent spectral changes, arguing favorably that the conformational change may be either a prerequisite and/or required component of the cold-induced insolubility of the cryoimmunoglobulin (Middaugh and Litman, 1976).

The existence of low temperature-induced conformational transitions appears to be a rather widespread phenomenon (Brandts, 1969; Mazhul' et al., 1970; Middaugh and MacElroy, 1976). Such transitions have also been shown to occasionally result in the cold-induced aggregation of proteins (Graves et al., 1965; Cowman et al., 1967). In addition, such conformational changes have been observed for immunoglobulin molecules under certain conditions of pH (Troitsky et al., 1973; Zav'yalov et al., 1975).

It is known that single amino acid replacements in polypeptide chains frequently produce significant changes in the thermal stability of the altered proteins (Langridge, 1968a,b; Lode et al., 1976). Since the well-known heterogeneity that exists within immunoglobulin classes is known to arise from slight differences in primary structure (e.g., Adetugbo et al., 1977), an occasional variant possessing altered conformational sensitivity at low temperatures is not improbable. The occurrence of immunoglobulins that display an unusual sensitivity to higher temperatures (pyroglobulins) is consistent with such speculations (Klein, 1973; Zinneman and Seal, 1971), as is the existence of some IgM proteins that display both cryoglobulin and pyroglobulin properties (Meltzer and Franklin, 1966; Wang et al., 1976).

Some evidence exists that conformational changes may occur in other cryoimmunoglobulins at low temperatures. Klein and Dorrington (1976) have recently observed by CD and UV difference spectroscopy, a conformational change at low temperatures for a cryoprecipitable λ-light chain isolated from a noncryoimmunoglobulin IgG1 myeloma protein. Saluk and Clem (1975) concluded from hydrodynamic measurements that, in the case of a human IgG3 myeloma protein, a low temperature-induced conformational transition was a necessary component of the cryoprecipitation event. Brouet et al. (1974) detected tryptophan exposure by UV difference spectroscopy at low temperatures for a cryoprecipitable Bence-Jones protein. No change in secondary structure was detected by CD analysis, although actual experimental data were not presented. Preliminary examinations of three other IgM and two IgG cryoglobulins in this laboratory by measurements of CD and fluorescence have failed to reveal evidence for low temperature-induced conformational changes. Thus, our observations cannot necessarily be extrapolated to all monoclonal cryoimmunoglobulins.

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References

Adetugbo, K., Milstein, C., and Secher, D. S. (1977), *Nature (London) 265*, 299.

Brandts, J. F. (1969), *Biol. Macromol. 2*, 213.

Brouet, J.-C., Clauvel, J.-P., Danon, F., Klein, M., and Seligmann, M. (1974), *Am. J. Med. 57*, 775.

Cathou, R. E., and Dorrington, K. J. (1975), *Subunits Biol. Systems, Part C 7*, 91.

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

Chen, M. C., and Lord, R. C. (1974), *J. Am. Chem. Soc. 96*, 4750.

Cowman, R. A., Swaisgood, H. E., and Speck, M. L. (1967),

J. Bacteriol. 94, 942.
Crumpton, J. J., and Wilkinson, J. M. (1963), *Biochem. J. 88,* 228.
Davies, D. R., Padlan, E. A., and Segal, D. M. (1975), *Annu. Rev. Biochem. 44,* 639.
Dorrington, K. J., Bennich, H., and Turner, M. W. (1972), *Biochem. Biophys. Res. Commun. 47,* 512.
Dorrington, K. J., and Smith, B. R. (1972), *Biochim. Biophys. Acta 263,* 70.
Frushour, B. G., and Koenig, J. L. (1975), in Advances in Infrared and Raman Spectroscopy, Clark, R. J. H., and Hester, R. E., Ed., London, Heyden & Son, Ltd.
Gally, J. A., and Edelman, G. M. (1964), *Biopolym. Symp., No. 1,* 367.
Ghose, A. C. (1971), *Biochem. Biophys. Res. Commun. 45,* 1144.
Goodman, M., and Toniolo, C. (1968), *Biopolymers 6,* 1673.
Gratzer, W. B. (1967), in Poly-α-Amino Acids. Protein Models for Conformational Studies, Biological Macromolecules Series, Vol. 1, Fasman, G. D., Ed., New York, N.Y., Marcel Dekker, p 177.
Graves, D. J., Sealock, R. W., and Wang, J. H. (1965), *Biochemistry 4,* 290.
Greenfield, N., and Fasman, G. D. (1969), *Biochemistry 8,* 4523.
Grey, H. M., and Kohler, P. F. (1973), *Semin. Hematol. 10,* 87.
Hammes, G. G., and Schullery, S. E. (1968), *Biochemistry 7,* 3882.
Holowka, D. A., and Cathou, R. E. (1976), *Biochemistry 15,* 3379.
Kincaid, Jr., H. L., and Jirgensons, B. (1972), *Biochim. Biophys. Acta 271,* 23.
Klein, M. (1973), *Immunochemistry 10,* 673.
Klein, M., and Dorrington, K. J. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 273.
Kronman, M. J., and Holmes, L. G. (1971), *Photochem. Photobiol. 14,* 113.
Langridge, J. (1968a), *Mol. Gen. Genet. 103,* 116.
Langridge, J. (1968a), *J. Bacteriol. 96,* 1711.
Leach, S. J., and Scheraga, H. A. (1960), *J. Am. Chem. Soc. 82,* 4790.
Lerner, A. B., Barnum, C. P., and Watson, C. J. (1947), *Am. J. Med. Sci. 214,* 416.
Litman, G. W., Frommel, D., Rosenberg, A., and Good, R. A. (1971), *Biochim. Biophys. Acta 236,* 647.
Litman, G. W., Litman, R. S., Good, R. A., and Rosenberg, A. (1973), *Biochemistry 12,* 2004.
Lode, E. T., Murray, C. L., and Rabinowitz, J. C. (1976), *J. Biol. Chem. 251,* 1675.
Longworth, J. W., McLaughlin, C. L., and Solomon, A. (1976), *Biochemistry 15,* 2953.
Mazhul', V. M., Chernitskii, Ye. A., and Konev, S. V. (1970), *Biofizika 15,* 5.
Meltzer, M., and Franklin, E. C. (1966), *Am. J. Med. 40,* 828.
Middaugh, C. R., and Litman, G. W. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1716.
Middaugh, C. R., and MacElroy, R. D. (1976), *J. Biochem. (Tokyo) 79,* 1331.
Painter, P. C., and Koenig, J. L. (1975), *Biopolymers 14,* 457.
Pearce, K. N. (1975), *Eur. J. Biochem. 58,* 23.
Pezolet, M., Pigeon-Gosselin, M., and Coulombe, L. (1976), *Biochim. Biophys. Acta 453,* 502.
Plaut, A. G., and Tomasi, Jr., T. B. (1970), *Proc. Natl. Acad. Sci. U.S.A. 65,* 318.
Poljak, R. J. (1975), *Nature (London) 256,* 373.
Pollet, R., Rossi, G., and Edelhoch, H. (1972), *J. Biol. Chem. 247,* 5921.
Putnam, F. W., Florent, G., Paul, C., Shinoda, T., and Shimizu, A. (1973), *Science 182,* 287.
Pysch, E. S. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 825.
Rosenheck, K., and Doty, P. (1961), *Proc. Natl. Acad. Sci. U.S.A. 47,* 1775.
Ross, D. L., and Jirgensons, B. (1968), *J. Biol. Chem. 243,* 2829.
Saluk, P. H., and Clem, W. (1975), *Immunochemistry 12,* 29.
Schneider, A. S., and Harmatz, D. (1976), *Biochemistry 15,* 4158.
Sonenberg, M. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1051.
Spande, T. F., and Witkop, B. (1967), *Methods Enzymol. 11,* 498.
Stevens, L., Townsend, R., Timasheff, S. N., Fasman, G. D., and Potter, J. (1968), *Biochemistry 7,* 3717.
Strickland, E. H. (1974), *CRC Crit. Rev. Biochem. 2,* 113.
Thomas, Jr., G. J. (1971), in Physical Techniques in Biological Research, Vol. 1A, Optical Techniques, Oster, G., Ed., New York, N.Y., Academic Press.
Thomas, Jr., G. J., and Barylski, J. R. (1970), *Appl. Spectrosc. 24,* 463.
Timasheff, S. N., Susi, H., and Stevens, L. (1967), *J. Biol. Chem. 242,* 5467.
Troitsky, G. V., Zav'yalov, V. P., and Kiryukhin, I. F. (1973), *Biochim. Biophys. Acta 322,* 53.
Urry, D. W., Hinners, T. A., and Masotti, L. (1970), *Arch. Biochem. Biophys. 137,* 214.
Wang, A. C., Fudenberg, H. H., Wang, I. Y., and Watanabe, A. (1976), *Scand. J. Immunol. 5,* 311.
Woods, A. H., and O'Bar, P. R. (1970), *Science 167,* 179.
Zav'yalov, V. P., Troitsky, G. V., Demchenko, A. P., and Generalov, I. V. (1975), *Biochim. Biophys. Acta 386,* 155.
Zinneman H. H., and Seal, U. S. (1971), *Rev. Eur. Etud. Clin. Biol. 16,* 668.