Nucleation-controlled Polymerization of Human Monoclonal Immunoglobulin G Cryoglobulins*

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The kinetics of the polymerization of human monoclonal cryoimmunoglobulins at low temperature was investigated in temperature jump experiments by monitoring the changes in turbidity resulting from the scattering of incident light by the polymers. Above a critical concentration between 2 and 3 mg/ml, depending on the ionic strength, the kinetics were characterized by a concentration-dependent lag phase and initial rate of self-assembly. Under equilibrium conditions which favored polymerization, the only stable intermediate detected by analytical ultracentrifugation was the dimer. Although purified monomers were unable to self-associate at 4 °C, addition of trace amounts of autologous dimers promoted polymerization. The apparent rate of polymerization was shown to be slow \( (k = 4.7 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}) \), and the process was governed by an equilibrium constant of \( 4.6 \times 10^{4} \text{ M}^{-1} \). The initial rate of self-assembly was proportional to the product of the monomer concentration and the concentration of promoter (i.e. dimer). The rate of depolymerization was three orders of magnitude greater than the rate of polymerization and was proportional to the concentration of polymers present. These results suggest that the polymerization of monoclonal cryoimmunoglobulins is a nucleation-controlled process in which dimerization is the rate-limiting step. Kinetic studies on the polymerization of Fab and F(ab')2 fragments from cryoimmunoglobulins and a comparison of cryogel ultrastructure by electron microscopy suggested that the interaction site between monomers is located in the Fab region. Since the polymerization of monomers was only induced by autologous dimers and not dimers from other cryoimmunoglobulins, it was concluded that the hyper-variable regions play a specific role in the condensation reaction. The fact that one cryoimmunoglobulin has a well defined antibody activity against streptolysin O argued against a low temperature-induced auto-antidiotypic mechanism. Reduction of the interchain disulfide bonds of the Fab fragments abolished their ability to polymerize, probably by inducing a conformational change a considerable distance away in the variable domains of the molecules.

Cryoglobulins are a clinically important group of immuno-

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Isolation and Purification of Cryoglobulins and Normal Immunoglobulins—Monoclonal human cryoglobulins (IgGlCac, IgG2k, IgM, and IgG3k Psv) were isolated from a population of myeloma patients by several successive cycles of precipitation at 4 °C and solubilization at 37 °C. The cryoprecipitates were extensively washed after each cycle in 10 mM Tris-HCl/0.15 M NaCl, pH 7.8, containing 0.2% sodium azide (Buffer TBS). Noncryoprecipitating monoclonal IgG and polyclonal IgG were prepared by ammonium sulfate precipitation followed by ion exchange chromatography on DEAE-cellulose (DE-52, Whatman) equilibrated in 20 mM NaCl, 10 mM Tris-HCl, pH 7.8.

Preparations of Immunoglobulin Subunits—Fab and Fc fragments were prepared by pepsin digestion, as described by Nisonoff et al. (23). Monoclonal immunoglobulins at 10 mg/ml were mildly reduced at 37 °C with 10 mM dithioerythritol (Sigma) in Buffer TBS, pH 8.6, for 30 min under nitrogen and alkylated with 24 mM iodoacetamide (Sigma) or with [35S]iodoacetamide (Amersham Corp.). Heavy and light chains were separated by gel filtration on a column of Sephadex G-100 equilibrated in 1 M acetic acid and were renatured by extensive dialysis against 4 mM Na acetate buffer, pH 5.4 (36). Hinge peptides were obtained by trypptic digestion of IgG labeled H chains and analyzed by high voltage electrophoresis according to Frangione (39).

Covalent and Noncovariant Reassembly of IgGlCac—The noncovariant reassociation of reduced and alkylated H and L chains was achieved by dialyzing an equimolar mixture of H and L chains in 1 M acetic acid against 4 mM acetate buffer, pH 5.4, at room temperature. The molecules were renatured and dialyzed against Buffer TBS at room temperature. The oxidative reassembly of the molecule was achieved in two ways. IgGlCac was reduced at 7 mg/ml with 10 mM dithioerythritol at 37 °C and reoxidized by dialysis against Buffer TBS, pH 8.6, at room temperature. Alternatively, H and L chains were separately separated from the reduced but not alkylated molecule as previously described, recombined, and then renatured and reoxidized by progressive dialysis against Buffer TBS, pH 8.6, in the presence of a disulfide interchange system (oxidized and reduced glutathione), as described by Petersen and Dorrington (25).

Preparation of Cryoglobulin Polymers—Cold-induced polymerization of IgGlCac and IgG3k Psv was achieved by cooling a solution of resolubilized proteins at 25 mg/ml from 40 °C to 22 °C. Alternatively, nonspecific aggregation of cryoglobulins and normal polyclonal IgG was achieved by heating protein solutions at 10 mg/ml for 10 min at 63 °C. The polymer-containing samples were then fractioned on a Sephacryl S-300 superfine (Pharmacia Fine Chemicals) column equilibrated in Buffer TBS at room temperature. The column was calibrated with standards of known molecular weight (IgM pentamer, IgM monomer, F(ab′)2, and albumin). Chemically cross-linked oligomers were prepared by adding to a 15 mg/ml solution of cryoglobulin monomer with 10 mM dimethyl suberimidate (Pierce Chemical Co.) for 30 min at room temperature. The reaction was stopped by the addition of ethanolamine.

Electrophoresis and Immunological Techniques—The purity of the monoclonal and polyclonal proteins and their fragments was assessed by electrophoresis, immunoelectrophoresis, and immunodiffusion, using monospecific and polyvalent antisera and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 7.5% gels containing 0.1% sodium dodecyl sulfate. When cryoglobulins were to be tested, immunoelectrophoresis and immunodiffusion were carried out at 37 °C. The molecular weight of cross-linked monomers was determined by SDS-polyacrylamide gel electrophoresis in 4% gels.

Analytical Ultracentrifugation—Sedimentation coefficients of native cryoglobulins, cryoglobulin oligomers, and cryoglobulin fragments were measured in a Beckman model E analytical ultracentrifuge operated at 60,000 rpm with a titanium rotor at 37 °C to the cryoglobulin solution or at lower temperatures in order to monitor the cold-induced formation of high molecular weight components. Sedimentation was followed using schlieren optics at protein concentrations above 2 mg/ml and with the photoelectric scanning system at 290 nm for concentrations below 2 mg/ml. Sedimentation coefficients were calculated in the usual way (5).

Turbidity Measurements—The kinetics of cold-induced polymerization of monomeric cryoglobulins was followed by monitoring changes in the absorbance at 380 nm resulting from the scattering of incident light due to the formation of polymers. Measurements were carried out on a Cary 118 spectrophotometer using full scale settings of 0.02 to 2 absorbance units. The temperature of the experimental cell (pathlength, 0.438 or 1.0 cm) was maintained at 4 °C by using a thermostatted cell holder connected to a circulating water bath (Lauda K2/H), and the temperature was continuously monitored with a Yellow Springs Instrument Co. model 425C thermostater equipped with a small nylon-covered thermistor probe. During preliminary studies, a wavelength scan of several solutions of cryoglobulin was carried out on a Perkin-Elmer model 403 spectrophotometer. Different concentrations of polymers were dissolved in ammonium sulfate, and the absorbance at 380 nm was measured for 30 min. The absorbance was proportional to the concentration of polymers. Under these conditions, it was shown that A(tmax) is an exponential function of the total protein concentration and is proportional to the quantity of high polymers found at 4 °C for protein concentrations ranging from 2-20 mg/ml.

Kinetics of Polymerization and Depolymerization—Temperature jump experiments were performed to determine the rates of polymerization and depolymerization of IgGlCac and its fragments. To initiate polymerization, 2.0 ml of cryoglobulin in Buffer TBS was warmed to 37 °C for 1 h, rapidly filtered (Millipore filter, 0.45 μm), and immediately placed in a prechilled cuvette maintained at 4 °C. Thermal equilibration was reproducibly reached in 10 ± 1 min. In order to study the polymerization of cryoglobulin monomers promoted by the addition of oligomers, increasing quantities of a solution of oligomers were added to the samples at 4 °C, so that the final protein concentration would be held constant at 3.5 mg/ml. The solution was then rapidly placed in the prechilled cuvette and the kinetics recorded. To study the kinetics of depolymerization, samples of polymerized cryoglobulin, maintained at 4 °C, were injected with a prechilled syringe into the experimental cell which had been prewarmed at a predetermined temperature so that the final temperature observed after addition of 1 ml of the cold cryoglobulin solution would be identical with that of the cell holder in which the cell was placed. Using this technique, the time required to obtain thermal equilibration was 10-15 s, irrespective of the amplitude of the temperature jump. The cell compartment was flushed with air throughout the experiment in the presence of anhydrous calcium sulfate in order to prevent condensation.

Initial rates of polymerization and depolymerization were determined graphically.

Electron Microscopy—The native IgGlCac and its proteolytic fragments were allowed to gel at 4 °C. About 1 ml of each gel was shaken by a sharp blow, and gel fragments of approximately 1 mm3 were transferred into cold fixative containing 3% glutaraldehyde. Fixation was allowed to proceed overnight and was followed by thorough washing in three changes of 0.1 m cacodylate buffer, pH 7.4, at 4 °C. The gel fragments were then post-fixed with 1% OsO4, in the previous buffer for 1 h at 4 °C. After this second fixation, the samples were washed and dehydrated in solutions of increasing ethanol concentrations and finally embedded in Epon. Ultrathin sections of about 50 nm were cut and stained on the grids with uranyl acetate followed by lead citrate. Sections were made conductive with a carbon layer about 5 nm thick in a high vacuum evaporator and were examined in a Philips EM 200 electron microscope equipped with an anticontamination device.

RESULTS

Immunchemical Characterization of Monoclonal Cryoglobulins—The IgGlCac used in the present study has a well documented antibody activity against streptolysin O (34). The V region subgroups of its γ and κ chains were determined by sequence analysis and were shown to be VγIII and VκII, respectively. The V region subgroups of the IgG2k Zie were VγIII and VκI, whereas PAV IgG3k Psv belongs to the VγIII and VκI variable frameworks. The 14C-labeled hinge peptides, obtained by trypptic digestion of the H chains of the IgGs were identical with their normal counterparts, as judged by mobility in high voltage electrophoresis performed at two pH values (6.5 and 3.5). The general physical-chemical parameters influencing the cryoprecipitation of IgGl cryoglobulins have been previously reported (16, 20). The optimal pH for cryoprecipitation for
the three cryoglobulins was near 7.4, and the amount of precipitate decreased when the ionic strength increased above physiological conditions. The IgG1k and the IgG3k gave a cryogel when cryoprecipitation was induced by cooling an aggregate-free solution of these proteins at high molar concentrations (10^4 M). The IgG2a cryoglobulin crystallized.

**Ultrastructural Studies**—Electron microscopy of the cryogels obtained with the native IgG1k Cac and its Fab fragments revealed a periodic tubular structure. The native IgG gave well aligned bundles of microtubules with a periodic structure and, in cross-section, the annuli were formed of a double ring with an external diameter of 30 nm and an internal diameter of 11 nm. These rods, following gel disruption, were homogeneous in diameter but varied in length. These tubular structures tended to form tangled bundles of long filaments. In contrast, the proteolytic fragments yielded disorder microtubules which in cross-section were formed of a single annulus composed of 12-14 globular sub-units with an external diameter of 19 nm. Although a similar structure was observed for the Fab cryogel, the ring structures appeared to be more fragile and to have an external diameter significantly smaller than that observed with the native cryoglobulin or its Fab fragments (Fig. 1).

**Kinetics of Cold-induced Polymerization**—The kinetics of the cold-induced polymerization of the IgG1k cryoglobulin at 4 °C was studied under physiological conditions at different protein concentrations (Fig. 2A). As described under "Materials and Methods," the absorbance at 330 nm was linearly proportional to the mass concentration of IgG polymers formed at low temperatures, and changes in light scattering were used to monitor the cryoprecipitation phenomenon. Turbidimetric measurements have been successfully used to monitor the temperature-dependent polymerization reactions of different proteins, such as tubulin (13) and cryoglobulins (32, 33). Below a critical concentration of approximately 2 mg/ml, no cryoprecipitation was observed with IgG1k Cac. Above this critical concentration, a monotonic increase in absorbance was observed after a short concentration-dependent lag phase following thermal equilibration (10 min for the lowest cryoglobulin concentration). The duration of the initial lag phase decreased as the protein concentration increased and correspondingly, the initial rate of the reaction determined graphically after the lag period increased. As shown in Fig. 2B, the polymerization phenomenon at 4 °C for 2 h (---) and subsequently depolymerized by rapid rewarming to 37 °C (----). Cycles of polymerization-depolymerization were repeated twice.

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**Fig. 1.** Electron micrographs of longitudinal and cross-sections of cryogels obtained with the intact IgG Cac cryoglobulin (a, b), its Fab (c, d), and Fab(·)2 (e, f) proteolytic fragments. Solid bars represent a distance of 100 nm in a, c, and e, and of 50 nm in b, d, and f. Bundles of aligned IgG fibers and the corresponding double-ring structures are shown in a and b, respectively. In d, the arrow indicates the presence of structures compatible with loose Fab annuli.

**Fig. 2.** Kinetics of the cold-induced polymerization. A, changes in turbidity at 330 nm as a function of time for various solutions of native IgG Cac at different concentrations in Buffer TBS. Protein concentrations in mg/ml were: a, 6.4; b, 4.2; c, 2.9; d, 2.2; e, 1.4; f, 0.2. The temperature was maintained at 4 °C after the initial temperature jump. B, reversibility of the cold-induced self-association. A sample of native IgG Cac at 5.7 mg/ml in Buffer TBS was polymerized at 4 °C for 2 h (---) and subsequently depolymerized by rapid rewarming to 37 °C (----). Cycles of polymerization-depolymerization were repeated twice.
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A nucleation event was further supported by the following experiments. The polymerization of the native IgG at 16 mg/ml was induced by lowering the temperature from 37 °C to 20 °C in less than 5 min. This relatively small drop in temperature was chosen in order to induce a slow polymerization. The turbidity of the solution was continuously monitored at 330 nm, and 0.5-ml aliquots were withdrawn every second hour during the initial polymerization phase and analyzed at 20 °C in the analytical ultracentrifuge. High polymers responsible for the light scattering sedimented to the bottom of the ultracentrifuge cell, and the dimer was the only stable intermediate detected in significant amounts during polymerization (data not shown).

Since the approach to equilibrium of the polymerization reaction seemed to involve a dimer as the stable intermediate acting as a putative promoter, it was necessary to isolate a preparation of aggregate-free monomer capable of polymerizing onto preformed oligomers as well as preparations of oligomers of various sizes which could be assessed for their ability to initiate the growth process. To this end, a solution of solubilized native cryoglobulin at 15 mg/ml was cooled to room temperature and applied to a calibrated Sephacryl S-300 column. The elution profile (Fig. 4) showed a distinct peak of high polymers, larger than IgM, followed by a major peak of monomer eluting at $V_c/V_t = 0.6$, characterized by a marked shoulder (fraction II) on its ascending limb corresponding to a $V_c/V_t$ smaller than that of 8 S IgM. As shown in Fig. 4, the fractions corresponding to the first peak (fraction I), the shoulder (fraction II), and the descending portion of the monomer peak (fraction III) were concentrated and analyzed by analytical ultracentrifugation at 37 °C in Buffer TBS. The high molecular weight fraction consisted of a heterogeneous population of polymers as judged by UV scanning at 2 mg/ml (data not shown). The schlieren pattern of fraction II concentrated to 5 mg/ml revealed the presence of symmetrical peaks with sedimentation rates of 6.7 S and 9 S (Fig. 3C). The first component was identified as monomer (48%) and the second as dimer (52%) in slow equilibrium with the monomer. The descending portion of the monomer peak isolated by gel filtration was concentrated to 8 mg/ml and was shown to be totally devoid of oligomers (Fig. 3B). Both the polymer and dimer preparations were able to cryoprecipitate in the cold. In contrast, the monomer preparation was unable to precipitate at 4 °C within the time course of the polymerization experiments. When this preparation was concentrated to over 20 mg/ml (i.e. half the serum concentration of the native monoclonal cryoglobulin), a small amount of cryoprecipitate was observed after 1 week at 4 °C.

Rate of Polymerization as a Function of Initiator and Monomer Concentrations—When high molecular weight polymers were added at 37 °C to a final mass concentration of 1% to a solution of monomer at a concentration as low as 2 mg/ml, the low temperature-induced polymerization was restored.

One characteristic of a self-nucleated condensation polymerization is that the initial rate of polymerization is directly proportional to the concentration of the initiator (13, 24). In order to test this prediction and to determine the minimal size of the promotor, increasing quantities of native dimer were added to a fixed concentration of monomer (Fig. 5A), and the temperature was reduced to 4 °C. No change in $A_{230\text{nm}}$ was observed in the absence of dimer, whereas a typical condensation polymerization reaction was observed upon addition of increasing concentrations of dimer. Above a critical concentration of dimer, the initial lag period rapidly decreased, and the initial rate of the reaction increased as a linear function of the dimer concentration. Similar findings were observed with IgGx (Fig. 5B). The various samples were allowed to stand at 4 °C for 1 week, and the cryoprecipitates formed were collected by centrifugation; the amount of cryoglobulin contained in the precipitate or remaining in the supernatant was determined spectrophotometrically after dilution in 0.25 M acetic acid. The data clearly indicated that the quantity of cryoprecipitate could only be accounted for if co-precipitation of the monomer had occurred.

An additional prediction of a condensation-polymerization reaction is that the initial rate of elongation is directly proportional to the monomer concentration above a critical concentration (13, 24). In order to verify this property, a fixed quantity of purified dimer was added at 37 °C to increasing concentrations of monomer. The mixture was then rapidly cooled, and the polymerization reaction was monitored at 330 nm. As shown in Fig. 6, the initial rate of polymerization, determined graphically for two different initiator concentrations $(1.7 \times 10^{-5}$ and $1.1 \times 10^{-6}$ M, respectively), increased as a linear function of the molar concentration of monomer. A 1.54-fold increase in the dimer concentration resulted in a 1.58-fold increase in both the slope and the y intercept of the curve obtained for the lowest seed concentration.

Assuming that the polymerization-depolymerization reaction occurs at the end of the linear polymer of cryoglobulin, the initial rate of assembly is the sum of the rates of polymerization and depolymerization and can be described by the following equation

$$A_{230\text{nm}} = \frac{1}{2} A_{230\text{nm}} \text{monomer} + \frac{1}{2} A_{230\text{nm}} \text{dimer}.$$
where $[M]$ and $[D]$ represent the monomer concentration and the number concentration of dimer, respectively, and $k_+$ and $k_-$ are the apparent rate constants for polymerization and depolymerization, respectively. The constant $c$ is a proportionality constant relating the $A_{330}$ units to monomer units polymerized. This proportionality constant was estimated to be $0.7 \times A_{330}$ units/um of monomers polymerized (see “Materials and Methods”). According to the preceding equation, the $y$ intercept in Fig. 6 corresponds to $-(1/c) \cdot k_- \cdot [D]$, and the slope is given by $(1/c)k_+ \cdot [D]$. Therefore, the first order rate constant for depolymerization ($k_-$) and the second order rate constant for polymerization ($k_+$) could be determined graphically and were found to be $1.02 \times 10^{-5}$ s$^{-1}$ and $4.72 \times 10^{-4}$ M$^{-1}$ s$^{-1}$, respectively, at 4°C. The equilibrium constant $K = k_+/k_-$ was, therefore, $4.6 \times 10^{10}$ M$^{-1}$. The monomer concentration at which the rate of polymerization is equal to the rate of depolymerization corresponds to the x intercept and was found to be $2.17 \times 10^{-5}$ M (i.e. 3.25 mg/ml) and independent of the promotor concentration. This point represents the equilibrium monomer concentration $M_e$ at which $d[M]/dt = 0$. Thus, $M_e = k_+/k_- = 1/K$, where $K$ is the equilibrium association constant (i.e. $4.6 \times 10^{10}$ M$^{-1}$). When similar polymerization reactions were carried out at lower ionic strength (i.e. 50 mM versus 150 mM NaCl) which was known to enhance the cryophenomenon, the equilibrium monomer concentration was decreased to about $6.7 \times 10^{-5}$ M (1 mg/ml), and the initial rates of polymerization were increased by one order of magnitude (data not shown).

### Specificity of the Cold-induced Polymerization

In order to test the specificity of the polymerization of the IgGlk monomer onto autologous native dimers used as promoters, we tested the ability of other initiators to induce the nucleation-controlled reaction at low temperature. Soluble high molecular weight polymers obtained by heat aggregation of either the autologous cryoglobulin or polyclonal IgG were prepared by gel filtration. Cross-linked IgGlk oligomers were obtained by reacting IgGlk monomers at 10 mg/ml with suberimidate. Under optimal conditions, 50% of the monomer was covalently cross-linked in the form of dimers, trimers, or tetramers, as judged by SDS-polyacrylamide gel analysis (data not shown). None of these preparations was able to initiate the temperature-dependent polymerization, even at appropriate initiator concentrations (i.e. over 10%). Furthermore, 10% IgGlk Cac dimers were unable to initiate the polymerization of two other monomers (IgGlk Zie and IgGlk Pav) known to cryoprecipitate in the presence of their autologous dimers.

It was also shown that the polymerization reaction is Fc independent. Both the F(ab')$_2$ and Fab fragments were able to form gel through a nucleation-controlled polymerization in the cold albeit at higher concentrations than the parent molecule (Fig. 7). It was possible to demonstrate that the addition of as little as 1% of IgGlk Cac dimers induced an instantaneous
polymerization of a 3.9 mg/ml solution of F(ab')2 monomers which had been previously shown to be unable to cryoprecipitate at 4 °C (Fig. 7).

Depolymerization of Cryoglobulin Tubular Structures—As shown in Fig. 2B, the cold-induced assembly is totally reversible at 37 °C. The depolymerization kinetics of solutions of cryoglobulins containing various quantities of tubular polymers of an unequal length and diameter were studied by temperature jump experiments (Fig. 8A). Following a brief lag phase of 10-15 s, corresponding to the time required for thermal equilibration, the rate of disassembly was about three orders of magnitude faster than the rate of polymerization. If the initial part of the reaction corresponding to the lag phase was ignored, the kinetics approximately followed pseudo-first order kinetics with a single relaxation time of 27 s. Nevertheless, curve-fitting analysis using a computer program showed that the reaction was more complex since the experimental data could not be accounted for by the sum of three first order processes (data not shown). The initial rate of depolymerization was directly proportional to the mass concentration of polymer determined at 4 °C (Fig. 8A). In a second series of experiments, it was shown that the initial rate of depolymerization for a fixed concentration of polymers increased with the amplitude of the temperature jump (Fig. 8B).

Molecular Localization of the Site of Monomer-Monomer Interaction—The previously described experiments strongly suggest that the self-nucleating event is indeed the dimerization of the cryoglobulin monomer. This dimerization step is thermodynamically unfavorable. It was not possible to demonstrate any temperature-dependent conformational changes in the intact IgG molecule or its Fab fragment. The temperature-dependent changes observed by circular dichroism for Fab Cac were not significantly different from those observed for the Fab fragment of a noncryoprecipitating monoclonal IgG1k (data not shown). By difference spectroscopy, we showed that lowering the temperature of a solution of IgG1k Cac or its Fab fragment induced red-shifted difference spectra. However, it was shown that the changes in molar absorbance at two different fixed wavelengths varied as a linear function of the temperature, as expected for a solvent effect and not a conformational change (data not shown).

It has been clearly shown that the Fab and F(ab')2 fragments of the molecule are capable of cryoprecipitating and forming tubular structures similar to the parent molecule. This implies that the region involved in the polymerization is contained within the Fab fragment. It should be stressed that higher moir concentrations of Fab fragments were required to initiate the formation of a cryogel, whereas F(ab')2 fragments had an ability to cryoprecipitate comparable to that of the intact IgG. The fact that only autologous dimers can induce the specific nucleation of both the IgG monomer (Fig. 5A) and the F(ab')2 fragment (Fig. 7) strongly suggests that the region involved in monomer-monomer association is restricted to the complementarity-determining segments of the V regions. Mild reduction of the inter-H-L disulfide bridge of the native IgG and its proteolytic fragments completely abolished this specific interaction. When this bridge was reoxidized in the absence of dissociation of the H and L chains, the cryoprecipitability of the molecule was restored (Fig. 2A). In contrast, the recombinant molecules obtained by noncovalent reassociation and reoxidation of isolated H and L chains were unable to polymerize even at 7 mg/ml, and the addition of
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Autologous dimers to the reduced and alkylated molecule could not trigger polymerization.

DISCUSSION

Although the mechanism of cold-induced polymerization of monoclonal cryoglobulins had not been elucidated, results from previous experiments suggested that the precipitation at low temperatures of these proteins might be a nucleation-controlled event (32, 33). The objectives of the present study were 2-fold: 1) to test for a condensation-polymerization mechanism in order to establish a model for the in vitro assembly of monoclonal cryoglobulins, and 2) to localize the region of the molecule involved in the monomer-monomer interaction promoted at low temperatures. The study of two monoclonal cryoglobulins of different subclasses (IgGlx, IgG3x) in parallel has clearly shown that cold-induced polymerization is indeed mediated by a nucleation event. The existence of a critical concentration below which the IgGlx cryoglobulin does not polymerize and a concentration-dependent lag phase and initial rate are strongly suggestive of such a mechanism. Similar observations have been made for a monoclonal IgG2x cryoglobulin (32, 33). It has been shown that a solution of cryoglobulin monomer, freed of oligomers by gel filtration, is unable to polymerize even at high concentrations, but that the addition of trace amounts of soluble polymers (seeds) at 3° C initiates the polymerization at 4° C. It was further demonstrated that the smallest species capable of acting as a promoter is the native dimer obtained by cryoprecipitation of the monomer at 4° C. The kinetic predictions for a nucleation-controlled polymerization have been confirmed in the case of monoclonal cryoglobulins by the following findings. Under equilibrium conditions favoring polymerization, the initially stable intermediate detectable by analytical ultracentrifugation is the dimer. No other discrete oligomeric species smaller than the large tubular structures are found in significant amounts. At a fixed concentration of monomer, the initial rate of polymerization is directly proportional to the concentration of dimer added. At a fixed concentration of dimer, the initial rate of assembly increases in direct proportion to the concentration of cryoglobulin monomer. The dimeric promoter depolymerizes upon dilution when the concentration of monomer is below the critical concentration. Finally, the initial rate of depolymerization following a temperature jump at 28° C is directly proportional to the polymer concentration.

Taken together, these results suggest that the cold-induced condensation polymerization of monoclonal cryoglobulins can be described as follows.

1) The dimerization of cryoglobulin monomers constitutes a thermodynamically unfavorable nucleation event. The rate of dimerization depends on the monomer concentration and is the limiting factor in the polymerization kinetics. This accounts for the concentration dependence of the lag phase and of the initial rate of the reaction. This finding explains the irreproducibility of the kinetic data obtained with different preparations of the same molecule which usually contain variable amounts of oligomers and why they depend on the techniques which have been used to isolate, solubilize, and centrifuge the cryoglobulin solution. Nevertheless, the detailed mechanism of the dimerization process remains unclear. The formation of dimers has been shown to be necessary for the cold-induced assembly of an IgGl Fab fragment (29) and for that of a cryoprecipitating human λ chain (17). Although a thermal transition has been proposed to explain the initial step of the polymerization pathway (17, 20, 31) circular dichroism and difference spectroscopy studies performed on both the native IgG and its Fab fragment at various temperatures failed to detect significant conformational changes. A similar observation has also been reported by other groups (21, 32). Conversely, as reported in this paper, a decrease in ionic strength resulted in a marked increase in the rate of polymerization and a significant decrease in the monomer critical concentration. However, a conformational change occurring in a limited region of the molecule which would not affect the spatial orientation of aromatic chromophores could be raised by these methods of detection. It has been established that an increase in ionic strength can reversibly abolish the monomer-monomer interactions at low temperatures (17, 21). These results indicate that electrostatic interactions between Fab fragments are an essential feature of the phenomenon, and since these electrostatic interactions are temperature independent, it is reasonable to assume that a redistribution of charged amino acid side chains have been induced at low temperature.

2) This rate-limiting nucleation event initiates the thermodynamically favorable temperature-dependent elongation of the tubular structures, as judged by the electron microscopic studies of the cryoglobulin. These growth processes are nevertheless slow, with an apparent forward rate constant for the elongation step at 4.7 X 10^{-14} M^{-1} s^{-1}. They probably result from the addition of monomeric subunits, initially onto the dimer and subsequently at the extremities of the growing microtubule. Further interactions between tubular structures lead to the alignment of microtubules into well ordered bundles or to the formation of entangled filaments. These results are analogous to those reported by Wilson and Makinen (40) for the fiber-to-crystal transition of deoxygenated sickle cell hemoglobin. These authors showed that gels consist of randomly oriented groups of fibers in contrast to the well ordered network of filaments in deoxy Hb S crystals. It is likely that the high viscosity of the gel phase has impeded the crystallization process of the two monoclonal cryoglobulins used in our study. In that respect, it is noteworthy that the crystallization of four human cryoglobulins (IgGlx Doh (35), IgGlx Kol (6), IgG1a Mcg, and IgGlx Zie (8)) has allowed their three-dimensional analysis by x-ray diffraction. Nevertheless, because of these multiple intermicrotubule interactions, any rigorous analysis of the kinetics of polymerization of cryoglobulins becomes extremely complex.

3) The depolymerization step is exceedingly rapid as compared to the polymerization step, and it has not been possible to elucidate the mechanism in detail because of the complexity of the reaction, due to the fact that the initial solution of tubular polymers is not homogeneous. Although the putative depolymerization by release of monomer from the ends of the tubule should obey zero order kinetics, a pseudo-first order rate will be superimposed due to the time-dependent disappearance of tubular structures heterogeneous in length. This explains why the initial rate of depolymerization is apparently a linear function of the initial mass concentration of polymers.

It has been clearly demonstrated by ultrastructural studies and by the use of proteolytic fragments that the cryophenomenon is Fab dependent. One remarkable finding is that the cryoglobulin structure of the F(ab')_2 fragment is similar to that of the parent molecule, although the external diameter of F(ab')_2 tubules is significantly smaller than that of IgG rods (19 nm versus 30 nm). IgG microtubules are formed of two concentric tubes, the smaller one being approximately the size of F(ab')_2 cross-sections. Therefore, the difference of about 11 nm in external diameter may be accounted for by the lack of the Fc fragment (7 nm), assuming that the IgG molecules are radially arranged within the tube, Fabs forming the internal tube and Fcs the external one. Lateral associations between aligned IgG microtubules might result from Fc-Fc interactions (26), since these well ordered structures are not seen in the cryoglobulins.
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proteolytic fragments. The dimensions of the annular section of the hollow Fab'12 rods are similar to those previously reported in the literature for other monoclonal cryoglobulins or cryocrystals (3, 14, 27, 37, 39). The observation that the Fab fragment can form similar structures, but with a slightly different and more fragile organization, indicates that multivalency is an important factor for stabilization of these supramolecular structures. This Fab-Fab interaction has been clearly demonstrated in the crystal lattice of IgG2 Zie cryoglobulin by electron microscopy (27). These results invalidate the hypothesis that a structural anomaly of the hinge (i.e., deletion (35) or extra S-S bond (33)) forms the molecular basis for the unusual thermal properties of monoclonal cryoglobulin. This crucial finding has recently been established by high resolution x-ray diffraction analysis (18).

In addition, we have shown that the nucleation-controlled polymerization is a phenomenon specifically and exclusively inducible by autologous native promoters. This crucial finding clearly indicates that hypervariable regions are directly involved in the recognition sites between cryoglobulin monomers and that aggregation of the cryoglobulin, chemical modification of lysing groups by bifunctional cross-linking agents, or transient acid denaturation of the polypeptide chains results in an irreversible loss of the thermal properties of the molecule, probably by modifying the limited region(s) involved in the phenomenon. Confirming our previous experiments on a cryoprecipitating human lambda chain (17), t change in the tertiary structure of the molecule due to the cleavage of the COOH-terminal causes inter H-L disulfide bridge-induced conformational changes at a distance in the variable region of the Fab fragment leading to a loss of its thermal sensitivity. Recently, it has been shown that some monoclonal cryoglobulins may express an autoantibody activity directed against themselves. Along this line, the possible anti-IgG activity of some monoclonal IgG cryoglobulin has been previously reported (11, 37), and a cryoprecipitating monoclonal IgM with cold agglutinin activity has been shown to react with its own N-acetylneuraminoyl residues (38). In IgG Kol crystals, the hypervariable segments of one molecule are in close contact with the hinge peptide of a neighboring molecule, as in an antigen-autoantibody complex (18). More recently, the presence of anti-idiotypic antibodies in mixed cryoglobulins has been suggested (10). In the cases of IgG1 Cac cryoglobulin, it is most improbable that this molecule, selected for this study because of its known anti-streptolysin O activity, is also an auto-anti-idiotype. Therefore, the polymerization of monomers is probably mediated by lateral surface interactions between Fab fragments.

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