Oligomerization and conformation change in solutions of calf lens γ_{II}-crystallin

Results from $1/T_1$ nuclear magnetic relaxation dispersion profiles

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ABSTRACT From analyses of the magnetic field dependence of $1/T_1$ (nuclear magnetic relaxation dispersion [NMRD] profiles) of water protons in solutions of highly purified calf lens γ_{II}-crystallin, we find that monomers form oligomers at relatively low concentrations, which increase in size with increasing concentration and decreasing temperature. At ~16% by volume and −4°C, the mean oligomeric molecular weight is ~120-fold greater than the monomeric value of 20 kD. Below this concentration, there is no indication of any substantive change in conformation of the monomeric subunits. At higher concentrations, the tertiary structure of the monomer appears to reconfigure rather abruptly, but reversibly, as evidenced by the appearance of spectra-like ^{14}N peaks in the NMRD profiles. The magnitudes of these peaks, known to arise from cross-relaxation of water protons through access to amide (NH) moieties of the protein backbone, indicate that the high concentration conformation is not compact, but open and extended in a manner that allows enhanced interaction with solvent. The data are analogous to those found for homogenates of calf and chicken lens (Beaulieu, C. F., J. I. Clark, R. D. Brown III, M. Spiller, and S. H. Koenig. 1988. *Magn. Reson. Med.* 8:47–57; Beaulieu, C. F., R. D. Brown III, J. I. Clark, M. Spiller, and S. H. Koenig. 1989. *Magn. Reson. Med.* 10:62–72.). This unusually large dependence of oligomeric size and conformation on concentration in the physiological range is suggested as the mechanism by which osmotic equilibrium is maintained, at minimal metabolic expense, in the presence of large gradients of protein concentration in the lens in vivo (cf Vérétout and Tardieu. 1989. *Eur. Biophys. J.* 17:61–68). Finally, the results of the NMRD data provide a ready explanation of the low temperature phase transition, and "cold-cataract" separation of phases, observed in γ_{II}-crystallin solutions; we suggest that the phases that separate are the two major conformers detected by NMRD.

INTRODUCTION

The large index of refraction of the vertebrate eye lens arises from a high concentration of proteins, called crystallins, in the cytoplasm of specialized fiber cells of the avascular lens tissue. These are cells that lack nuclei, mitochondria, and other organelles that might compromise lens transparency (cf Wistow and Piatigorsky, 1988). The crystallin sizes for a given species range in mammals, for example, from α-crystallins, of high molecular weight, ~800 kD, to β-crystallins, of intermediate size, to the many smaller γ-crystallins, ~20 kD. All vertebrate lenses contain a multiplicity of crystallin types, the genetic expression of which is carefully regulated as the lens develops. The result is a mature lens with a gradient of total protein concentration coupled with species-specific variations in crystallin composition from the lens center to its periphery.

There have been several recent suggestions that crystallin solutions must have unusual osmotic properties that have evolved to minimize the gradients of osmotic pressure that would otherwise accrue from the gradients of protein concentration in the lens (Koenig et al., 1989; Magid et al., 1989; Vérétout and Tardieu, 1989). Such behavior would be reflected in unusual intermolecular association of crystallins at high concentration because, to first order, osmotic pressure is proportional to the number of solute molecules per unit volume of solution. In the present paper, we demonstrate such unusual association in solutions of calf γ_{II}-crystallin, thus extending our earlier work on lens homogenates (Beaulieu et al., 1988, 1989).

Most of the standard experimental techniques that provide information on protein association and aggregation, i.e., protein–protein interactions, are generally of value over a restricted range of protein concentration; by contrast, the technique used here is equally useful over the range 3–30% protein. We measure the magnetic field dependence of the longitudinal magnetic relaxation rate $1/T_1$ of solvent protons, known as a $1/T_1$ nuclear magnetic relaxation dispersion (NMRD) profile. The presence of solute diamagnetic globular protein induces changes in the NMRD profile of solvent water protons from which the rotational relaxation time of solute macromolecules can be inferred (Koenig and Schillinger,

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1969; Hallenga and Koenig, 1976; Koenig and Brown, 1987). When the protein is not compact and globular, but more extended and open (such as can occur with heat denaturation), a spectra-like set of features (^{14}N peaks) is added to the NMRD profile that is known to arise from cross-relaxation between solvent protons and the ^{14}N nuclei of backbone amide (NH) moieties (Winter and Kimmich, 1982*a*, *b*; Koenig et al., 1984; Koenig, 1988).

From measurements of NMRD profiles of homogenates of calf lens nucleus and cortex over a wide range of protein concentration, and more limited data on chicken lens homogenates, we have recently demonstrated (Beaulieu et al., 1988, 1989) that there is an unusual dependence of the state of association of heterogeneous crystallins on concentration and temperature. The crystallins tend to oligomerize, even at relatively low concentrations. At higher concentrations, much of the protein in the homogenate appears to undergo an alteration in tertiary structure: solute macromolecules reorganize into large, extended, and open polymeric configurations that permit access of solvent to backbone NH groups to an extent that may be unique to eye lens proteins.

We now report NMRD studies of solutions of a highly purified crystallin, calf γ_{II} (γB in the uniform nomenclature of Aarts et al. [1988]), as a function of concentration and temperature. This protein, which makes up about one-third of the γ-crystallins in the nuclear region of calf lens, is extremely well characterized: its primary sequence is known (Croft, 1972) and its crystal structure solved to 1.9 Å (Wistow et al., 1983). Moreover, solutions of calf γ_{II}-crystallin, like native calf lens nuclear homogenates, exhibit a reversible first-order phase transition (cf Siezen et al., 1985) at temperatures and concentrations convenient for NMRD studies and which, in our view, also indicates the presence of unusual intermolecular protein interactions in solutions of γ_{II}-crystallin. We find that the association behavior of pure calf γ_{II}-crystallin is analogous to that of samples of (heterogeneous) calf lens homogenates (Beaulieu et al., 1988, 1989); however, because the solute is a homogeneous protein, the results are more readily quantitated.

MATERIALS AND METHODS

Calf γ_{II}-crystallin

Two solutions of γ_{II}-crystallin were furnished by Dr. John A. Thomson, prepared to better than 99% purity by methods previously given (Thomson et al., 1987). The more concentrated sample was 24.4% vol/vol (334 mg/ml); the second sample, diluted with distilled water from an aliquot of the first, was 9.5% vol/vol (130 mg/ml). Samples of intermediate concentration were generated by mixing aliquots of these two in different proportions. A ~3% sample was obtained by diluting part of the 9.5% sample with deionized water. Concentrations were determined optically by Dr. Thomson, using a specific absorption coefficient $A^{0.1\%}_{1cm} = 2.4$ at 280 nm (Thomson et al., 1987). Throughout the text and figures, concentrations of samples that were not measured directly, but derived by known dilutions, are indicated as being approximate.

Measurement and interpretation of NMRD profiles

$1/T_1$ of solvent water protons can be measured at any value of magnetic field over the range 0.01–50 MHz proton Larmor frequency, corresponding to 0.00024–1.2 T, with the automated field-cycling relaxometer developed in our laboratory (Koenig and Brown, 1987). Comparable instrumentation is not available commercially although instrumentation with similar capabilities is available in a limited number of other laboratories.

A sample, in a stoppered test tube, is surrounded by circulating freon, allowing measurements in the range −10–35°C, with the temperature regulated to within ±0.1°C to stabilize the relaxation rates to within the uncertainty of ±0.5% with which they can generally be measured.

Measurements of $1/T_1$ NMRD profiles have proven to be particularly useful for studying aggregation of diamagnetic globular proteins in solution: e.g., oligomerization of sickle hemoglobin in the presence of oxygen (Lindstrom et al., 1976); its gelation in the absence of oxygen (Lindstrom and Koenig, 1974); and, very recently, the monomer-dimer equilibrium of lysozyme (Raeymaekers et al., 1989).

The Cole–Cole fit to the monotonic background

We use the four-parameter Cole–Cole expression (Cole and Cole, 1941) to characterize the monotonic parts of the NMRD profiles, both for the information they contain and to use as a subtractive background so that the structured regions of the profiles can be expanded. Rewritten for $1/T_1$ NMRD data, it becomes (Hallenga and Koenig, 1976)

$$\frac{1}{T_1} = \frac{1}{T_{1w}} + D + A(\nu_c)\,\mathrm{Re}\left(\frac{1}{1 + (i\nu/\nu_c)^{\beta/2}}\right), \qquad (1)$$

where Re means "the real part of," $1/T_{1w}$ is the contribution of solvent, D and A (which is a function of ν_c) are the amplitudes of constant and dispersive contributions to the NMRD profile, and ν is the magnetic field in units of the proton Larmor frequency. The curve has an inflection point (which corresponds to the half amplitude point of the A component) at:

$$\nu/\nu_c = 1, \qquad (2)$$

and β is a parameter that determines the slope of the profile at the inflection.

$1/\nu_c$ is related to τ_R, the rotational relaxation time that enters into the usual theory for relaxation by proton–proton interactions (Bloembergen et al., 1948). For spherical proteins (Koenig and Schillinger, 1969),

$$1/\nu_c \simeq 2\pi\sqrt{3}\tau_R;\ \tau_R = 4\pi\eta r^3/3kT. \qquad (3)$$

Here r is the radius of the hydrated solute protein entities, T is the absolute temperature, and η is the (temperature dependent) microscopic viscosity as experienced by the solute particles. In the dilute limit, $\eta = \eta_0$, the viscosity of the neat solvent, and η/η_0 increases with protein content due to hydrodynamic effects, as discussed by Koenig (1980). The dependence of ν_c on viscosity, temperature, and protein size has been well studied and amply verified; how close the equivalence relation, Eq. 3, is to an equality has not been considered in detail to date. This

point, not germane to the conclusions of the present work, is nonetheless considered below.

A highly simplified model of diamagnetic relaxation (Koenig and Schillinger, 1969; Hallenga and Koenig, 1976) would give $\beta = 2$, resulting in a Lorentzian profile and A linear in $1/\nu_c$. The experimental data generally do not deviate substantially from this model view. For nonassociating protein molecules, ν_c is relatively insensitive to protein concentration below ~10% by volume (Koenig, 1980). We use this criterion to demonstrate oligomerization of γ_{II}-crystallin.

RESULTS

Concentration dependence of the $1/T_1$ NMRD profiles

Fig. 1 *A* shows the $1/T_1$ NMRD profiles of solutions of γ_{II}-crystallin, at 25°C, for a range of protein concentration. $1/T_1$ decreases monotonically with increasing magnetic field for protein concentrations $\lesssim$16% vol/vol. At higher concentrations, a structured region appears between 0.5 and 4 MHz in the otherwise monotonic NMRD profiles. This region is shown expanded (on a linear scale) in Fig. 1 *B*. The rather sudden onset of peaks at higher concentration, centered at 2.1 and 2.9 MHz is much as found earlier for lens homogenates (Beaulieu et al., 1988, 1989). In addition, there is structure on either side of 1 MHz that first becomes apparent in the 20.8% sample. The onset of peaks in the NMRD profile at the higher protein is accompanied by a steep increase in relaxation rates at low fields (Fig. 1 *A*): the ~30% increase in concentration, from ~16 to 20.8% vol/vol, results in an almost threefold increase in low field rates; the next 20% increase produces another twofold increase.

The solid curves (Fig. 1 *A*), for γ_{II} concentrations $\lesssim$ 16% vol/vol, derive from least squares comparisons of the data with the Cole–Cole expression, Eq. 1. The dotted line is the Cole–Cole fit to the data of the ~3% sample, which has a significantly lower ionic strength than the other samples. For concentrations $\gtrsim$ 16% vol/vol, a single Cole–Cole expression is only adequate to describe the monotonic part of the NMRD profiles below 5 MHz, which suffices as a background for examining the peaks. The extensions of these fits to higher fields, Fig. 1 *A*, simply connect the data points and are meant to serve as visual guides. Derived values of ν_c are shown by arrows; a shift of ν_c to lower fields with increasing concentration (and possibly a loss of the inflection at the higher concentrations [*dotted arrows*]) is also clear.

Temperature dependence of the $1/T_1$ NMRD profiles

Fig. 2, *A* and *B*, show $1/T_1$ NMRD profiles of the 9.5 and 24.4% vol/vol samples, respectively, at several temperatures between 35° and −7°C. The solid lines are fits to the

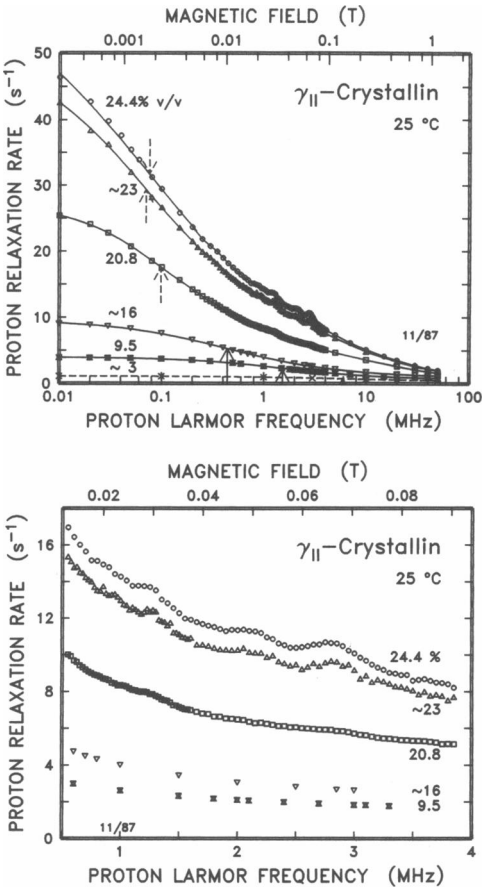

FIGURE 1 (*A*) $1/T_1$ NMRD profiles of γ_{II}-crystallins, at 25°C, for concentrations of: 24.4(○), ~23(△), 20.8(□), ~16(▽), 9.5(▣), and ~3% (×) by volume. The curves through the data points (*solid* for 9.5–24.4% vol/vol, *dotted* for the 3% sample, see Materials), derive from a least squares comparison of the data points, excluding the structured region, with the Cole–Cole expression, Eq. 1. The arrows indicate the derived inflection points of the curves, with ν_c = 5.94, 1.53, and 0.44 MHz, for concentrations, ~3%, 9.5%, and ~16% vol/vol, respectively; dotted arrows indicate ν_c ~0.1 MHz for the 20.8% sample and $\lesssim$0.07 MHz for the two most concentrated samples, where the existence of an inflection depends critically on the accuracy of the data at 0.01 MHz. (*B*) An expansion of the region between 0.5 and 4 MHz, emphasizing the rather sudden onset of peaks in the NMRD profiles of (*A*) with increasing protein concentration. The structure in the region 0.9–1.7 MHz is the first to appear as the protein concentration increases. Pronounced peaks at 2.1 and 2.9 MHz are seen at higher concentrations.

Cole–Cole expression, Eq. 1, as in Fig. 1 *A*, with arrows indicating ν_c of the dispersions in Fig. 2 *A* (where the inflection points are unequivocal). NMRD profiles were obtained as a function of temperature for all the samples of Fig. 1 *A*. All the samples were either cloudy or opaque at −7°C ("cold-cataract" [Siezen et al., 1985]), conditions that were completely reversible. As exemplified by the data of Fig. 2, *A* and *B*, the NMRD profiles appear

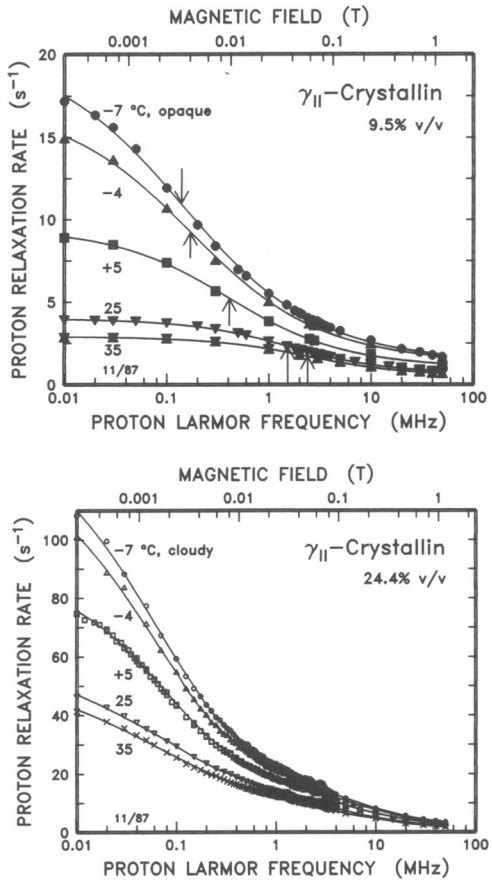

FIGURE 2 (*A*) $1/T_1$ NMRD profiles of γ_{II}-crystallin, 9.5% vol/vol, at 35°(⧗), 25°(▼), 5°(■), −4°(▲), and −7°(●). The solid curves through the data points derive from a least squares fit to the Cole–Cole expression, Eq. 1. The fits give ν_c = 2.40, 1.53, 0.41, 0.17, and 0.14 MHz, respectively, with decreasing temperature (*arrows*). Upon cooling, the sample became opaque near −7°C. (*B*) $1/T_1$ NMRD profiles of γ_{II}-crystallin, 24.4% vol/vol, at 35°(×), 25°(▽), 5°(□), −4°(△), and −7°C (○). The sample became cloudy at −7°C. The solid curves through the data points are fits that allow expansion of the structured region but (see text) are more complex than a single Cole–Cole fit.

unaffected by the transition to the opaque state, indicating that the dynamics of the solvent and solute are not influenced by the spatial density fluctuations, of the order of a wavelength of visible light, that arise from the known phase separation at low temperatures.

ANALYSIS

Low concentrations, below ~16% vol/vol

The value of ν_c, the inflection point of the $1/T_1$ NMRD profiles, depends on protein size, on viscosity, and on temperature, as in Eq. 3. For nonassociating solute protein (or if self-association is independent of temperature, an unlikely occurence for a reversible equilibrium), the slope of $1/\nu_c$ vs. η_0/T on a double logarithmic plot should be unity. Fig. 3 *A* shows the values of $1/\nu_c$ for γ_{II}-crystallin, at several concentrations, plotted against η_0/T. For comparison, and as a reference standard, values for $1/\nu_c$ for 3.4% vol/vol human carbonic anhydrase B (HCAB) are included from Hallenga and Koenig (1976). This protein, of 30 kD dry weight (comparable to γ_{II}-crystallin), is known to remain monomeric as high as 8% vol/vol (Wells et al., 1979); its ν_c at 3.4% vol/vol clearly displays the behavior of a monomeric protein over the

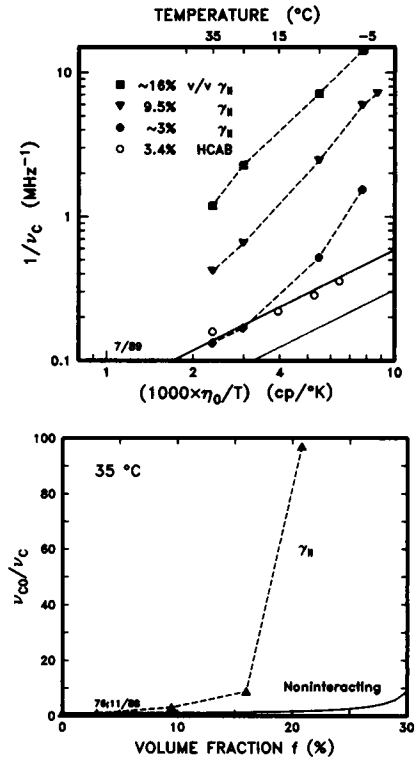

FIGURE 3 (*A*) The variation of $1/\nu_c$ with η_0/T for three concentrations of γ_{II}-crystallin (~16(■), 9.5(▼), and ~3% (●) vol/vol). Similar data are shown for 3.4% vol/vol human carbonic anhydrase B (HCAB) (○) (Hallenga and Koenig, 1976), a noninteracting globular protein used here as a standard. The upper (*heavier*) solid line associated with the data for HCAB is the linear dependence expected from theory (cf Eq. 3). The lower (*lighter*) solid line is the actual prediction of Eq. 3, using a hydrated molecular weight of 33 kD for HCAB (see text). The increase of $1/\nu_c$ of the γ_{II}-crystallins with increasing concentration at fixed temperature, and the greater slopes of the dashed curves compared with the solid line, show oligomerization of γ_{II}-crystallin with increasing concentration and with decreasing temperature. (*B*) The solid curve shows the concentration dependence of $1/\nu_c$, relative to its low concentration value $1/\nu_{c0}$, at 35°C, anticipated for noninteracting globular proteins from purely hydrodynamic effects. The concentration is expressed as volume fraction of the solution. (After Lindstrom et al. [1976] and Koenig [1980]). The set of points (▲) and the dashed curve show results obtained here for γ_{II}-crystallin.

temperature range 0.5–35°C, as indicated by the agreement of the data with the upper (*heavier*) solid line of unity slope. This line was drawn as a fit to the data for HCAB. The lower (*lighter*) solid line is derived from Eq. 3, using the hydrated molecular weight of HCAB (33 kD), and gives an indication of the accuracy of the equivalence relation in a particular case.

By contrast, all the data for γ_{II}-crystallin indicate oligomerization with decreasing temperature at fixed concentration, and with increasing concentration at fixed temperature. Specifically, a small amount of oligomerization is found at the highest temperature for the ~3% sample, sufficient to give the 20 kD γ_{II} solute molecules an average rotational relaxation time equal to that of HCAB, with a molecular weight of 30 kD. For 9.5 and ~16% vol/vol γ_{II}-crystallin, $1/\nu_c$ varies approximately as $(\eta_0/T)^2$, indicating an increase in average oligomeric size that is roughly linear in $1/T$.

This dramatic variation of $1/\nu_c$ with concentration (expressed as volume fraction f) for γ_{II}-crystallin, at 35°C, is demonstrated in Fig. 3 *B*. The (*upper*) dashed line connecting the data points shows the variation of $1/\nu_c$, relative to its value $1/\nu_{c0}$ in the dilute limit, as a function of f. The (*lower*) solid curve shows the variation of $1/\nu_c$ with f expected for noninteracting protein molecules; i.e., considering only hydrodynamic effects. This solid curve represents the predictions of a simple hydrodynamic theory (Koenig, 1980) based on data for hemoglobin (Lindstrom et al., 1976) and is valid for uncharged spherical proteins of any molecular weight. For charged proteins the hydrodynamic theory should be an *overestimate* of the variation of ν_c with f (cf Fig. 3 of Lindstrom and Koenig, 1974).

After correction for the minor hydrodynamic effects, Fig. 3 *B*, one can derive a mean molecular weight for the solute γ_{II} aggregates from the NMRD profiles, assuming that the aggregates are not too aspherical. The distribution of oligomeric sizes, however, cannot be determined. From Fig. 3 *A*, one concludes that as the γ_{II} concentration increases from ~3 to ~16% vol/vol, at fixed temperature, $1/\nu_c$ increases roughly 10-fold, indicating a comparable increase in mean volume of solute particle, Eq. 3. The hydrodynamic corrections are $\lesssim$20%. In addition, for fixed concentration, $1/\nu_c$ of γ_{II}-crystallin increases roughly 10-fold as the temperature is lowered from 35° to −4°C. Thus, the average size of the oligomerized γ_{II}-crystallin of ~16% vol/vol, at −4°C, is ~100 times larger than monomeric 30 kD HCAB, namely ~3,000, or ~2,400 kD after correction for hydrodynamic effects (Fig. 3 *B*). Similarly, from Fig. 3, *A* and *B*, we derive the average oligomeric weight of γ_{II}-crystallin, at 25°C, for the ~3%, 9.5%, and ~16% vol/vol samples in Fig. 1 *A*: ~30 kD, ~100 kD, and ~320 kD respectively.

Despite the extensive association of the γ_{II} molecules discussed above, there is no indication of ^{14}N peaks in the NMRD profiles at these concentrations between 5° and 35°C.

High concentrations, above ~16% vol/vol

The values of ν_c for concentrations of γ_{II}-crystallin above ~16%, at 25°C (*dotted arrows* in Fig. 1 *A*), have a large uncertainty because the existence of any inflection in the monotonic, low field part of the NMRD profiles depends heavily on the precision of the data at 0.01 MHz. Nonetheless, we can assign a lower limit to the average molecular weight of the oligomeric solute molecules: at 20.8% protein, with $\nu_c \lesssim 0.1$ MHz, the mean weight of the aggregates, considered spherical, is $\gtrsim$60 times larger than that of the ~3% sample (average of 30 kD) at 25°C, Fig. 3 *A*, or $\gtrsim$1,800 kD. For the two higher concentrations, which represent at most a 15% increase in volume fraction over the 20.8% sample, ν_c is $\lesssim$0.07 MHz, indicating a ~40% increase of the mean molecular weight of the aggregates compared with the 20.8% sample, to a lower limit of ~2,500 kD.

The magnitudes of the ^{14}N peaks at 25°C, seen only at these higher γ_{II} concentrations, increase as the temperature is lowered, scaling approximately with the monotonic background of the NMRD profiles at 24.4% (cf Fig. 2 *B*). Fig. 4 shows the structured region of the 25° and 5°C profiles of Fig. 2 *B* expanded after subtraction of the monotonic background. For comparison, the peaks

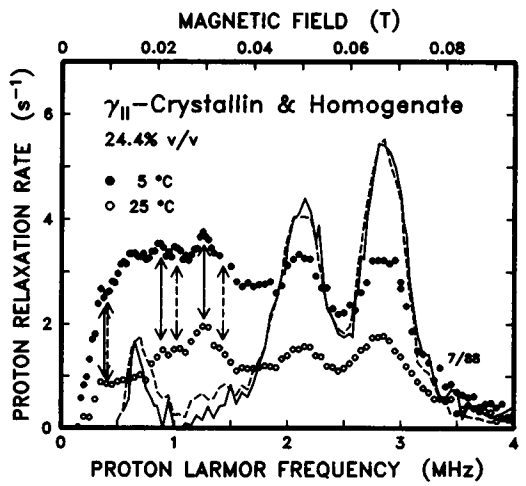

FIGURE 4 The structured region of Fig. 2 *B*, expanded, after subtraction of the monotonic background, at 25°(○) and 5°C(●). Comparable results for native calf lens homogenate (34.4% protein), at 25°(*dashed lines*), and 5°C (*solid lines*) (after Beaulieu et al., 1989) are also shown. The dashed and solid arrows indicate a pair of ^{14}N triplets, which (to date) have only been reported for γ_{II}-crystallin.

observed for native calf lens nuclear homogenate, 34% vol/vol (Beaulieu et al., 1989; Koenig, 1988), are also shown; of note is the absence of any dependence on temperature. The triplet of lines near 0.75, 2.1, and 2.9 MHz (theory shows that the lowest line is at the difference frequency of the upper two [cf Koenig, 1988]), is ostensibly the same in both samples (actually an overlapping of a pair of similar triplets, Beaulieu et al., 1988; Koenig, 1988), arising from ^{14}N nuclei in analogous environments. In addition, in γ_{II}-crystallin, there is significant cross-relaxation in the approximate range 0.9–1.7 MHz, which appears to be another pair of triplet peaks: 0.38, 0.88, and 1.26 MHz; and 0.41, 1.02, and 1.43 MHz (*solid and dashed arrows,* respectively). The data indicate the existence of at least two significantly different classes of environments for NH moieties in γ_{II}-crystallin, each in turn resolvable into two subclasses which are readily accessible to solvent water at high protein concentrations, but not below ~16% vol/vol.

The total area under the peaks of Fig. 4 for γ_{II}-crystallin, at 24.4% vol/vol and 5°C (and for the ~23% sample, not shown), is about the same as for the native nuclear lens homogenate, indicating that water has access to comparably many NH moieties. From the theory of the ^{14}N peaks (Koenig, 1988), one infers that essentially a majority of the backbone NH moieties are exposed to solvent water in γ_{II}-crystallin solutions at the higher concentrations measured here. Consequently, above ~16% protein, the solution conformation of γ_{II}-crystallin must change dramatically from a compact, globular structure, highly oligomeric, to an open and extended configuration, shown previously to be mimicked (except for its irreversible nature) by the heat denaturation of hen egg white (Beaulieu et al., 1989).

DISCUSSION

γ_{II}-crystallin intermolecular interactions

In summary, the NMRD data presented here imply the following solution behavior of γ_{II}-crystallin: calf γ_{II}-crystallin is a compact globular protein with a strong tendency to oligomerize, both with increasing concentration and with decreasing temperature Monomers predominate only at low concentrations ($\lesssim$3% protein) and high temperatures. At higher protein concentrations (~16% vol/vol) and lower temperatures (5°C) (cf Fig. 3 *A*), oligomers with a mean size as large as 30-mers can occur with no indication of a substantive change in the conformation of the monomeric units. However, above a certain concentration, which depends on temperature (~16% vol/vol at 25°C), the monomers themselves must reconfigure in a manner that produces an altered NMRD profile: the underlying monotonic form goes from that associated with solute globular proteins to that exhibited by a variety of polymeric, extended solutes (cf Beaulieu et al., 1988, 1989; Koenig and Brown, 1988); and triplets of ^{14}N peaks appear in the region 0.5–4 MHz. These peaks are known to arise from cross-relaxation between solvent protons and ^{14}N nuclei of the NH moieties of solute protein (Winter and Kimmich, 1982*a*, *b*; Koenig et al., 1984; Koenig, 1988; Beaulieu et al., 1988, 1989), from which we infer that much of the backbone of the primary polymeric structure is accessible to solvent. A natural explanation is that the initially compact γ_{II} monomers, themselves approximate quadruple repeats of a ~5-kD, predominately β-sheet, motif, unfold and reassemble into an extended, interconnected configuration. At low concentrations, the monomeric units are ostensibly maintained compact by the series of *intramolecular* hydrogen bonds and alternating charge pairs (salt bridges) that also stabilize the monomers in the solid state (Wistow et al., 1983). At higher concentrations, these same interactions can presumably link different molecules into an extended *intermolecular* network, a two- or three-dimensional open array. At any given temperature and protein concentration, there is a reversible equilibrium among a distribution of oligomers of the compact monomer and a distribution of extended polymeric conformers.

The $1/T_1$ NMRD results for calf γ_{II}-crystallin are surprisingly similar to those for calf lens nuclear homogenates, including the occurrence of a low temperature transition to opacity. There is a difference in the monotonic backgrounds (cf Fig. 1 of Beaulieu et al., 1988), readily attributable to a contribution from the large α-crystallins to the NMRD profiles of the homogenates, as well as a difference in the details of the structure of the cross-relaxation peaks, as evidenced in Fig. 4. However, it is the similarity of these results to each other, and to calf lens cortical homogenates and chicken lens homogenates (Beaulieu et al., 1989) that is intriguing. Clearly, similar oligomerization of lens proteins, with conformational reorganization of the fundamental monomeric units at the higher protein concentrations, occurs in all these instances, behavior that has not been encountered in studies of other globular protein systems over the years. Among the implications of these results is the prediction of an unusual concentration dependence of the osmotic pressure for each of these heterogeneous crystallin systems.

Relation to osmotic pressure

Vérétout and Tardieu (1989) have recently reported the concentration dependence of the "colloidal" osmotic pressure (the part that arises from solute macromolecules) for

solutions of calf α- and (mixed) γ-crystallins. They find that α-crystallin behaves classically, in the sense that deviations from ideality give a dependence that is greater than that expected for ideal solutions and can be accounted for by excluded volume effects at constant molecular weight plus the repulsive intermolecular interactions expected from identical solute particles of like charge. On the other hand, for the mixture of γ-crystallins, they found that the osmotic pressure increases much less rapidly with concentration than the ideal case, indicating "attractive solute–solute interactions." They conjecture that this attractive interaction produces an "uneven distribution" of the monomeric particles; however, the data are explained more readily if the attraction is sufficient to lead to oligomerization and a decrease in particle number with increasing concentration. A cursory quantitative comparison shows that our results, Fig. 3, can readily account for the osmotic pressure data, Fig. 1 of Vérétout and Tardieu (1989) and Magid et al. (1989). Further support of our interpretation of the osmotic pressure results come from the work of Siezen and Owen (1983) who, from chromatographic studies at relatively low protein concentrations, find "indefinite self-association" in calf γ-crystallin mixtures but little interaction between α-crystallin molecules and either α- or γ-crystallin molecules.

From the point of view of lens function, there are few reasons that would make self-aggregating proteins advantageous. Aggregation does not alter the index of refraction and has the disadvantage that it can limit the high solubility needed to reach the protein concentration of native lens tissues. What aggregation will do, however, is decrease the buildup of osmotic pressure that would otherwise occur if the crystallins remained monomeric at the high concentrations needed to produce a large refractive index. And, indeed, the rapid increase in solute size sets in, for both γ_{II}-crystallin and lens homogenates, at total crystallin concentrations comparable to the protein content of other tissues. Could this be an evolutionary feature driven by the need to minimize the metabolic energy that would otherwise be expended in maintaining osmotic equilibrium in the eye lens in vivo?

The "cold cataract" transition

Solutions of calf γ_{II}-crystallin exhibit a reversible first-order phase transition, and spatial separation of two phases, at a critical temperature, T_C, near 7°C and a critical concentration f_C at ~17% vol/vol protein, both somewhat sensitive to buffer composition. The thermodynamic coexistence curve and a spinodal decomposition have been reported (cf Siezen et al., 1985; Thomson et al., 1987). We suggest that it is not coincidental that f_C for γ_{II}-crystallin is near the concentration at which the ^{14}N peaks appear, and we associate the separating phases with the two conformational forms of γ_{II}-crystallin that we have found from the NMRD data. Thus, above T_C, any solution of γ_{II}-crystallin would be clear and homogeneous, an equilibrium of monomers, compact oligomers, and extended, conformationally altered aggregates. Below f_C, the compact molecular forms would dominate and above f_C, the extended, open conformers would dominate. Below T_C, a miscibility gap ostensibly exists between molecules in the compact and extended conformations. Thus, the conclusions from the NMRD data provide a ready framework for the interpretation of the phase transition in γ_{II}-crystallin solutions.

There are strong physical arguments that make it unlikely that a first-order phase transition can occur in a protein solution if all the solute entities are identical; i.e., if the transition were the analogue of a liquid-gas transition of a monatomic or monomolecular gas. The usual arguments for a liquid-gas transition of like particles in a vacuum carry over to like particles (proteins) in water (the vacuum); these arguments are thermodynamic, and therefore the difference in dynamics of the particles (Newton's laws vs. diffusion) becomes irrelevant. There are some differences: the effects of excluded volume in the "gas" phase would be more important for protein solutions, but this mainly alters entropy (by reducing the accessible volume for each molecule) and produces deviations from ideal-gas behavior, e.g., as found for the virial coefficients in the osmotic pressure of α-crystallin by Vérétout and Tardieu (1989). For a phase transition to occur, there must be an attractive interaction between particles, $\sim T_C$, when the concentration is $\sim f_C$ (cf Landau and Lifshitz, 1938; Pryde, 1966). This cannot be readily achieved in protein solutions since all protein molecules are similarly charged and the dominant protein–protein interaction would be repulsive (or zero at the isoelectric point). In addition, due to screening by the large dielectric constant of solvent water and Debye screening from the solute ions, the intermolecular interactions would be reduced considerably. Attractive interactions in gasses, in the final analysis, result from unscreened electric dipolar interactions, generally induced in one molecule by another. These are intrinsically much weaker than monopolar forces and therefore, for screened proteins in solution, these must be $\ll kT$, precluding phase transitions at the temperatures of interest in the absence of oligomerization. Oligomerization, as pictured here, results from interactions at close range, with solvent excluded, so that the attractive forces which stabilize the oligomers can become large.

Blankschtein et al. (1985), following earlier work of Ben-Shaul and Gelbart (1982), studied the thermodynamics of self-associating micellar solutions, taking into account multiple equilibria among the monomeric units

and micelles of all sizes (all with the same free energy), and applied it to the coexistence curve for the phase separation of C_8-lecithin micelles in water. Given the similarity of the view derived from the NMRD experiments on γ_{II}-crystallin with the behavior of the system analyzed by Blankschtein et al. (1985), we are confident that an analogous theoretical thermodynamic analysis of the self-associating, phase-separating γ_{II}-crystallin system, including the compact-to-open conformational change, would be equally successful. The resulting oligomerization at the lower concentrations will influence the sign of the osmotic virial coefficients, as observed for γ_{II}-crystallin by Vérétout and Tardieu (1989). Thus, one can readily combine the results of NMRD and osmotic pressure data with the thermodynamics of γ_{II}-crystallin solutions to produce a self-consistent molecular picture.

Relation to the solid state

Oligomerization of γ_{II}-crystallin at low concentration is consistent with the fact that attempts to measure the solution coexistence curve of the γ_{II}-crystallin phases (Thomson et al., 1987) were complicated by the tendency for protein crystals to form. These authors concluded that one point on the liquidus (the crystal-liquid phase boundary), at 4°C, is at a concentration less than that of the liquid-liquid coexistence curve. Related to this, Sergeev et al. (1988), using amino acid sequence data and analysis of electron density maps of the crystal structure, concluded that in crystals of calf lens γ_{II}-crystallin the intermolecular contact areas comprise ~33% of the total accessible surface area. Together with the experience of Thomson et al. (1987), these results suggest that the monomeric form of γ_{II}-crystallin in solution is much like that in the crystal; thus extensive oligomerization in solution and the readiness with which crystals form are two aspects of the same intermolecular interactions. The conformational transition at physiological total protein concentrations in solution is, however, an additional and unrelated phenomenon.

Our conjectures regarding oligomerization and conformation change in solutions of homogeneous lens proteins, and their extension to heterogeneous systems that are models for lens homogenates, can be addressed, perhaps uniquely, by NMRD studies of other pure crystallins and carefully chosen crystallin mixtures in solution.

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