Thermodynamic and Conformational Studies on an Immunoglobulin Light Chain Which Reversibly Precipitates at Low Temperatures†

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ABSTRACT: A λ light chain, isolated from an immunoglobulin G molecule, was found to reversibly precipitate at low temperatures. This cryoprecipitation was a function of pH, ionic strength, protein concentration, and time as well as temperature. The λ chain underwent a cooperative conformational change as the temperature was lowered from 26 to 0 °C as judged by ultraviolet difference spectroscopy and circular dichroism. Normal λ chains showed no conformational change. By difference spectroscopy it was possible to calculate the equilibrium constant governing the conformational change. The change was strongly exothermic ($\Delta H \sim -80$ kcal mol^{-1}) and accompanied by a large decrease in entropy ($\Delta S \sim -280$ eu). The midpoint of the transition was dependent on the initial protein concentration, suggesting that only the noncovalent dimer of the λ chain exhibited the conformational change. The existence of a monomer-dimer equilibrium ($K_A \sim 4 \times 10^5$ M^{-1}) was confirmed by sedimentation velocity. No conformational change was observed by circular dichroism at concentrations where greater than 95% of λ chain was in the form of a monomer. Although high ionic strength inhibited the cryoprecipitation, it had no effect on the conformational change. Stabilization of the dimer by forming an interchain disulfide bond between two monomers abolished both the conformational change and cryoprecipitation. A fragment corresponding to the constant region was isolated from both peptic and tryptic digests of the λ chain. This fragment neither cryoprecipitated nor showed temperature dependence conformational changes. It proved impossible to isolate a fragment corresponding to the variable region. Both qualitative and quantitative models are presented to account for the behavior of the λ chain at low temperatures.

Cryoglobulins are a clinically important group of immunoglobulins that reversibly precipitate or form gels upon cooling. Since the early studies of Lerner and Watson (1947), several immunochemical investigations of human cryoglobulins (Ritzman and Lewin, 1961; Meltzer and Franklin, 1966; Grey and Kohler, 1973) have formed the basis for their classification into three major groups (Klein et al., 1972): (1) monoclonal cryoglobulins, most of which belong to the IgM[1] or IgG class, although IgA and Bence-Jones protein cryoglobulins have been reported; (2) mixed cryoglobulins, usually IgM-IgG immune complexes, in which the IgM is a monoclonal antibody against polyclonal IgG; (3) mixed polyclonal cryoglobulins in which polyclonal antibodies against various antigens can sometimes be demonstrated. Correlations have been established between the immunochemical type of cryoglobulins and the clinical symptoms as well as the underlying disease processes (Brouet et al., 1974).

Previous studies have shown that cyroprecipitation of monoclonal or mixed cryoglobulins was dependent upon protein concentration, temperature, pH, and ionic strength (Meltzer and Franklin, 1966; Saha et al., 1968, 1970; Klein et al., 1972; Saluk and Clem, 1975). This property is not associated either with any particular H-chain class or subclass or with any specific L-chain type. Isolated Fc regions of IgG or IgM cryoglobulins did not cryoprecipitate (Saha et al., 1970; Middaugh et al., 1976), whereas the peptic F(ab')$_2$ fragments of cryoprecipitating IgG and IgA retain this property (Saha et al., 1970; Grey and Kohler, 1973; Pruzanski et al., 1973). The papain Fab fragment of one IgG cryoglobulin exhibited a temperature-dependent self-association (Saha et al., 1968). There is some evidence that the formation of a cryoprecipitate by an IgG3 could be a consequence of temperature-induced conformational changes in the Fab fragment prior to its binding to the autologous Fc fragment (Saluk and Clem, 1975). Chemical analyses of H and L chains of monoclonal IgG cryoglobulins or the monoclonal IgM components of mixed cryoglobulins have shown that unblocked L chains are preferentially associated with H chains of V_HI subgroup (Wang et al., 1974). These various findings suggest that the critical region of the molecule responsible for the cold precipitation resides in the variable domains.

The physicochemical mechanisms underlying cryoprecipitation are poorly understood. In the present paper we describe the results of detailed studies on the behavior of a cryoprecipitating human λ L chain at low temperatures. This protein was selected because it represents the simplest molecular model available. The λ chain, isolated from a noncryoprecipitating IgG1 myeloma protein (patient Do), in common with other L chains, is composed of two globular, compact domains (Edelman et al., 1969). One domain corresponds to the variable region (V_λ) and the other to the constant region (C_λ). The domains are linked by a "switch" region sensitive to proteolysis, thus allowing the preparation of V_λ and C_λ fragments (Solomon and McLaughlin, 1969; Bjork et al., 1971; Karlsson et al., 1972). Utilizing this property we have attempted to localize the thermal transition to a particular domain.

Materials and Methods

Isolation of IgG and λ Chains. Plasma containing high levels of monoclonal IgG1λ proteins was obtained from several patients with multiple myeloma. The IgG1λ were isolated by

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[1] Abbreviations used: IgM, IgG, IgA, immunoglobulins M, G, and A; L, light chain; H, heavy chain; CD, circular dichroism; Do, patient from whose serum the noncryoprecipitating IgG was isolated; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

ammonium sulfate precipitation followed by ion-exchange chromatography as described previously (Ellerson et al., 1976). The IgG was reduced at 10 mg/ml with 10 mM dithioerythritol (Sigma) in a 0.1 M Tris-HCl buffer, pH 8.6, under nitrogen for 1 h at room temperature and was alkylated with 24 mM iodoacetamide (Sigma, twice crystallized) or iodoacetic acid (Sigma) or acrylonitrile (BDH). In some experiments radioalkylation was achieved using [^{14}C]iodoacetamide (Amersham/Searle, 57 mCi/mmol) or [^{3}H]iodoacetic acid (Amersham/Searle, 70 mCi/mmol). λ chains were separated from H chains by gel filtration on a column of Sephadex G-100 equilibrated in 1 M acetic acid containing 0.025 M NaCl. The eluted L chains were dialyzed first against distilled water and then against 4 mM acetate buffer, pH 5.2. They were then concentrated in 8/32 Visking tubing under negative pressure at 4 °C or in an Amicon pressure concentration cell using a UM 10 membrane. In order to prepare the covalent dimer of Do L chain, Do IgG was reduced under the same conditions as described above but reduction was stopped by lowering the pH to 2.5 by dropwise addition of a 4 M propionic acid solution. The sample was then immediately applied to a Sephadex G-100 column equilibrated in 1 M propionic acid. The reduced L chains were pooled and allowed to reoxidize during dialysis first against two changes of distilled water for 3 h each, then for 6 h against 4 mM acetate buffer, pH 5.4, and finally for 6 h against 10 mM Tris-HCl, pH 8.0. After oxidation, covalent dimers were separated from residual reduced chains by gel filtration on a Sephadex G-100 column equilibrated in 1 M acetic acid. The purified covalent dimers were dialyzed against distilled water for 6 h and then against 4 mM acetate buffer, pH 5.2, for 6 h prior to being concentrated.

Studies of the Physiochemical Parameters Influencing the Cryoprecipitability of Do λ Chain. Influence of Temperature. The temperature at which cryoprecipitation occurs has been determined for a given concentration of the L chain as the temperature at which turbidity of the solution or a light scattering at 320 nm was noticed. It can also be defined more accurately as the temperature corresponding to the midpoint of the thermal transition observed in the spectroscopic studies described below.

Influence of pH and Ionic Strength. To 0.5-ml aliquots of an L chain solution at 7.4×10^{-5} M[2] in distilled water were added equal aliquots of acetate or phosphate buffers of constant ionic strength, ranging from pH 3.8 to pH 7.8. Three series of duplicate experiments were performed so that the final ionic strengths were μ = 0.005, 0.05, and 0.1, respectively. After warming to 37 °C for 30 min, the samples were allowed to cryoprecipitate at 4 °C for 18 h. The tubes were then spun at 12 000 rpm for 30 min at 4 °C and the absorbance of each supernatant was read in a spectrophotometer at 280 nm. The quantity of precipitated light chain was evaluated from the difference in absorbance of the solution before and after cryoprecipitation. The pH of each supernatant was checked at 4 °C, using a Radiometer pH meter.

Influence of Time and Concentration. One-milliliter aliquots of a light-chain solution at varying concentrations in 4 mM acetate buffer, pH 5.2, were allowed to precipitate at 4 °C for varying times (10 min, 30 min, 2 h, and 18 h). The quantity of precipitated light chain was evaluated as described above.

Proteolytic Cleavage of Do L Chain. Peptic digestion proceeded as follows: The L chain at 10 mg/ml in 25 mM acetate buffer, pH 4.5, was digested at 37 °C for 1 h at an enzyme-to-substrate ratio of 1/150 (w/w). Digestion was stopped by raising the pH to 8.2 by dropwise addition of a 2 M Tris solution. The digest became clear and free of precipitate. The fragments were fractionated on a DEAE column (Whatman DE 52) equilibrated with 5 mM Tris-HCl buffer, pH 8.0, using a linear salt gradient (0 to 0.2 M NaCl). The peptic C_λ fragment was recycled on a Sephadex G-75 column in 150 mM NaCl–10 mM Tris-HCl buffer, pH 8.0, and dialyzed against 4 mM acetate buffer, pH 5.2.

Insolubilized trypsin was prepared by coupling trypsin (Worthington, treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone) directly to the Agarose matrix by periodination and subsequent reduction with sodium borohydride in the presence of benzamidine (Royer et al., 1975). Approximately 2 mg of trypsin was coupled per ml of packed Sepharose. Tryptic digestion was performed batchwise at pH 8.0 at room temperature, using 0.5 g of packed trypsin–Sepharose per ml of L chain solution at 10 mg/ml. After 60 s, the liquid phase was removed by filtration through a Nalgene filter unit (Falcon Plastics, Oxnard, Calif). The filtrate was fractionated on a G-100 column equilibrated in 1 M acetic acid. The protein eluted at the V_e/V_t corresponding to halves of L chain was dialyzed in Dialya-Por tubing (National Scientific, Cleveland, Ohio) against 5 mM Tris-HCl buffer, pH 8, and further purification was achieved by ion-exchange chromatography as previously described.

Analytical Ultracentrifugation. Sedimentation coefficients were measured in a Beckman Model E analytical ultracentrifuge operated at 60 000 rpm with a titanium rotor at 30 °C, in order to prevent cryoprecipitation of Do light chain which occurs at 20 °C at high concentrations. For this series of experiments, the solvent was 50 mM NaCl–4 mM acetate buffer, pH 5.2. Sedimentation was followed using Schlieren optics at protein concentration above 2 mg/ml and with the photoelectric scanning system set at 280 nm for concentrations below 2 mg/ml. Sedimentation coefficients were calculated in the usual way (Chervenka, 1970). In certain cases, sedimentation velocity experiments were performed at 4 °C, using varying concentrations of Do L chain in 4 mM acetate buffer, pH 5.2, or in 0.1 M NaCl–4 mM acetate buffer, pH 5.2. The rotor containing the sample was equilibrated at 4 °C for at least 18 h prior to the run.

Difference Spectroscopy. Difference spectra were obtained on a Cary 118 ratio-recording double-beam spectrophotometer (Varian Inst) equipped with jacketted cell holders using full scale settings of 0.02 and 0.05 absorbancy unit. The temperatures of the reference and the sample cells were controlled independently through the use of separate circulating water baths and continuously monitored with calibrated thermistors. Equal volumes of centrifuged and filtered (Millipore filter, 0.45 μm) solutions of L chains at varying concentrations in 4 mM acetate buffer, pH 5.2, or in 0.1 M NaCl–4 mM acetate buffer, pH 5.2, were placed in matched 1.0-cm rectangular quartz cuvettes (Hellma). The cell compartment was flushed with nitrogen throughout the experiment in the presence of anhydrous calcium sulfate to prevent condensation. A baseline was established between 320 and 250 nm with both cuvettes held at 26 °C and subtracted from all subsequent spectra. The temperature of the reference compartment was held constant at 26 °C. The temperature in the experimental cuvette was lowered progressively to −9 °C and then brought back to 26 °C. Difference spectra were recorded in duplicate at each temperature. The total time required for each complete experiment was less than 12 h. The temperature and the corre-

[2] All molar concentrations of L chain are given as moles of monomer per liter.

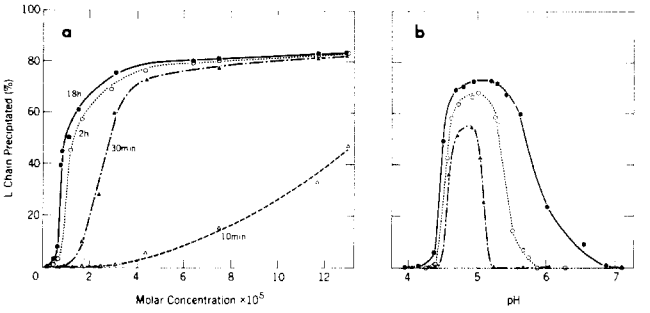

FIGURE 1: (a) Influence of molar protein concentration (expressed as moles of monomer/liter) and time on the cryoprecipitation of Do λ chain at 4 °C. The buffer was 4 mM acetate, pH 5.2. (b) Effects of pH and ionic strength on the cryoprecipitation of Do L chain at 4 °C. The initial protein concentration was 3.7×10^{-5} mol of monomer/l. and $\mu = 0.005$ (●), 0.05 (○), or 0.1 (▲).

sponding change in absorbance were recorded until evidence of light scattering was observed at 320 nm.

Circular Dichroism. CD spectra were obtained with an ORD/CD 15 spectropolarimeter (Durrum/Japan Spectroscopic Co.), equipped with a SS-20 CD modification (Sproul Scientific Inst.) and a thermoregulated cell holder. The performance characteristics of this instrument have been described previously (Dorrington and Smith, 1972). Between 320 and 250 nm, protein concentrations of 1.0 to 1.4 mg/ml in cells of 1.00-cm pathlength were used. Below 250 nm, the protein concentrations were between 0.1 and 0.2 mg/ml in cells of 0.10-cm pathlength, except in one experiment where the λ chain was at a concentration of 0.02 mg/ml in a 1.00-cm cuvette. The temperature in the cuvette was decreased under conditions previously described and was monitored with a thermistor placed in the cell neck. Following temperature equilibration spectra were accumulated using a time constant of 16 s and a scanning speed of 3.8 nm/min. The results are presented as molar ellipticity (deg cm^2 dmol^{-1}) at each wavelength, using the following equation:

$$[\theta]_\lambda = \frac{100\theta}{cl} \qquad (1)$$

where c is the molar protein concentration, l the pathlength in cm, and θ is the observed ellipticity in degrees. The protein solutions were in 4 mM acetate buffer, pH 5.2, and were filtered through cellulose ester membranes (Millipore, pore size 0.45 μm) prior to spectral analysis. No Lorentz corrections for the dispersion of the solvent refractive index have been made.

Protein concentrations were determined spectrophotometrically using a value of $E_{1cm}^{1\%} = 12.5$ at 280 nm for all λ chains and 14.6 for the Do C_λ fragment (Karlsson et al., 1972).

Electrophoresis and Immunological Techniques. The purity of the L chains and their fragments was assessed by cellulose acetate electrophoresis at pH 8.6, immunoelectrophoresis, and sodium dodecyl sulfate–polyacrylamide electrophoresis in 12% gels containing 0.1% sodium dodecyl sulfate (Maizel, 1971). For molecular weight determinations all samples were boiled in 2% sodium dodecyl sulfate at pH 8.0 for 2 min, then reduced with 40 mM dithioerythritol for 30 min at 37 °C, and alkylated with 88 mM iodoacetamide. A standard curve was obtained by plotting log molecular weight against the electrophoretic mobility of proteins of known molecular weight. Gels were stained overnight with Coomassie brilliant blue or Amido black. Antisera against Do λ chain were prepared by injecting rabbits subcutaneously with 1 mg of proteins emulsified in Freund's complete adjuvant. The rabbits were boosted with 0.5 mg of protein every week for 6 weeks. Antisera against the variable region were obtained by adsorbing the homologous antisera with heterologous λ chains. Immunodiffusion analyses were performed in 1.5% agar in 0.15 M NaCl. The isoelectric points of Do L chain and its covalent dimer were determined by thin-layer gel electrofocusing in polyacrylamide gels containing carrier ampholytes covering the range pH 4 to 7 at 20 °C using an LKB 2117 Multiphor apparatus.

Results

Factors Affecting Cryoprecipitation. Preliminary experiments indicated that the overall structure of Do L chain was typical of λ chains in general. The protein was antigenically normal when compared with several other λ chains using three different rabbit antisera against heterologous λ chains. The molecular weight as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was 22 500. The number of half-cystines, estimated as cysteic acid on amino acid analysis, was found to be five. Radioalkylation experiments showed that only the carboxy-terminal cysteine was labeled following reduction of the parent IgG, indicating that the two intrachain disulfide bonds were intact. The L chain did not contain any carbohydrate as judged by amino acid analysis and by the anthrone test.

The isolated Do L chain, renatured in 4 mM acetate buffer, pH 5.2, precipitated upon cooling and redissolved upon warming. This behavior could be modified by varying the protein concentration, temperature, pH, ionic strength, and time. As the protein concentration was increased, the higher was the temperature at which precipitation occurred: e.g., 4 °C at 8×10^{-6} M and 20 °C at 1.2×10^{-4} M. At 4 °C, pH 5.2, and $\mu = 0.004$, the amount of protein precipitated was a function of time and initial protein concentration (Figure 1a). After a given time at 4 °C, the amount of precipitate increased as a function of total protein concentration, although some L chain remained in solution even at an initial concentration of 2×10^{-4} M. Below 2×10^{-6} M no precipitate could be detected after 18 h at 4 °C. The influence of pH and ionic strength was determined at a protein concentration (3.7×10^{-5} M) similar to that used in the spectroscopic studies. The amounts of L chain precipitated between pH 4 and 7 at three different ionic strengths (0.005, 0.05, 0.1) are shown in Figure 1b. For each ionic strength there is an optimal pH, near to the isoelectric point (pI = 5.4), at which the amount of precipitate was maximal. An increase in ionic strength decreased the amount of protein precipitated at the optimal pH, shifted this pH to slightly lower values (pH 5.2 at $\mu = 0.005$ and pH 4.9 at 0.1), and resulted in a narrowing of the pH range over which precipitation occurred. No precipitation was observed below pH 4.2 or above pH 7.0, and at pH 5.2 it was possible to choose a low ionic strength (0.005) at which precipitation occurred and a high ionic strength (0.1) at which it did not.

A fragment corresponding to the C_λ region of Do L chain was prepared in high yield by both tryptic and peptic cleavage at 37 °C. Although this fragment was relatively resistant to further proteolysis, it proved impossible to prepare a V_λ fragment. The C_λ fragments produced with the two enzymes had a normal β_2 electrophoretic mobility and were identical by immunochemical analysis. With an antiserum against a heterologous λ chain, the fragments gave a reaction of identity with intact Do λ chain and normal λ chains. A reaction of partial identity with Do λ chain was observed using a homologous antiserum, indicating that the C_λ fragments were antigenically deficient compared with the parent protein. Both

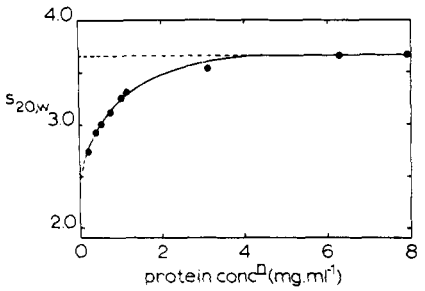

FIGURE 2: Sedimentation coefficient of Do λ chain as a function of initial protein concentration.

fragments had a molecular weight near 11 500 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Neither of these fragments cryoprecipitated under various conditions even at concentrations 100-fold higher than the minimum concentration at which the intact L chain precipitated.

Ultracentrifuge Studies. When Do L chain at 7 mg ml^{-1} was subjected to sedimentation velocity at 20 °C, three Schlieren peaks were observed with apparent sedimentation rates of 3.3, 6.0, and 9.0 S. These values were consistent with the formation of discrete polymeric species as intermediates in the cryoprecipitation process: probably a dimer, a hexamer, and a dodecamer, respectively. In order to avoid this polymerization, experiments were performed at 30 °C. At this temperature, single symmetrical sedimenting boundaries were seen. The concentration dependence of $s_{20,w}$ for Do L chain is shown in Figure 2. The increase of $s_{20,w}$ to a plateau value as the total protein concentration was raised was typical of a self-associating system in this case involving a monomer-dimer equilibrium. The sedimentation coefficient of the monomer, $s_M°$, determined by extrapolating the weight-average sedimentation coefficient, $\bar{s}$, at low concentrations to infinite dilution, was 2.4 S. The coefficient for the dimer, $s_M°$, estimated by extrapolating the linear portion of the curve obtained at high protein concentrations was 3.65 S. Assuming that s_M and s_D are independent of concentration, then the equilibrium constant governing dimerization, K_A, can be estimated from the following equations (Nichol et al., 1964):

$$\bar{s} = (1 - \alpha)s_D + \alpha s_M \quad (2)$$

$$K_A = \frac{(1 - \alpha)M}{2\alpha^2 C} \quad (3)$$

where $\bar{s}$ is the second moment sedimentation coefficient; α, the weight fraction of monomer; $1 - \alpha$, the weight fraction of dimer; M, the molecular weight of the monomer; and C, the total protein concentration in g l.$^{-1}$. K_A was calculated to be in the range 10^4 to 10^5 M^{-1} at pH 5.2. Values of K_A calculated by eq 3 varied with concentration and extrapolated to 3×10^4 M^{-1} at infinite dilution. From these data, the concentration of monomer, C_M, can be evaluated at any concentration of L chain according to the relationship

$$C = C_M + K_A C_M^2 \quad (4)$$

where C is the total concentration.

Do L chain in 0.1 M NaCl-4 mM acetate buffer, pH 5.2, did not precipitate when kept at 4 °C for up to a week. Only a single peak corresponding to an equilibrium mixture of monomer and dimer was observed by sedimentation velocity in this solvent at 4 °C at concentrations between 9×10^{-6} and 9×10^{-5} M.

Absorption Difference Spectra. When the temperature of a Do L chain solution was lowered relative to a reference solution held at 26 °C, a complex difference spectrum was observed between 320 and 250 nm. The intensity of the spectrum increased as the temperature was progressively lowered (Figure 3). Difference peaks were seen at 305, 298, 292, 288, 286, 274, and 266 nm. Since the absorption properties of chromophores are influenced by temperature-induced changes in solvent polarizability (Donovan, 1973), it was necessary to follow changes in molar extinction coefficient, $\Delta\epsilon$, at several wavelengths to detect a conformational transition in the presence of the nonspecific solvent effects. Figure 4 shows the variation

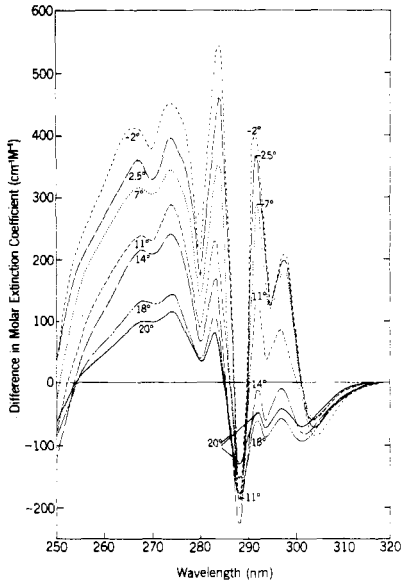

FIGURE 3: Absorption difference spectra generated, between 250 and 320 nm, when a solution of Do λ chain was cooled to the temperatures indicated relative to a reference sample maintained at 26 °C. The protein concentration was 3.48×10^{-5} mol of monomer/l. in 0.1 M NaCl-4 mM acetate buffer, pH 5.2.

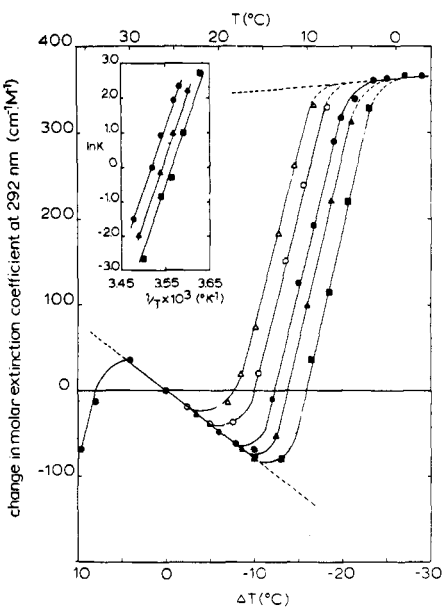

FIGURE 4: Change in molar extinction coefficient at 292 nm as a function of temperature for different concentrations of Do λ chain. Protein concentrations were 6×10^{-5} (△), 4×10^{-5} (○), 3.48×10^{-5} (●), 2×10^{-5} (▲), and 1.05×10^{-5} (■) mol of monomer/l. Buffer was 4 mM acetate, pH 5.2, except for 3.48×10^{-5} M which also contained 0.1 M NaCl. Insert shows the linear dependence of ln K on the reciprocal of the absolute temperature for three of the concentrations. For further details, see Results.

TABLE I: Thermodynamic Parameters for the Low-Temperature Transition of Do L Chain Obtained by Difference Spectroscopy.

Molar Protein Concn ($\times 10^5$)[a]	Temp (K)	K_{app}[b]	ΔG[c] (cal)	ΔS (eu)	ΔH (kcal mol^{-1})
3.48	289	0.029	2039	−281	
	287	0.233	834	−280	
	284	0.967	19	−280	79.5
	282.5	1.988	−386	−280	
	280	6.938	−1079	−280	
	279	11.33	−1347	−280	
2.04	286.5	0.114	1237	−282	
	283	0.889	66	−281	72.4
	280.5	2.706	−555	−281	
	278	10.43	−1298	−281	
1.05	286	0.057	1625	−283	
	282.5	0.532	354	−283	
	280.5	0.729	177	−284	78.2
	278.5	2.783	−566	−283	
	276	16.00	−1519	−282	

[a] Calculated using the molecular weight of the monomer [b] Equilibrium constant governing reaction: $L_N \rightleftharpoons L_T$. [c] Free energy change calculated from relationship: $\Delta G - RT \ln K_{app}$.

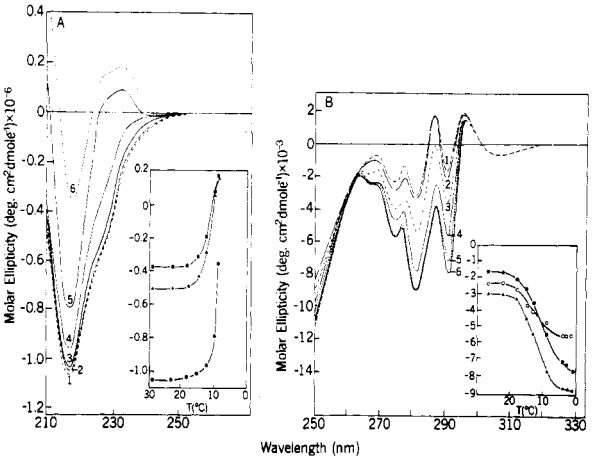

FIGURE 5: Effect of temperature on the circular dichroism of Do λ chain in 4 mM acetate (pH 5.2) (A) between 210 and 250 nm and (B) between 250 and 320 nm. Spectrum 1, 26 to 22 °C; 2, 18 °C; 3, 12 °C; 4, 9 °C; 5, 6 °C; and 6, 1.75 °C. Insert in A shows changes in molar ellipticities at 217 (●), 228 (▲), and 232 nm (■) as a function of temperature. Insert in B shows the effect of temperature on the ellipticities at 275 (○), 282 (▲), and 291 nm (●).

in $\Delta\epsilon$ at 292 nm as a function of temperature. Qualitatively similar data were obtained by following $\Delta\epsilon$ at either 297 or 286 nm. At small temperature differences $\Delta\epsilon_{292}$ varies as a linear function of temperature due mainly to solvent effects. As the temperature is lowered further, $\Delta\epsilon_{292}$ shows a steep sigmoidal change suggestive of a cooperative structural change. The temperature at which this change occurred was a function of protein concentration: the higher the concentration, the higher the temperature at which the thermal transition took place (Figure 4). At low ionic strength, the whole of the transition could not be followed because of precipitation in the cuvette. Difference spectra were therefore recorded in the presence of 0.1 M NaCl which was known to inhibit cryoprecipitation (see above). A transition, similar to those observed in the absence of salt, was seen and the complete change in extinction could be measured by decreasing the temperature to −2.5 °C. The low-temperature side of the transition was characterized by a linear dependence of $\Delta\epsilon_{292}$ upon T. These data indicated that Do L chain existed in two conformers: L_N at relatively high temperatures and L_T at low temperatures. The reversibility of the equilibrium, $L_N \rightleftharpoons L_T$, was shown by raising the temperature from −2.5 to 26 °C; the difference spectrum disappeared. Control experiments with two noncryoprecipitating λ chains over the same temperature range gave $\Delta\epsilon$ values which were a linear function of T at all wavelengths. Interestingly, a disulfide-linked dimer of Do L chain also failed to show a thermal transition.

The apparent equilibrium constant, K_{app}, for the reaction $L_N \rightleftharpoons L_T$ can be determined from each transition curve according to the relationship:

$$K_{app} = \frac{[L_T]}{[L_N]} = \frac{\Delta\epsilon - \Delta\epsilon_N}{\Delta\epsilon_T - \Delta\epsilon} \quad (5)$$

The values of K_{app} increased as T decreased and ΔG consequently became more negative (Table I). The insert in Figure 4 shows that $\ln K_{app}$ was a linear function of the reciprocal of the absolute temperature. The enthalpy change, ΔH, obtained from the slope of these lines was −79.5 kcal mol^{-1} and independent of protein concentration over the range studied. The entropy change was also constant at −280 eu (Table I).

Circular Dichroism. When a solution of Do L chain was cooled progressively from 26 to 0 °C, dramatic changes in the CD spectrum were observed between 310 and 200 nm (Figure 5). At 26 °C the CD spectrum above 250 nm, which includes contributions from aromatic chromophores and cystine, was typical of human λ chains (Kincaid and Jirgensons, 1972). This region showed a weak band centered near 305 nm and two well-resolved bands at 291 and 282 nm. Upon cooling the optical activity became more intense and the negative bands above 263 nm (an isobestic point) were better resolved. No new CD bands were seen. When the changes in molar ellipticity at 291, 282, and 275 nm were plotted as a function of T, a series of sigmoidal curves was obtained (Figure 5). Below 250 nm the optical activity arises primarily from the peptide chromophore, although contributions from the far-ultraviolet transitions of aromatic groups cannot be ignored. In this region Do L chain, in common with other L chains (Kincaid and Jirgensons, 1972), shows a principal band at 217 nm and shoulder near 230 nm. As the temperature was lowered, the ellipticity at 230 nm became progressively less negative and eventually a positive band was apparent at this wavelength (Figure 5). These changes were accompanied by a progressive loss of ellipticity at 217 nm. As seen at the longer wavelengths, the changes at 217, 228, and 230 nm were not a linear function of temperature. When these experiments were repeated with the L chain at a concentration (0.02 mg ml^{-1}) where 95% of the protein was in the form of the monomer, no significant changes were observed as the temperature was lowered (Figure 6). The CD changes did not result from aggregation upon cooling since identical effects were observed in the presence of 0.1 M NaCl. This concentration of salt did not significantly affect the CD spectrum of Do L chain at 26 °C.

The CD changes above 250 nm (Figure 5) were used to calculate K_{app} and the thermodynamic parameters for the transition as described for the difference spectral data (eq 5). The values of ΔH and ΔS were found to be −74.5 kcal mol^{-1} and −260 eu, respectively, in good agreement with those obtained spectrophotometrically. Further evidence that both spectral techniques were following the same conformational transition was obtained by plotting the fractional change in either $\Delta\epsilon_{292}$ or $[\theta]_{291}$ as a function of T (Figure 7). At similar

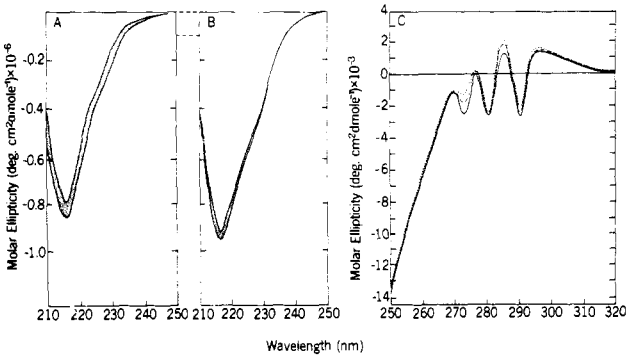

FIGURE 6: Lack of any significant effect of lowering the temperature from 26 to 2 °C on the circular dichroism of (A) Dori λ chain, between 210 and 250 nm, at a concentration of 0.02 mg ml^{-1}, where 95% of the protein was in the monomeric form; (B) covalent dimer of Do λ chain between 210 and 250 nm; (C) covalent dimer of Do λ chain between 250 and 320 nm. The buffer was 4 mM acetate, pH 5.2. In A and B the spectra obtained at the various temperatures between 26 and 2 °C lie within the shaded area.

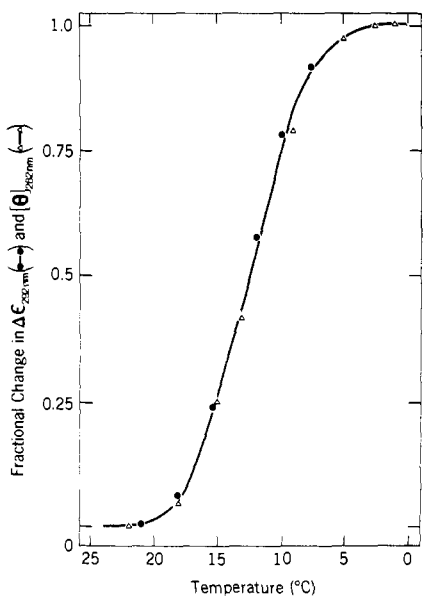

FIGURE 7: Fractional change in molar extinction coefficient at 292 nm and molar ellipticity at 282 nm plotted as a function of temperature for the same concentration of Do λ chain (1.05 × 10^{-5} mol of monomer/l.) in 4 mM acetate buffer, pH 5.2.

concentrations of L chain, the two sets of data are superimposable.

The covalent dimer of Do L chain showed none of the cooperative CD changes exhibited by the reduced and alkylated protein (Figure 6). The CD spectra of the two forms were similar but the changes observed with the covalent dimer upon lowering the temperature were all linear. Similarly, no evidence for a conformational change could be obtained with the peptic or tryptic C_λ fragment.

Control CD experiments with three noncryoprecipitating λ chains showed small linear changes in the ellipticity associated with aromatic transitions as the temperature was lowered but no significant alterations in optical activity below 250 nm.

Discussion

The data presented above have been synthesized to give a model describing the behavior of Do L chain at low temperatures. The features of this model, shown in Figure 8, are as follows: (i) the L chain can exist either as a monomer (L) or

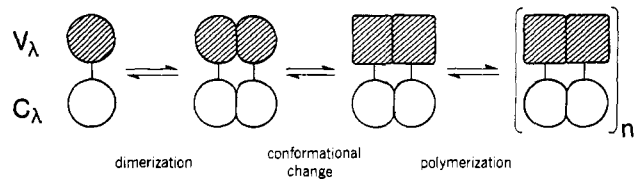

FIGURE 8: Schematic model showing putative behavior of Do λ chain at low temperatures. The conformational change is shown restricted to the variable regions but the evidence for this is not conclusive. For further details, see Discussion.

a dimer (D_N), the relative proportions being related to the total protein concentration; (ii) the dimer, but not the monomer, can undergo a reversible conformational change as the temperature is lowered, $D_N \rightleftharpoons D_T$; (iii) the low-temperature conformer, D_T, can precipitate reversibly. This process probably involves well-defined polymeric intermediates; (iv) polymerization, but not the conformational change, is inhibited at high ionic strength. The evidence for this model will be summarized below.

The sedimentation velocity data clearly showed that Do L chain exists as a monomer–dimer system, $L + L \rightleftharpoons D$, with an association constant between 10^4 and 10^5 M^{-1}. Do L chain is not unique in this respect since other human L chains have been shown to self-associate in this way (Stevenson and Dorrington, 1970; Green, 1973). The K_A governing the dimerization of a human κ chain at 25 °C and pH 5.1 has been estimated from the data of Green (1973) to be 2.5 × 10^4 M^{-1} which is entirely consistent with our own value. The estimated value of 3.65 S for the sedimentation coefficient of Do L dimer agrees closely with the theoretical value calculated from the coefficient of the monomer using eq 6:

$$s_D^\circ = s_M^\circ n^{2/3} f_M/f_D \qquad (6)$$

For our system, the number of protomers, n, is 2 and the ratio of the frictional coefficients for the monomer and dimer, f_M/f_D, is 0.97 (Cathou and Dorrington, 1975). The theoretical value for s_D° is therefore 3.65 (i.e., 2.35 × 1.587 × 0.97). The sedimentation coefficients of the Do monomer and dimer are essentially the same as those obtained for a normal λ chain, i.e., 2.3 and 3.6 S, respectively (Karlsson et al., 1972). These findings imply that the hydrodynamic properties of the Do L chain under noncryoprecipitating conditions are very similar to those of its normal counterparts.

Evidence for a low-temperature-induced conformational change came from the difference spectral and CD studies. Although noncryoprecipitating λ chains as well as Do L chain showed absorption changes as the temperature was lowered, those of the former were a linear function of T and can be ascribed to changes in solvent polarizability. The changes observed for Do L chain were initially linear but showed a sharp sigmoidal change as the temperature was lowered below a critical point. The temperature of the midpoint of the transition, T_m, decreased as the total protein concentration was lowered (discussed below). The positive changes in $\Delta\epsilon$ ("red shift") suggest that aromatic chromophores are being transferred to a less polar environment as a result of the conformational change (Donovan, 1969). The difference peak at 292 nm can be assigned to the 0-0 ^1L_b electronic transition of indole, and the magnitude of the total change in $\Delta\epsilon_{292nm}$ corresponds to the burial of 0.3 tryptophanyl side chain per monomer (Donovan, 1969).[3] The positive peak at 298 nm may arise

[3] The fractional change implies that the perturbation involves only a portion of the chromophore or that the change in environment is only 0.3 times as effective as the transfer from water to the protein interior.

from charge perturbation of the indole group involving either an increase in negative or decrease in positive charge density (Ananthanarayanan and Bigelow, 1969a,b). Changes in electrostatic environment may also account for the absorption differences at 305 nm (Lehrer and Barker, 1973). The difference peaks observed with Do L chain at lower wavelengths (283, 275, and 265 nm) could have arisen by the perturbation of tyrosine, phenylalanine as well as tryptophan transitions.

Temperature-dependent cooperative changes in the environment of aromatic chromophores were also apparent in the CD spectrum of Do L chain above 250 nm. As in the difference spectra, normal L chains showed changes in ellipticities which were a linear function of temperature and probably reflected solvent effects on the transitions.[4] The complexity of the CD changes seen with Do L chain suggests that the conformational transition involves a wide range of aromatic transitions (and perhaps cystine) and reflected alterations in the tertiary folding of the molecule. The CD changes below 250 nm probably also correlated with changes in aromatic chromophore environment. The 217-nm band is thought to be contributed by peptide chromophores in a β conformation (Cathou and Dorrington, 1975) and large changes were observed in this region upon cooling. However, these changes may have occurred passively due to the appearance of one or more new transitions exhibiting positive ellipticity in the 225- to 235-nm region. The wavelength position of these latter bands is probably too high to be attributed to the peptide group. Aromatic chromophores are known to have transitions in this region. On the basis of thermodynamic considerations discussed below, we feel that cooling does not induce a gross conformational change in Do L chain and consequently does not involve large changes in secondary structure.

The conformational changes detected by the optical techniques were completely reversible and did not arise because of aggregation. Similar changes were seen at ionic strengths at which polymerization was completely abolished as judged by sedimentation velocity.

One of the interesting features of the behavior of Do L chain was the apparent obligatory involvement of the noncovalent dimer in the cryoprecipitation. The experimental evidence for this involvement was as follows: (1) the variation in the temperature of the midpoint of the thermal transition with total protein concentration as measured by difference spectroscopy; (2) the absence of far-ultraviolet CD changes under conditions where Do L chain was predominantly in the form of monomer; (3) no precipitate formed, at the lowest experimentally attainable temperatures, when the L chain concentration was low.

The following model system incorporates the features discussed above. The model consists of three protein species, L, D_N, and D_T, interrelated as follows:

$$2L \overset{K_A}{\rightleftharpoons} D_N \overset{K_T}{\rightleftharpoons} D_T$$

The equations governing the molar concentrations of the species are:

$$\frac{[D_N]}{[L]^2} = K_A \tag{7}$$

$$\frac{[D_T]}{[D_N]} = K_T \tag{8}$$

[4] These changes could also reflect an increased rigidity of the molecules at low temperatures leading to a sharpening of the CD bands (Moscowitz et al., 1963).

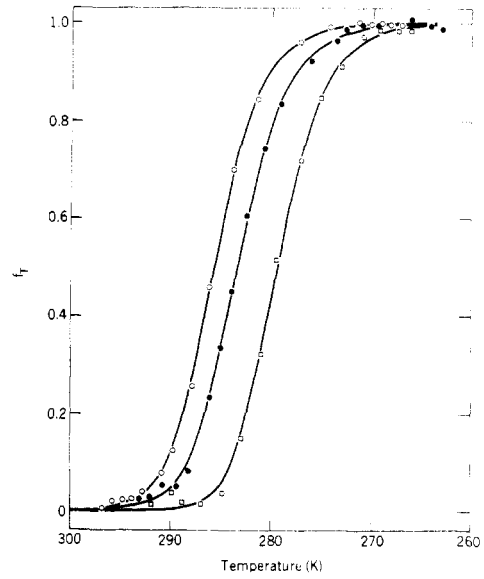

FIGURE 9: Comparison of the observed and calculated values of f_T as a function of temperature for Do λ chain illustrating that the behavior of the experimental system is well described by the model (see Discussion). Data points obtained by difference spectroscopy are shown for three concentrations of λ chain: 2.29×10^{-5} (○), 5.74×10^{-5} (●), and 7.22×10^{-5} (□) mol of monomer/l. of 0.1 M NaCl–4 mM acetate buffer, pH 5.2. The curves represent computer-calculated values of f_T using eq 10 and values for the parameters given in Table II.

$$[L] + 2[D_N] + 2[D_T] = [P] \tag{9}$$

where [P] is the total protein concentration expressed as moles of monomer per liter. When these equations are solved for $[D_T]$, the result is:

$$f_T = 2[D_T]/[P]$$

$$= \left\{\frac{1 + 4(1 + K_T)K[P] - \sqrt{1 + 8(1 + K_T)K[P]}}{4(1 + K_T)K_A[P]}\right\} \times \left\{\frac{K_T}{1 + K_T}\right\} \tag{10}$$

The quantity f_T may be measured experimentally using eq 5, written in the form

$$f_T = (\Delta\epsilon - \Delta\epsilon_N)/(\Delta\epsilon_T - \Delta\epsilon_N) \tag{5a}$$

While we have considered the general case in which both K_T and K_A are functions of temperature, a simpler model in which only K_T is temperature dependent seems to fit the experimental data reasonably well. In this model, it is assumed that

$$(d \ln K_T)/dT = \Delta H/RT^2 \tag{11a}$$

or

$$\ln K_T = \frac{-\Delta H}{RT_c}\left(\frac{T_c}{T} - 1\right) \tag{11b}$$

where ΔH and T_c are constants; when $T = T_c$, $K_T = 1.0$.

Substituting eq 11b and 5a into eq 10 produces a theoretical model for the system which is amenable to experimental verification. Assuming a value of 4×10^4 M^{-1} for K_A and utilizing experimental values for $\Delta\epsilon$ and T, it was possible to estimate ΔH, T_c, $\Delta\epsilon_N$, and $\Delta\epsilon_T$ for three different L chain concentrations. A computerized nonlinear least-squares minimization routine (Gaushaus) was used to estimate these parameters and their standard deviations. The results are summarized in Table II. Figure 9 shows plots of the observed and calculated values of f_T illustrating that the behavior of the

TABLE II: Values for Various Parameters Used in the Model System Estimated Using a Nonlinear Least-Squares Minimization Routine with Experimentally Obtained Values of $\Delta\epsilon$ and T.

Parameter	Total Protein Concn[a] 2.29×10^{-5}	5.74×10^{-5}	7.22×10^{-5}
ΔH (kcal)	-85 ± 10	-76 ± 5	-81 ± 4
T_c (K)	281.2 ± 0.3	284.8 ± 0.2	286.9 ± 0.2
$\Delta\epsilon_N$ slope[b]	5.8 ± 0.8	7.7 ± 1.3	6.3 ± 1.0
$\Delta\epsilon_N$ intercept[b]	-1700 ± 200	-2300 ± 400	-1900 ± 300
$\Delta\epsilon_T$ slope[b]	-7.4 ± 2.2	-0.78 ± 0.8	-4.0 ± 0.5
$\Delta\epsilon_T$ intercept[b]	2300 ± 600	600 ± 200	1400 ± 100

[a] Expressed as moles of monomer per liter. [b] In the calculations it was assumed that $\Delta\epsilon_N$ and $\Delta\epsilon_T$ were linear functions of temperature.

experimental system is well described by the model; the midpoint of the transition is clearly a function of protein concentration.

The formation of the low-temperature conformer of Do L chains was a strongly exothermic reaction. To a first approximation the experimentally determined values of ΔH and ΔS were independent of temperature and total protein concentration. The lack of any marked dependence of ΔH on temperature implies that the heat capacities of D_N and D_T are similar. This in turn suggests either that the observed conformational change is quite limited or that, if the change is substantial, it does not involve large alterations in the disposition of hydrophobic side chains (Tanford, 1968). The sign of both ΔH and ΔS are consistent with the formation of hydrogen bonds as the temperature is lowered. Since the monomer does not undergo this transition, these putative H bonds are probably formed between the two monomers constituting the dimer. However, since one of the consequences of the conformational change is the ability of D_T to cryoprecipitate at low ionic strength, it seems probable that this change results in a redistribution of charged groups on the surface of the dimer thus facilitating association. The sensitivity of the precipitation to pH and ionic strength strongly points to electrostatic interactions between dimers. There are some data which argue against nonspecific isoelectric effects: (1) normal L chains do not precipitate at low temperature at their isoelectric points; (2) the disulfide-linked dimer of Do L chain does not cryoprecipitate even though it has a pI within 0.2 unit of the noncovalent dimer (unpublished observation). The sedimentation velocity experiments performed at 20 °C in the absence of salt indicated that this association involves discrete intermediates, probably trimers and hexamers of D_T; i.e., $2L \rightleftharpoons D_N \rightleftharpoons D_T \rightleftharpoons (D_T)_6 \rightleftharpoons (D_T)_n$.

Unambiguous evaluation of the roles played by V_L and C_L in the cryoprecipitation phenomenon was not possible since it proved impossible to isolate a fragment corresponding to intact V_L because of its extreme sensitivity to trypsin and pepsin. The fragment representing the entire C_L region of Do L chain did not undergo any conformational change at low temperature. Although this suggests that the V region is responsible for the observed change, a cooperative involvement of V_L and C_L cannot be formally excluded. Support for the involvement of V_L comes from other studies in this laboratory on another λ chain cryoglobulin; isolated V_L reversibly precipitates at low temperature. Sequence studies on Do L chain indicate that this protein represents a new V_λ-region subgroup (Klein, Hofmann, and Dorrington, to be published). However, cryoglobulin behavior is not uniquely associated with any particular V-region subgroup or indeed any one isotype of H or L chain.

Several investigators have shown that reduction of interchain disulfide bonds of dimeric Bence-Jones cryoglobulins resulted in a loss of cryoprecipitability (Alper, 1966; Meinke et al., 1974; Brouet et al., 1974). The opposite effect was observed with Do L chain; the covalent dimer neither precipitated nor showed a conformational change at low temperature. However, if the structures responsible for the cryoprecipitation are localized to the V regions, the relevance of disulfide bonds situated at the COOH terminus of the dimer is difficult to understand. Schiffer et al. (1973) have shown by x-ray crystallographic analysis that the two monomers in a covalently linked λ chain dimer do not have the same conformation. This specific quaternary relationship between the monomers may be essential for cryoprecipitation in the proteins sensitive to reduction but inimicable to this process in Do L chain. These data strongly suggest that the behavior of one domain may be modulated by interactions with its neighbors.

Acknowledgments

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Different Metal-Binding Properties of the Two Sites of Human Transferrin†

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ABSTRACT: Transferrin, the serum iron-transport protein which can bind two metal ions at physiologic pH, binds just one Fe^{3+}, VO^{2+}, or Cr^{3+} ion at pH 6.0. Fe^{3+} and VO^{2+} appear to be bound at the same site, designated A, based on electron paramagnetic resonance (EPR) spectra of VO^{2+}-transferrin and $(Fe^{3+})_1(VO^{2+})_1$-transferrin. The EPR spectra of $(Cr^{3+})_1(VO^{2+})_1$-transferrin and of $(Cr^{3+})_1(Fe^{3+})_1$-transferrin indicate that Cr^{3+} is bound to site B at pH 6.0. Transferrin was labeled at site A with ^{59}Fe at pH 6.0 and at site B with ^{55}Fe at pH 7.5. When the pH of the resulting preparation was lowered to 6.3 and the dissociated iron was separated by gel filtration, about ten times as much ^{55}Fe as ^{59}Fe was lost. The same EPR and isotopic-labeling experiments showed that Fe^{3+} added to transferrin at pH 7.5 binds to site A with about 90% selectivity.

Transferrin is the protein responsible for carrying iron in the blood (Aisen and Brown, 1975; Morgan, 1974; Aisen, 1973). Each molecule of weight near 80 000 consists of a single peptide chain containing two metal-binding sites of remarkably similar thermodynamic and spectroscopic properties (Aasa et al., 1963; Aisen et al., 1966; Binford and Foster, 1974; Gafni and Steinberg, 1974). Since the suggestion of Fletcher and Huehns (1967, 1968; Fletcher, 1969) that each site of transferrin may have a different physiologic function, there has been a great deal of interest in any distinguishing properties of the two sites. The interpretation of the EPR[1] spectrum of diferric transferrin has been somewhat controversial (Aasa and Aisen, 1968; Aasa, 1972), but there appear to be two kinds of spectroscopic behavior in the presence of perchlorate (Price and Gibson, 1972). A very clear distinction between the sites is evident in the EPR spectra of the vanadyl (VO^{2+}) (Cannon and Chasteen, 1975) and Cr^{3+} (Aisen et al., 1969; Harris et al., 1975) complexes of transferrin.

It has recently been demonstrated by Princiotto and Zapolski (1975) and by Lestas (1976) that the two iron-binding constants of transferrin have significantly different pH dependences. It was shown that one site loses its iron as the pH is reduced to near six, but the other site retains its iron until the pH is reduced to approximately five.

It was our hope that using the effect of pH upon metal binding, it would be possible to label transferrin at each site with a different radioisotope of iron. Protein so labeled would

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl; EPR, electron paramagnetic resonance.