

**Proton relaxation rates of water in dilute solutions of  $\beta$ -lactoglobulin.  
Determination of cross relaxation and correlation with structural changes by the  
use of two genetic variants of a self-associating globular protein**

In order to relate resonance relaxation behavior to protein structural states, pulse Fourier transform NMR was employed to obtain water proton longitudinal and transverse relaxation rates ( $R_1$  and  $R_{1\rho} = R_2$ ) of bovine  $\beta$ -lactoglobulins A and B in buffered solutions. Measurements at concentrations from 5 to 100 mg/ml were made at pH 4.65, 6.2 and 8.0, at 30 and 2°C, to monitor specific structural changes. The parameters characterizing the concentration dependence of the observed  $R_1$  and  $R_2$  were used to derive a number of quantities relating to protein-influenced water, including a hydration parameter  $h$ . Changes in  $h$  under the different sets of conditions were correlated with (a) the irreversible denaturation of this protein at pH 8.0, 2°C and (b) the dimer  $\rightleftharpoons$  octamer association at pH 4.65, 2°C. Corresponding correlation times, however, were low, indicating cross relaxation which had not manifested itself as nonexponential relaxation because of the large amount of water present. Differences in the extent of the dimer  $\rightleftharpoons$  octamer association between genetic variants A and B allowed an evaluation of dynamics and extent of hydration from  $R_2$  alone, assuming the absence of intermolecular interactions. Derived parameters were in agreement with hydrodynamic and X-ray values in the literature. Cross relaxation was likewise evaluated and was found to contribute to  $R_1$  to a large extent. The results show that changes in proton relaxation rates in solutions of a globular protein occurring as genetic variants with different physical properties (such as  $\beta$ -lactoglobulin) can be utilized to detect variations in hydration corresponding to changes in molecular association and conformation, as well as to obtain cross relaxation and structural data.

### Introduction

The state of water in systems of biomacromolecules has been the subject of intensive study in recent decades [1-3]. One of the investigative methods employed has been nuclear magnetic resonance and, in particular, nuclear spin relaxation [4,5]. Beall et al. [6], among others, have suggested that changes in observed proton relaxation rates of cellular water relative to those of bulk water may be attributable to the conformational state as well

as to the nature and concentration of macromolecules in the cell. There has been little unanimity on the general subject because of the complex nature of hydration phenomena and their relationships to NMR relaxation, and because of complicating factors such as cross relaxation between protein and water protons. Examination of water-protein interactions by measurement of proton spin relaxation of water in a well-defined system of a protein capable of undergoing structural changes should, however, afford opportunity to observe corre-

lations between these changes and the measured relaxations.

A suitable protein for this purpose is  $\beta$ -lactoglobulin, which occurs in two genetic variants, A and B, possessing physical properties nearly identical except for the extents of specific structural changes [7-13]. We report here measurements of the longitudinal ( $R_1$ ) and transverse ( $R_2$ ) proton relaxation rates of water in buffered solutions as a function of protein concentration, with pH and temperature varied to allow examination of several of the protein structural states for concomitant differential behavior between the two variants. These data are evaluated first in terms of very simple model assumptions for the water-macromolecule interaction, with the aim of determining (i) whether significant changes in this interaction can be found for dilute solutions of this model protein as a result of environmental changes and (ii) whether such changes can be shown to reflect the respective molecular states of the protein, as reported in the literature from studies by other methods under the same conditions. Next, the consequences of considering cross relaxation are examined. As a special alternative to such other methods as those using magnetic field dependence of NMR relaxation [14,15] or measurements on solvent  $^{17}\text{O}$  and  $^2\text{H}$  nuclei [15-17], we explore the combined use of proton NMR data from the two genetic variants, making use of their differing association behavior. The results of cross relaxation evaluated in this way are compared with results from hydrodynamics, X-ray diffraction and small-angle scattering, and deuterium NMR relaxation.

Preliminary reports of this work were presented at the 174th and 176th National Meetings of the American Chemical Society, 1977 and 1978.

## Materials and Methods

*Materials.*  $\beta$ -Lactoglobulins A and B, furnished by R.E. Townend of this laboratory, were the recrystallized lyophilized products, prepared from the milk of homozygous A/A and B/B cows by the method of Aschaffenburg and Drewry [18]. To exclude the possibility that observations might be affected by paramagnetic enhancement due to the presence of heavy-metal ions, some experiments

were carried out both with and without prior treatment of the preparations with EDTA. In no case was a significant difference found. Distilled water from an all-glass still was used without further treatment. The buffers employed were: pH 4.65, 0.1 M acetate ( $\beta$ -lactoglobulin B) and 0.3 M acetate ( $\beta$ -lactoglobulin A); pH 6.2, 0.1 M phosphate ( $\beta$ -lactoglobulin A) and 0.1 M acetate ( $\beta$ -lactoglobulin B); pH 8.0, 0.1 M glycylglycine/0.1 M KCl, adjusted to pH by addition of KOH. All buffer salts were the potassium salts of the respective acids and were Baker analyzed reagents\*. Glycylglycine was purchased from Calbiochem, and benzene- $d_6$  and acetone- $d_6$  (99.5 atom %) were purchased from Wilmad Glass Co., Inc.

*Measurements.* Protein solutions, prepared 1 day before use, were exhaustively dialyzed overnight against buffer at 0-5°C, except for the study at pH 8.0. In that case, all manipulations, including filling of the sample cells, were carried out at room temperature, and the low-temperature measurements were made last. All dilutions were made with the appropriate dialyzate. Protein concentrations were determined with the use of an absorption coefficient of  $0.96 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$  at 278 nm [19].

Proton NMR spectra were obtained by pulse Fourier transform spectroscopy at 90 MHz (Bruker WH-90,  $R_1$  measurements) and at 60 MHz (JEOL FX60Q spectrometer,  $R_2$  measurements). Since the high concentration of water in a dilute solution produces an intense signal, a single accumulation at the particular sample temperature (2°C or  $30 \pm 1^\circ\text{C}$ ) was sufficient for each spectrum. To avoid exceeding the dynamic range of the computer with consequent signal truncation, it was necessary to provide the Bruker WH-90 with a 20-db attenuator in the probe preamplifier, in addition to reducing the sample volume for both instruments by the use of a microcell assembly with an expendable 35- $\mu\text{l}$  sample bulb (Wilmad Glass Co., Inc.). The assembly included this microbulb, filled with the sample and inserted into either a 10-mm or 5-mm outer diameter sample tube containing deuterated

\* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

solvent (benzen- $d_6$  or acetone- $d_6$ ) to provide an external heteronuclear lock signal.

*Determination of relaxation rates.* Proton longitudinal relaxation rates  $R_1$  were measured by the inversion-recovery method [20], where the repetition time  $T$  in the usual pulse sequence (... $T$ ... $180^\circ$ ... $\tau$ ... $90^\circ$ ...) was chosen to be at least 5-times the longitudinal relaxation time  $T_1$  ( $\equiv R_1^{-1}$ ). Under the conditions of this method, the relation of the peak intensity  $A_\tau$  to the pulse delay time  $\tau$  becomes:

$$\ln(1 - A_\tau/A_\infty) = \ln(1 - A_0/A_\infty) - R_1\tau \quad (1)$$

where  $A_0$  is the peak intensity at  $\tau = 0$ , and  $A_\infty$  is the limiting and maximum peak intensity at  $\tau_\infty$ . Five sets of values of  $A_\tau$  as a function of  $\tau$  were determined. In a departure from conventional practice, the value of  $A_\infty$  used was the average of five independent measurements at long  $\tau$  taken alternately with the measurements for variable  $\tau$ , in order to provide proper statistical weighting in the linear regression of  $\ln(1 - A_\tau/A_\infty)$  on  $\tau$ , from which the coefficient ( $-R_1$ ) was evaluated (cf. Fig. 1A). Each such determination of  $R_1$  was replicated four times and the results were averaged. This procedure was repeated for each of a minimum of five concentrations under each set of conditions of temperature ( $30^\circ\text{C}$  and  $2^\circ\text{C}$ ) and pH (4.65, 6.2 and 8.0) investigated.

Proton transverse relaxation rates  $R_2$  were determined on the JEOL FX60Q by spin-locking measurement [21] of  $R_{1\rho}$ , the longitudinal relaxation rate in the rotating frame.  $R_{1\rho}$  equals  $R_2$  in dilute solutions of low viscosity whenever the magnitude of  $R_{1\rho}$  is independent of  $H_{1\rho}$ , the spin-locking radio-frequency field in the rotating frame; this was found to be the case, within the limits of experimental error, in the present work. The evaluation of  $R_2$  was as described above for  $R_1$ , except that the pertinent relation between peak intensity  $A_\tau$  and decay time  $\tau$  in this case becomes:

$$\ln A_\tau = \ln A_0 - R_2\tau \quad (2)$$

where  $A_0$  is the maximum peak intensity (cf. Fig. 1B).

*Data reduction.* The theory of Zimmerman and Brittin [22] implies a linear dependence of  $R_1$  or  $R_2$  on macromolecular concentration if one as-

sumes a simple two-state, fast-exchange model, where the states refer to 'bound' (protein-influenced) and 'free' (bulk) water, respectively. (This simple theory is used as a point of departure; an extension to a more complex model including the effects of cross relaxation will be considered later.) The observed proton relaxation rate  $R_{\text{obs}}$  (regardless of mode,  $T_1$ ,  $T_{1\rho}$  or  $T_2$ ) can then be expressed as the sum of contributions from the relaxation rates of the two states (b for bound, f for free), each contribution being the product of the water proton relaxation rate ( $R_b$  or  $R_f$ ) and the fraction of protons ( $p_b$  or  $p_f$ ) in the respective state:

$$R_{\text{obs}} = p_b R_b + p_f R_f, \quad p_b + p_f = 1 \quad (3)$$

If  $h$  is the degree of hydration in grams of b-state water per gram of anhydrous solute, and  $c$  the concentration in grams of anhydrous solute per gram of total water, one has  $p_b = hc$ ,  $p_f = 1 - hc$ , and:

$$R_{\text{obs}} = R_b hc + R_f (1 - hc) = R_f + (R_b - R_f) hc \quad (4)$$

or

$$R_{\text{obs}} = R_f + kc, \quad k = (R_b - R_f) h \equiv h\Delta R \quad (5a,b)$$

where  $\Delta R$  is the total excess relaxation rate (i.e., the difference between the relaxation rates of the protein-influenced and free states of water involved).

For dilute protein solutions, the concentrations may be more conveniently expressed, with negligible error, in units proportional to molarity (e.g., mg/ml) instead of molality, as above. With due attention to the conversion of units, linear plots of  $R_{\text{obs}}$  vs.  $c$  (shown in Fig. 2A and B) then conform to Eqn. 5a, with extrapolation to infinite dilution giving the ordinate intercept  $R_f$ . The slope  $k$  is a characteristic parameter which may be viewed as a relaxation increment, in analogy to similar parameters related to such quantities as dielectric constant, refractive index and conductance. It is important, in principle, to distinguish between (i) this relaxation increment (strictly speaking  $dR_{\text{obs}}/dc$ ), (ii) the specific excess relaxation rate,  $(R_{\text{obs}} - R_f)/c$  (with which it is identical if the concentration dependence of  $R_{\text{obs}}$  is, indeed, linear), and (iii) the quantity  $h\Delta R$ , termed here the hydration product,

because it is the product of the hydration and the total excess relaxation rate. The hydration product equals the other two quantities only in the absence of cross relaxation (see further under Discussion); otherwise  $k$  equals an uncorrected hydration product, relating to an apparent hydration uncorrected for cross relaxation.

*Hydrations and correlation times.*  $R_f$  and  $k$  data derived from both  $R_1$  and  $R_2$  were utilized with suitable expressions which permit solution of a system of simultaneous equations for  $h$ . As suggested by Daskiewicz et al. [23],  $R_1$  and  $R_2$  of the bound-water fraction may both be expressed in terms of a common rotational correlation time,  $\tau_c$ . Subject to the assumptions stated initially, the appropriate expressions [24] are:

$$R_{1b} = 0.3\hbar^2\gamma^4b^{-6}\tau_c \left[ (1 + \omega_{0,1}^2\tau_c^2)^{-1} + 4(1 + 4\omega_{0,1}^2\tau_c^2)^{-1} \right] \quad (6)$$

and

$$R_{2b} = 0.15\hbar^2\gamma^4b^{-6}\tau_c \left[ 3 + 5(1 + \omega_{0,2}^2\tau_c^2)^{-1} + 2(1 + 4\omega_{0,2}^2\tau_c^2)^{-1} \right] \quad (7)$$

where  $\hbar$  is Planck's constant divided by  $2\pi$ , or  $1.0546 \cdot 10^{-27}$  erg  $\cdot$  s;  $\gamma$  is the nuclear gyromagnetic ratio,  $2.6752 \cdot 10^4$  gauss $^{-1} \cdot$  s $^{-1}$ ;  $b$  is the proton internuclear distance for water, 1.526 Å; and  $\omega_{0,1}$  and  $\omega_{0,2}$  are the angular resonance frequencies, in radians  $\cdot$  s $^{-1}$ , corresponding to 90.00 and 59.79 MHz, respectively. Eqns. 6 and 7, and the use of a common correlation time, involve the assumption of isotropic tumbling and thus represent an approximation which, however, is not unreasonable for a globular protein.

Two expressions of the form of Eqn. 5b, one for each mode of relaxation, contain a total of three unknowns ( $R_{1b}$ ,  $R_{2b}$ , and  $h$ ); two of these ( $R_{1b}$  and  $R_{2b}$ ) may be eliminated by means of Eqns. 6 and 7 in terms of  $\tau_c$ . The resulting system of two equations in two unknowns ( $\tau_c$  and  $h$ ), although not solvable for either unknown in closed form, can be evaluated by numerical approximation. Input data are the three constants ( $0.15 \hbar^2\gamma^4b^{-6}$ ),  $\omega_{0,1}^2$ ,  $\omega_{0,2}^2$ , and the data parameters  $k_1$ ,  $k_2$ ,  $R_{1f}$ , and  $R_{2f}$ . The required iterations are readily performed with adequate precision on a desk calculator with program storage.

An alternative approach would have been to use, in place of the  $R_2$  data, a set of  $R_1$  data at the second frequency; two applications of Eqn. 6 would then furnish the requisite number of simultaneous equations. It was found, however, that the two frequencies available (60 and 90 MHz) were too close to give data sufficiently different to allow solution for the unknowns with satisfactory precision, as might be surmised also from the data of Hallenga and Koenig [15], from which it is clear that frequency dispersion effects become relatively small for frequencies above 10 MHz.

A third approach, use of a separate set of data from a single mode of relaxation for each of two variants, was also implemented. It permits an explicit consideration of cross relaxation and is detailed under Discussion.

## Results and Discussion

### Relaxation rates

$R_1$  data for three selected concentrations of  $\beta$ -lactoglobulin B at pH 6.2 and 2°C are illustrated in Fig. 1A. The slope of the line fitted to data for the 4.01% solution, as a typical example, gives  $R_1 = 0.687$ , with a standard error of 0.003, or  $T_1 = 1.456 \pm 0.006$  s.  $R_2$  data for three selected concentrations of  $\beta$ -lactoglobulin A at pH 8.0 and 30°C, with  $A_r$  normalized to facilitate comparisons, are illustrated in Fig. 1B. The slope of the line fitted to values for the 2.46% solution, as a typical example, gives  $R_2 = 0.726$ , with a standard error of 0.033, or  $T_2 = 1.38 \pm 0.06$  s.

### Relaxation increments and free-water relaxation rates

Typical concentration dependences for  $R_1$  and  $R_2$  data are shown in Fig. 2A and B, respectively, and are seen to be linear, in agreement with the simple theory. Values of  $k_1$  and  $R_{1f}$ , and of  $k_2$  and  $R_{2f}$ , calculated by linear regression of the respective dependence of relaxation rates on protein concentration (Eqn. 5a), are listed in Table I. As expected [25], the relaxation rates of bulk water protons,  $R_{1f}$  and  $R_{2f}$ , increased at the lower temperature in each of these systems; the magnitude of the change did not differ significantly among the solvent buffers used. The relaxation increments  $k_1$  and  $k_2$  also changed in the same direction.

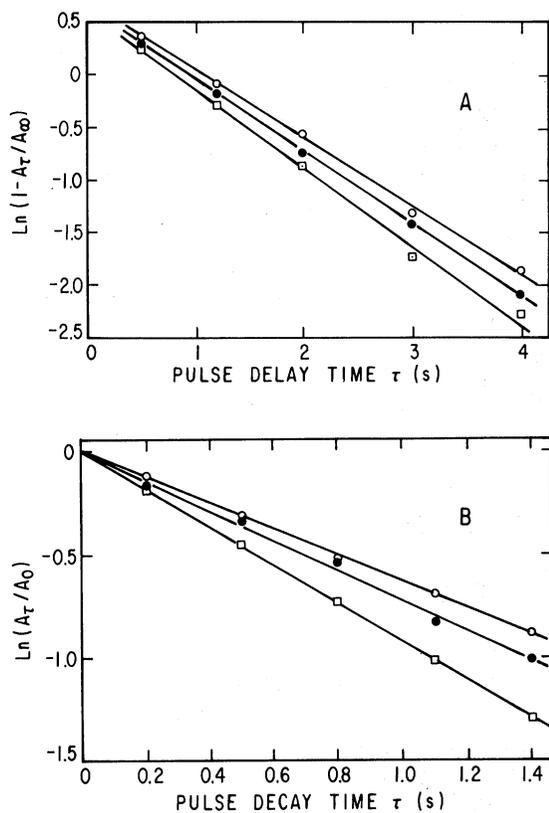


Fig. 1. Measurement of proton relaxation rates of water in dilute  $\beta$ -lactoglobulin solutions. Pulse NMR experiments performed as described under Materials and Methods. (A) Proton longitudinal relaxation rates ( $R_1$ ) of water in  $\beta$ -lactoglobulin B solutions at 2°C in 0.1 M acetate (pH 6.2), as function of pulse delay time. Protein concentrations: (○) 11.9 mg/ml; (●) 40.1 mg/ml; (□) 60.1 mg/ml. (B) Proton transverse relaxation rates ( $R_2$ ) of water in  $\beta$ -lactoglobulin A solutions at 30°C in 0.1 M glycylglycine/0.1 M KCl (pH 8.0), as function of decay time. Protein concentrations: (○) 16.4 mg/ml; (●) 24.6 mg/ml; (□) 55.4 mg/ml.

Values of  $k_1$ ,  $k_2$ ,  $R_{1f}$  and  $R_{2f}$  so found were used, as described, in the calculation of apparent degrees of hydration  $h$ , uncorrected for cross relaxation (also listed in Table I), as well as apparent rotational correlation times and longitudinal and transverse relaxation times for water in the bound state,  $R_{1b}$  and  $R_{2b}$  (not listed). In each case,  $R_{2b}$  increased while  $R_{1b}$  decreased at the lower temperature. This temperature effect is consistent with the absence of an exchange contribution, as is implicit in Eqns. 6 and 7 [4].

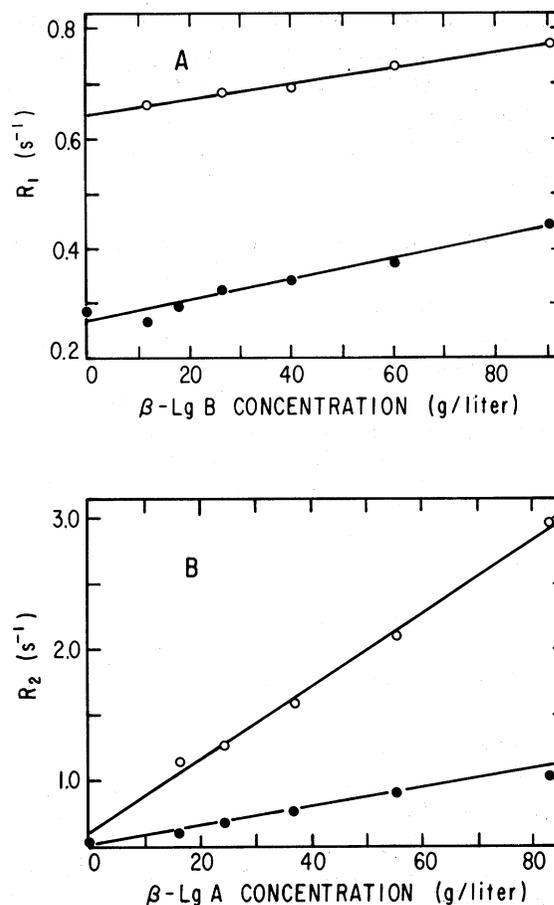


Fig. 2. Proton relaxation rates of water in  $\beta$ -lactoglobulin solutions as a function of protein concentration. The slope ( $k$ ) is the relaxation increment, and the intercept ( $R_f$ ) is the relaxation rate of free water, as described under Results. (A) Proton longitudinal relaxation rates ( $R_1$ ) of water as function of  $\beta$ -lactoglobulin B concentration at pH 6.2. Temperatures: (●) 30°C ( $k_1 = 1.94 \pm 0.18$ ,  $R_{1f} = 0.271 \pm 0.009$ ); (○) 2°C ( $k_1 = 1.49 \pm 0.11$ ,  $R_{1f} = 0.643 \pm 0.006$ ). (B) Proton transverse relaxation rates ( $R_2$ ) of water as function of  $\beta$ -lactoglobulin A concentration at pH 8.0. Temperatures: (●) 30°C ( $k_2 = 7.0 \pm 0.2$ ,  $R_{2f} = 0.53 \pm 0.01$ ); (○) 2°C ( $k_2 = 27.9 \pm 1.3$ ,  $R_{2f} = 0.61 \pm 0.06$ ).

#### Structural states and hydration

The temperatures at which measurements were made had been chosen because of known properties of the protein. At both pH 4.65 and 8.0 it is known to undergo significant structural changes, mainly in the cold; at pH 6.2, no change in structure accompanies the same temperature change. On inspection of the temperature and pH

TABLE I

RELAXATION INCREMENTS (LONGITUDINAL,  $k_1$ , AND TRANSVERSE,  $k_2$ ) AND SOLVENT RELAXATION RATES ( $R_{1f}$  AND  $R_{2f}$ ) FOR SOLUTIONS OF TWO VARIANTS OF  $\beta$ -LACTOGLOBULIN

Longitudinal measurements (at 90.0 MHz) and transverse measurements (at 59.79 MHz), made at two temperatures and three values of pH, as indicated. Values of the parameters are the results of linear least-square fits to points at five to seven concentrations, each representing the mean of quadruplicate relaxation rate determinations based on measurements at five time values each. Error terms indicate standard errors of the parameter.

pH	Variant	Temp. (°C)	Relaxation increment ( $s^{-1} \cdot g_{\text{water}} \cdot g_{\text{prot}}^{-1}$ )		Solvent relaxation rate ( $s^{-1}$ )		Uncorrected hydration ( $h$ ) ( $g_{\text{water}} \cdot g_{\text{prot}}^{-1}$ )
			$k_1$	$k_2$	$R_{1f}$	$R_{2f}$	
4.65	A	30	$1.52 \pm 0.25$	$8.9 \pm 1.5$	$0.307 \pm 0.011$	$0.23 \pm 0.07$	$0.070 \pm 0.012$
		2	$3.56 \pm 0.23$	$55.3 \pm 4.4$	$0.603 \pm 0.010$	$0.70 \pm 0.24$	$0.298 \pm 0.021$
4.65	B	30	$1.26 \pm 0.26$	$5.2 \pm 0.4$	$0.314 \pm 0.004$	$0.34 \pm 0.02$	$0.047 \pm 0.008$
		2	$2.18 \pm 0.24$	$27.7 \pm 2.6$	$0.644 \pm 0.011$	$0.60 \pm 0.16$	$0.163 \pm 0.017$
6.2	A	30	$1.92 \pm 0.26$	$4.5 \pm 0.2$	$0.301 \pm 0.007$	$0.31 \pm 0.01$	$0.056 \pm 0.005$
		2	—	$10.4 \pm 1.2$	—	$0.89 \pm 0.06$	$0.077 \pm 0.007$
6.2	B	30	$1.94 \pm 0.18$	—	$0.271 \pm 0.009$	—	—
		2	$1.49 \pm 0.11$	—	$0.643 \pm 0.006$	—	—
8.0	A	30	$1.77 \pm 0.13$	$7.0 \pm 0.2$	$0.264 \pm 0.005$	$0.53 \pm 0.01$	$0.064 \pm 0.004$
		2	$2.73 \pm 0.51$	$27.9 \pm 1.3$	$0.514 \pm 0.015$	$0.61 \pm 0.06$	$0.178 \pm 0.023$

dependence of the various parameters in Table I, it is noticeable that not only  $h$  but also the hydration product  $k_2$  appears to be suitable as at least a preliminary measure of hydration: allowing for the temperature effect (exhibited by itself at pH 6.2),  $k_2$  and  $h$  on going from 30 to 2°C at pH 4.65 display increases by factors of 2.7 and 3.1 for the A variant and of 2.3 and 2.5 for the B variant, while at pH 8.0 the A variant shows increases by factors of 1.7 for  $k_2$  and 2.0 for  $h$ . These indications of pronounced increases in apparent hydration in the cold at both pH 4.65 and 8.0 may be interpreted in terms of the known molecular behavior of  $\beta$ -lactoglobulin.

This protein has been reported to occur naturally in five genetic variants [26]. The A and B forms employed here undergo a variety of changes in conformation and state of association, summarized in Fig. 3. The present investigation focuses on two of these: a slow, irreversible denaturation [13] and a rapid dimer  $\rightleftharpoons$  octamer equilibrium [7,9–12], both occurring primarily in the cold. The 2-subunit, 36 700 dalton dimer is the kinetic unit persisting over a wide range of moderate conditions of pH from 3 to about 7 [9]; its structure has been reported in detail [32–33].

Above pH 6.5, the dimer is known to begin to dissociate [27–29] and then to denature irreversibly [28,30,31] while at first remaining in solution.

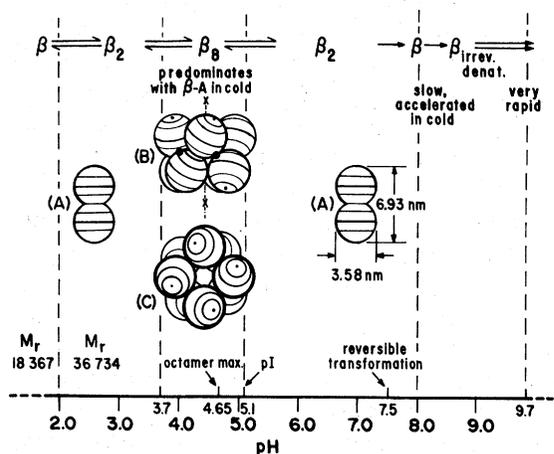


Fig. 3. Schematic representation of changes in the structure of  $\beta$ -lactoglobulins as a function of pH.  $\beta$ ,  $\beta$ -lactoglobulin. Insets (molecular models): (A) dimer; (B) octamer, with square decahedral faces on top and bottom;  $\bullet$ , octamer bonds;  $\times$ ----- $\times$ , tetrad axis; circular lines indicate monomer equators and parallels perpendicular to the dimer axes; (C), octamer, with square faces in front and back, tetrad axis perpendicular to plane of paper. (Based on data of Refs. 7–13, 27–33, 45.)

The time-dependent denaturation, referred to as 'cold denaturation' since it is accelerated in the cold as compared to room temperature, occurs about three times faster with  $\beta$ -lactoglobulin A than with  $\beta$ -lactoglobulin B [13]. This correlates well with the observed indication of increased hydration of  $\beta$ -lactoglobulin A on exposure to the cold, since both the concurrent dissociation and subsequent denaturation presumably involve increased availability of water-binding sites on the denatured protein molecule.

Between pH 3.7 and 5.1, self-association of  $\beta$ -lactoglobulin dimer to octamer occurs as temperature decreases. This is a rapid equilibrium process that is also well characterized [12]; it occurs to a greater extent with the A variant than with the others. Since self-association involves protein-protein interactions which should remove some of the potential water-binding sites on the dimer surface, a decrease in hydration might be expected. The observed increased hydration requires a more detailed examination of the molecular geometry. The octamer, as determined by Timasheff and Townend [10], is a closed ring, consisting of four dimers associated symmetrically about a tetrad axis; its general shape is that of a decahedron with a substantial central cavity (Fig. 3). Because of a sizable hole (approx. 1.4 nm diameter) in each of the two square faces of the decahedron (Fig. 3, inset C), the water in the interior of the octamer is readily accessible for fast exchange with the bulk water on the outside, as was assumed by the theory above.

An estimate of the amount of water contained in the cavity may be obtained from the geometric parameters [10], which indicate that an inscribed sphere, tangent to the interior van der Waals surfaces of the octamer, would have a volume of about 6.45 nm<sup>3</sup>. (This estimate neglects the spaces in corners and crevices of the cavity; these will be largely offset by the dimer-dimer contact areas between the eight monomer units made unavailable to water binding upon tetramerization at the twelve new protein-protein contact sites.) From the molecular weight and volume of water and the molecular weight of the protein [33], one finds that this cavity corresponds to 0.264 g H<sub>2</sub>O/g protein. It can, therefore, accommodate a considerable amount of water detectable by NMR, giving rise

to the increased hydration observed under the conditions where octamer formation occurs. It may now also be seen that the relative enhancements of the hydration parameters noted above (i.e., factors of 3.1 for  $h$  and 2.7 for  $k_2$  for the A variant vs. 2.5 and 2.3, respectively, for the B variant) are consistent with the known higher degree of octamer formation by  $\beta$ -lactoglobulin A compared with B [12].

There is thus qualitative agreement between the hydrations shown in Table I and the known structure of the corresponding states of the protein, but it remains to be examined how this agreement may be affected by a consideration of cross relaxation.

#### *Dynamics and hydration from transverse relaxation*

Values of apparent correlation times calculated from Eqns. 5b, 6, and 7 range from 1.4 to 7.1 ns, considerably lower than corresponding estimates obtained for  $\beta$ -lactoglobulin from the Debye-Einstein relation [25] for dielectric relaxation (i.e., 15–53 ns [34]) or fluorescence depolarization (20–78 ns [35]), or from NMR by means of a method which obviates the effects of cross relaxation (10–32 ns [17]). The lowered apparent correlation times may be considered a clear indication of cross relaxation [36], not evidenced here in the form of nonexponential relaxation (cf. Fig. 1A and B) because of the vast excess of bulk water protons in dilute solutions. Instead, it shows its presence by its effect on the apparent  $\tau_c$  and by increasing the apparent water proton relaxation rate,  $R_1$ , [36,37] and thus the values of  $k_1$ . These, as well as the validity of Eqn. 5b, require further examination, especially as there is definite evidence for the general occurrence of cross relaxation in protein systems [16,36–38].

Since, however, cross relaxation affects primarily longitudinal relaxation [16,38], one may start by dealing separately with the transverse relaxation, for which Eqn. 5b will continue to serve in conjunction now with the added information supplied by the availability of data for both genetic variants at pH 4.65. For this purpose, the extents of tetramerization for each variant at this pH at 2°C can be calculated from light-scattering data of Kumosinski and Timasheff [12] as 91.2% ( $\beta$ -lactoglobulin A) and 31.0% ( $\beta$ -lactoglobulin B).

This information allows the values of  $k_2$  from

Table I to be expressed in terms of the relative contributions of dimer and octamer and, together with the knowledge of the amount of water in the cavity and Eqn. 5b, provides two simultaneous equations for the two variants. In this way, a number of parameters of interest not affected by cross relaxation can be obtained (Appendix, A). These include the hydrations of dimer,  $h_D = 0.0276$  g/g, and octamer,  $h_O = 0.0540$  g/g, various correlation times, and the Stokes radius of the octamer,  $R_{s,O} = 3.82$  nm. Repetition of this calculation for 30°C with the separately determined values of  $k_2$  of Table I at that temperature, combined with the information [12] that the extents of tetramerization here are 26.9% ( $\beta$ -lactoglobulin A) and less than 0.02% ( $\beta$ -lactoglobulin B), gives  $h_D = 0.0223$  g/g,  $h_O = 0.0487$  g/g, and  $R_{s,O} = 3.75$  nm. The values of  $R_{s,O}$  from measurements at the two temperatures thus are in close agreement, as would be expected since the Stokes radius should be nearly temperature-independent. They are in general agreement with literature values of 4.33 nm, from sedimentation [40]; 4.44 nm, from small-angle X-ray scattering [40]; and 3.04 nm, from  $^2\text{H}$  NMR [17].

#### *Cross relaxation and longitudinal relaxation*

Longitudinal relaxation data can be utilized in similar manner when Eqn. 5b is modified by decomposition into a corrected term,  $k'_1 \equiv h'\Delta R$  (where  $h'$  is a hydration corrected for cross relaxation), and a term for a cross-relaxation increment,  $k_x$ , to account for the contribution of cross relaxation to  $k_1$ . The justification for such a procedure derives from a consideration of a cross-relaxation model which treats the water and protein protons as separate thermodynamic systems in magnetic interaction [16,26] (Appendix, B). This results in values at 2°C (see Appendix, C) for the  $k_x$  terms for octamer and dimer, respectively, of  $k_{x,O} = 3.71$  and  $k_{x,D} = 1.39$ . Since spin diffusion, which is the basis for cross relaxation, should be dependent on distance from particle center of mass to surface (see, e.g., Ref. 16), the pertinent distance for the fairly isotropic octamer should be approximately its Stokes radius, 4.33 nm [40], whereas for the dimer, consisting of two tangent, nearly spherical monomer subunits, it should be approximately the monomer radius, 1.79 nm [10]. The ratio of these

distances is 2.42, while the ratio  $k_{x,O}/k_{x,D} = 3.71/1.39$  is 2.67, and therefore in good agreement with the value estimated from the known geometry. (The 30°C values, based on  $k_1$  values and extents of tetramerization much smaller, and therefore containing larger relative errors, yield a ratio  $k_{x,O}/k_{x,D} = 1.86$ , which is still of the correct order of magnitude.)

To examine the phenomena at pH 8.0, we make a first assumption that  $k_x$  at 30°C may be approximated by its value for the dimer at pH 4.65, at the opposite side of the isoelectric point of 5.1, since additional cross relaxation due to higher pH should be largely offset by decreased spin diffusion with the then more disordered protein. For pH 4.65, the modified Eqn. 5b,  $k_1 = h_D\Delta R_1 + k_x$ , with  $h_D = 0.0223$  obtained from  $k_2$ , and with the  $k_1$  of Table I, gives  $k_x = 1.088$ . This value, substituted in the same equation applied to the pH 8.0 conditions, gives  $h_D\Delta R_1 = 0.69$  and, from Eqn. 6,  $\tau_c = 5.36$  ns, or a Stokes radius of 1.88 nm. Compared to the approx. 1.79 nm Stokes radius of the monomer [10], this implies that at this alkaline pH, even at room temperature, a substantial fraction of the protein exists as monomer. This is in agreement with the findings of Townend et al. [27] and Georges et al. [28], according to which the dimer at room temperature above about pH 7.0 begins to dissociate before alkaline denaturation takes place. Since this might weaken the above assumption regarding  $k_x$ , we look, alternatively, to the pH 6.2, 2°C data for confirmation. Used as before, these yield a  $k_x$  of 1.43 which, applied to the pH 8.0, 2°C data, gives  $\tau_c = 8.67$  ns, or a Stokes radius of 1.68 nm, and again the distinct indication is of a prevalence of monomer, in accord with the literature.

Cross-relaxation increments and hydrations calculated in this manner for the various forms of the protein under the different conditions, as well as the respective correlation times and Stokes radii, are listed in Table II (where  $h'$  designates values obtained by taking cross relaxation explicitly into account). Correlation times here range from 10.2 to 51.2 ns, more in keeping with the literature values cited above [17,34,35]. In view of the various approximations made and the high contribution of  $k_x$  to  $k_1$ , the values of  $h'$  listed must, however, be considered to be somewhat less accu-

TABLE II

PARAMETERS CALCULATED FOR  $\beta$ -LACTOGLOBULIN SOLUTIONS WITH CROSS RELAXATION TAKEN INTO ACCOUNT

Based on data of Table I and Eqns. 6, 7 and 11. For statistics, see legend to Table I.

pH	Variant or species	Temp. (°C)	$k_x$ ( $s^{-1}$ , $g_{\text{water}} \cdot g_{\text{prot}}^{-1}$ )	$h'$ ( $g_{\text{water}} \cdot g_{\text{prot}}^{-1}$ )	$\tau_c$ (ns)	$R_{\text{Stokes}}$ (nm)	
						this work	literature
4.65	A	30	$1.34 \pm 0.25$	$0.029 \pm 0.001$	$12.5 \pm 1.5$		
		2	$3.50 \pm 0.23$	$0.052 \pm 0.009$	$51.2 \pm 4.6$		
	B	30	$1.09 \pm 0.26$	$0.022 \pm 0.002$	$10.2 \pm 0.1$		
		2	$2.11 \pm 0.24$	$0.036 \pm 0.008$	$33.0 \pm 1.6$		
	Pure dimer	30	$(1.09 \pm 0.26)^a$	$(0.022 \pm 0.002)^a$	$10.2 \pm 0.1$		
		2	$1.39 \pm 0.32$	$0.028 \pm 0.010$	$23.6 \pm 0.1$		
	Pure octamer	30		$0.049 \pm 0.002$	$18.6 \pm 5.6$ $(42.2 \pm 12.4)^a$	3.75	4.33, 4.44 <sup>c</sup>
		2	$3.71 \pm 0.25$ $2.03 \pm 1.29^b$	$0.054 \pm 0.010$	$53.8 \pm 5.0$ $(102.6 \pm 38.4)^a$	3.82	
6.2	A or B	30	$1.79 \pm 0.18$	$0.019 \pm 0.001$	$10.2 \pm 0.1$		
		2	$3.52 \pm 0.19$	$0.021 \pm 0.002$	$23.6 \pm 0.1$		
8.0	A	30	$1.09 \pm 0.26$	$0.047 \pm 0.009$	$5.4 \pm 0.3$	1.88 <sup>c</sup>	1.79 <sup>f</sup>
		2	$2.08 \pm 0.51$	$0.103 \pm 0.007$	$12.4 \pm 0.6$	1.68 <sup>d</sup>	

<sup>a</sup> Pure octamer without cavity contribution.<sup>b</sup> Parameters of B variant.<sup>c</sup> From cross relaxation at pH 4.65.<sup>d</sup> From cross relaxation at pH 6.2.<sup>e</sup> From Ref. 40.<sup>f</sup> From Ref. 10.

rate than their standard errors would indicate.

The contributions of cross relaxation indicated by  $k_x$  (Table II) comprise the major portions of the values of  $k_1$  (Table I): for either variant, under all three pH conditions,  $k_x$  at 30°C amounts to about 90% of  $k_1$ . This could be expected, inasmuch as solutions of proteins ranging in molecular weight from 30 000 to 100 000, under various conditions of concentration and temperature, have been found to exhibit zero-field cross relaxation rates already roughly equal to water proton relaxation rates [16]. At higher frequencies, the ratio of cross relaxation to total proton relaxation must increase further, since cross relaxation, particularly for proteins of molecular weight above 20 000 and at resonant frequencies near 100 MHz, increases substantially with frequency [23,36], while the bound-water proton relaxation decreases (cf. Eqn. 7). This expectation should hold for the present data relating to a 37 000 dalton protein examined at 90 MHz. It is notable, however, that  $k_x$  at 2°C,

which for pH 4.67 and 6.2 amounts to more than 97% of  $k_1$ , for pH 8.0 shows a marked drop to 76%, in agreement with the predicted loss of spin diffusion in the more disordered state under these denaturation conditions.

#### Significance of parameters

In regard to the significance of the hydration parameter  $h$ , it is well recognized [2,4,41] that any technique used to measure hydration of proteins implies an operational definition of the water observed as 'bound' that pertains to that particular technique, as well as to the model used to analyze the data thus obtained. The results pertain to rotationally as well as irrotationally bound water, together with the effects of inter- as well as intramolecular contributions to relaxation. Cross-relaxation effects have already been mentioned. Anisotropic surface effects also may well play a role, as dealt with by Walmsley and Shporer [42] and Halle et al. [43].

Using these authors' approach, one can relate the hydration values shown in Table II to hydrodynamic hydration values in the literature by taking into account the effects of the anisotropic surface environment of the protein molecule. This involves the use of a scaling factor [42] or order parameter  $S$  [43], which is nearly independent of the specific protein, and which enters Eqns. 6 and 7 so as to change the  $R_b$  value by a factor of  $S^2$ , and consequently  $h$  (related to  $R_b$  by Eqn. 5b) by very nearly  $1/S^2$ . From the data of Koenig et al. [44], and the arguments of Walmsley and Shporer [42] as embodied in their Fig. 3, one obtains an estimate of  $S$  as approx. 0.21. Applying this, as indicated, to the values of  $h'$  of Table II, gives conventional hydrations from 0.43 to 0.50 for the pH 4.65 and 6.2 dimer at room temperature. This is squarely within the 0.30–0.54 range, clustering around 0.46, reported for  $\beta$ -lactoglobulin in the literature [2].

Without the use of  $S$ , the  $h'$  values of Table II, ranging from 0.019 for dimer at 30°C, pH 6.2, to 0.052 for the A variant at 2°C, pH 4.65, may be directly compared with results from a method based on deuterium NMR to avoid the effects of cross relaxation, which gave corresponding values from 0.015 to 0.043 (Table III, column II, of Ref. 17).

While the hydration values of Table II are smaller than the uncorrected ones of Table I (roughly half, or less), in either set the relationships between them under the various conditions are not greatly different. It is seen, therefore, that no matter in which way one prefers to interpret the data of Table I, one can obtain definite correlations with known structural information, and that, contrary to doubts expressed in the literature [15,16], relaxation measurements at the frequencies employed here contain considerable structural information relating to the solute protein. Furthermore, because we have been concerned mainly with changes rather than absolute values of  $h$ , other effects not specifically evaluated may largely cancel. Although absolute values obtained for a hydration parameter may be very dependent on model assumption, observed changes in such a parameter are less dependent on assumptions and can be equally useful in correlations with structural changes.

Returning to the questions posed at the outset, one sees that it is possible by a simple procedure to obtain useful hydration parameters which can account, at least in a qualitative fashion, for the effects of (i) cold-denaturation of  $\beta$ -lactoglobulin A at alkaline pH and (ii) of octamer formation of  $\beta$ -lactoglobulin at pH 4.65; (iii) give a quantitative account of the effect on relaxation of the marked difference between variants A and B in octamer formation; (iv) provide quantitative cross-relaxation information; and (v) confirm such conjectures as that of Beall et al. regarding correlation of water proton relaxation rate changes with changes in molecular states of a protein. It thus indicates that observed proton relaxation rate changes can be used by way of the parameter  $h$  or  $h'$  to evaluate concurrent structural changes in a given system.

## Appendix

### (A) Parameters at pH 4.65, 2°C from transverse relaxation increments and solvent relaxation rates

Summing the contributions to  $k_2$  of dimer and octamer for each variant according to Eqn. 5b, one has, from Table I, the two simultaneous equations:

$$k_{2,A} = 55.3 = 0.912h_O\Delta R_{2,O} + (1-0.912)h_D\Delta R_{2,D} \quad (8a)$$

and

$$k_{2,B} = 27.7 = 0.310h_O\Delta R_{2,O} + (1-0.310)h_D\Delta R_{2,D} \quad (8b)$$

where  $h_O$ , the hydration of the octamer, is that of the dimer,  $h_D$ , augmented by the contribution of the cavity; i.e.,  $h_O = h_D + 0.0264$ ; and  $\Delta R_{2,O}$  and  $\Delta R_{2,D}$  are the total excess transverse relaxation rates for octamer and dimer, respectively.  $R_{2,D}$  can be obtained by way of an estimate of the correlation time of the dimer,  $\tau_D$ , from the known dry volume of the latter, suitably corrected for its deviation from spherical shape.

The Einstein diffusion equation, expressing the rotary diffusion coefficient  $D_{rot}$  in terms of spherical volume  $V$ , provides a correlation time equal to  $V\eta/kT$  [25], where  $k$  is Boltzmann's constant,  $1.381 \cdot 10^{-16}$  erg/K, and  $\eta$  is the viscosity of solvent, approximated sufficiently by that of water, 0.01673 and 0.00801 poise at temperatures  $T$  of 275.2 and 303.2 K, respectively.  $V$  equals  $\bar{v}M/N$ , where  $\bar{v}$ , the partial specific volume of  $\beta$ -lacto-

globulin, is 0.751 ml/g [39],  $M$  is  $2 \cdot 18\,370$ , and  $N$  is Avogadro's number,  $6.022 \cdot 10^{23} \text{ mol}^{-1}$ . The correlation times for spheres then would be 20.18 ns at  $2^\circ\text{C}$  and 8.77 ns at  $30^\circ\text{C}$ . Application of the shape factor 1.168 for the elongated  $\beta$ -lactoglobulin dimer [35] gives values for  $\tau_D$  of 23.57 ns at  $2^\circ\text{C}$  and 10.24 ns at  $30^\circ\text{C}$ ; from Eqn. 8, together with the values of  $R_{2f}$  from Table I, one obtains for  $\Delta R_{2,D}$   $488.6 \text{ s}^{-1}$  at  $2^\circ\text{C}$  and  $231.8 \text{ s}^{-1}$  at  $30^\circ\text{C}$ .

Eqns. 8a and 8b can now be solved for the remaining unknowns, which are found to be  $h_D = 0.0276$  and  $\Delta R_{2,O} = 1096.8$ . It follows that  $h_O = 0.0276 + 0.264 = 0.0540$ . From  $\Delta R_{2,O}$  and  $R_{2f}$  from Table I at  $2^\circ\text{C}$ , one obtains  $R_{2b,O}$  and, by means of Eqn. 7, the correlation time  $\tau_{O,h}$  of the hydrated octamer, 53.82 ns. The latter may be regarded as the sum of weighted correlation times of dry octamer,  $\tau_O$ , and the cavity water,  $\tau_{cav} = V_{cav}\eta/kT$ . With the values of  $V_{cav}$  and  $\eta$  above,  $\tau_{cav}$  equals 2.839 ns at  $2^\circ\text{C}$  and 1.234 ns at  $30^\circ\text{C}$ . Thus, at  $2^\circ\text{C}$ ,  $53.82 = (0.0276/0.0540)\tau_O + (0.0264/0.0540)(2.839)$ , and  $\tau_O = 102.6$  ns. But from  $\tau_O = V\eta/kT = 4\pi R_{s,O}^3/3kT$ , where  $R_{s,O}$  is the Stokes radius of the octamer, follows  $R_{s,O} = 3.82$  nm.

#### (B) Cross-relaxation increment

The cross-relaxation model results in a set of simultaneous differential equations for the coupled magnetization decays of water and protein protons in terms of: corresponding reduced magnetizations,  $M_W(t)$  and  $M_P(t)$ , defined as  $M(t) \equiv (A_\infty - A_t)/2A_\infty$  (cf. Eqn. 1); corresponding longitudinal relaxation rates in the absence of cross relaxation,  $R_W$  and  $R_P$ ; the rate of magnetization transfer from water to protein protons,  $R_T$ ; and the ratio of water to protein protons,  $n_W/n_P$ . Standard methods lead to a double-exponential solution of the form [16,22,38]:

$$M_{W,P}(t) = C_{W,P}^+ \exp(-R^+t) + C_{W,P}^- \exp(-R^-t) \quad (9)$$

where  $R^+$  and  $R^-$  refer to two apparent relaxation rates, fast and slow respectively, which are the same for both kinds of proton and are given by:

$$2R^\pm = R_P + R_W + R_T n_W/n_P + R_T \pm \left[ (R_P - R_W + R_T n_W/n_P - R_T)^2 + 4R_T^2 n_W/n_P \right]^{1/2} \quad (10)$$

Judging from the data of Koenig et al. [16],

where neglecting the  $4R_T^2 n_W/n_P$  term in Eqn. 10 in comparison with the terms preceding it resulted in less than 5% error, we may approximate the component rates of the double-exponential relaxation by:

$$R^+ \approx R_P + R_T n_W/n_P \text{ and } R^- \approx R_W + R_T \quad (10a)$$

In dilute solutions,  $n_W \gg n_P$ , and  $R^+$  becomes so large that the first right-hand term of Eqn. 9 is small compared to the second term. (This, together with a pulse-width dependence, was the reason why the double exponential was not detectable in Fig. 1A and B.) The observed relaxation rate then becomes  $R \approx R_W + R_T$ , and Eqn. 3 for longitudinal relaxation can be rewritten as:

$$R_{obs} = p_b R_b + p_f R_f + R_T \quad (3')$$

Since for dilute solutions  $R_T$  should be proportional to the protein concentration  $c$ , or  $R_T \equiv k_x c$ , Eqns. 4 and 5b can be rewritten:

$$R_{obs} = R_f + [(h'\Delta R) + k_x]c = R_f + (k'_1 + k_x)c \quad (4')$$

where

$$k'_1 = h'\Delta R, \quad k_1 = k'_1 + k_x \quad (5b')$$

and the observed relaxation increment now equals the sum of the hydration product and the cross-relaxation increment. The term  $k_x$ , it should be noted, represents  $R_T$  only approximately, reflecting in addition the combined effects of the simplifying assumptions made above.

#### (C) Parameters at pH 4.65, $2^\circ\text{C}$ , from longitudinal relaxation increments and solvent relaxation rates

In analogy to Eqns. 8a and b, one has:

$$k_{1,A} = 3.56 = (0.912)(0.0540)\Delta R_{1,O} + (1 - 0.912)(0.0276)\Delta R_{1,D} + k_{x,A} \quad (11a)$$

and

$$k_{1,B} = 2.18 = (0.310)(0.0540)\Delta R_{1,O} + (1 - 0.310)(0.0276)\Delta R_{1,D} + k_{x,B} \quad (11b)$$

Here,  $\Delta R_{1,O}$  and  $\Delta R_{1,D}$  are found from Eqn. 6

with  $\tau_{O,h} = 53.82$  and  $\tau_D = 23.57$  ns, together with the appropriate  $R_{1f}$  from Table I, as 0.969 and  $2.936 \text{ s}^{-1}$ , respectively. With these values,  $k_{x,A} = 3.50$  and  $k_{x,B} = 2.11$ . Considering each of these, in turn, to be made up of contributions from octamer,  $k_{x,O'}$  and dimer,  $k_{x,D'}$  one has the two equations:

$$k_{x,a} = (0.912)k_{x,O} + (1.088)k_{x,D} \quad (12a)$$

$$k_{x,b} = (0.310)k_{x,O} = (0.690)k_{x,D} \quad (12b)$$

which yield  $k_{x,O} = 3.71$  and  $k_{x,D} = 1.39$ .

### Acknowledgments

The authors express their thanks to Dr. M.J. Kronman for valuable suggestions and to Dr. T.F. Kumosinski for critical discussions.

### References

- 1 Hazlewood, C.F. (ed.) (1973) *Ann. N.Y. Acad. Sci.* 204, Parts II and V, New York Academy of Sciences, New York
- 2 Kuntz, I.D. and Kauzmann, W. (1974) *Adv. Protein Chem.* 28, 239-345
- 3 Rowland, S.P. (ed.) (1980) *Water in Polymers*, pp. 11-322, American Chemical Society Symposium Series, Washington D.C.
- 4 Cooke, R. and Kuntz, I.D. (1974) *Annu. Rev. Biophys. Bioeng.* 3, 95-126
- 5 Lynch, L.J. and Webster, D.S. (1975) *J. Polym. Sci., Polym. Symp.* 49, 43-63
- 6 Beall, P.T., Hazlewood, C.F. and Rao, P.N. (1976) *Science* 192, 904-907
- 7 Timasheff, S.N. and Townend, R. (1961) *J. Am. Chem. Soc.* 83, 464-469
- 8 Timasheff, S.N. and Townend, R. (1962) *J. Dairy Sci.* 45, 259-266
- 9 Timasheff, S.N. (1964) in *Symposium on Foods: Proteins and Their Reactions* (Schultz, H.W. and Angelmeier, A.F., eds.), p. 174, Avi, Westport, CT
- 10 Timasheff, S.N. and Townend, R. (1964) *Nature (Lond.)* 203, 517-519
- 11 Townend, R. (1965) *Arch. Biochem. Biophys.* 109, 1-6
- 12 Kumosinski, T.F. and Timasheff, S.N. (1966) *J. Am. Chem. Soc.* 88, 5635-5642
- 13 Weissbluth, M.D. and Grieger, R.A. (1974) *Biochemistry* 13, 1285-1288
- 14 Grösch, L. and Noack, F. (1976) *Biochim. Biophys. Acta* 453, 218-232
- 15 Hallenga, K. and Koenig, S.H. (1976) *Biochemistry* 15, 4255-4264
- 16 Koenig, S.H., Bryant, R.G., Hallenga, K. and Jacob, G.S. (1978) *Biochemistry* 17, 4348-4358
- 17 Kumosinski, T.F. and Pessen, H. (1982) *Arch. Biochem. Biophys.* 218, 286-302
- 18 Aschaffenburg, R. and Drewry, J. (1955) *Nature (Lond.)* 176, 218-219
- 19 Townend, R., Winterbottom, R.J. and Timasheff, S.N. (1960) *J. Am. Chem. Soc.* 82, 3161-3168
- 20 Vold, R.L., Waugh, J.S., Klein, M.P. and Phelps, D.E. (1968) *J. Chem. Phys.* 48, 3831-3832
- 21 Farrar, T.C. and Becker, E.D. (1971) *Pulse and Fourier Transform NMR*, p. 92, Academic Press, New York
- 22 Zimmerman, J.R. and Brittin, W.E. (1957) *J. Phys. Chem.* 61, 1328-1333
- 23 Daskiewicz, O.K., Hennel, J.W., Lubas, B. and Szczepkowski, T.W. (1963) *Nature (Lond.)* 200, 1006-1008
- 24 Solomon, I. (1955) *Phys. Rev.* 99, 559-567
- 25 Poole, C.P. and Farach, H.A. (1971) *Relaxation in Magnetic Resonance*, p. 65, Academic Press, New York
- 26 Eigel, W.N., Butler, J.E., Ernstrom, C.A., Farrell, H.M., Jr., Harwalker, V.R., Jenness, R. and Whitney, R. McL. (1984) *J. Dairy Sci.* 67, 1599-1631
- 27 Townend, R., Weinberger, L. and Timasheff, S.N. (1960) *J. Am. Chem. Soc.* 82, 3175-3179
- 28 Georges, C., Guinand, S. and Tonnelat, J. (1962) *Biochim. Biophys. Acta* 59, 737-739
- 29 McKenzie, H.A. and Sawyer, W.H. (1967) *Nature (Lond.)* 214, 1101-1104
- 30 Roels, H. Préaux, G. and Lontie, R. (1971) *Biochimie* 53, 1085-1093
- 31 Brown, E.M. and Farrell, H.M., Jr. (1978) *Arch. Biochem. Biophys.* 185, 156-164
- 32 Green, D.W., Aschaffenburg, R., Camerman, A., Coppola, J.C., Dunnill, P., Simmons, R.M., Komorowski, E.S., Sawyer, L., Turner, E.M.C. and Woods, K.F. (1979) *J. Mol. Biol.* 131, 375-397
- 33 Dayhoff, M.O. (ed.) (1973) *Atlas of Protein Sequence and Structure*, Vol. 5, Suppl. 1, Nat. Biomed. Res. Found., Washington, D.C.
- 34 Cohn, E.J. and Edsall, J.T. (1943) *Proteins, Amino Acids, and Peptides*, p. 557, Reinhold Publishing Corp., New York
- 35 Wahl, P. and Timasheff, S.N. (1969) *Biochemistry* 8, 2945-2949
- 36 Kalk, A. and Berendsen, H.J.C. (1976) *J. Magn. Resonance* 24, 343-366
- 37 Shirley, W.M. and Bryant, R.G. (1982) *J. Am. Chem. Soc.* 104, 2910-2918
- 38 Edzes, H.T. and Samulski, E.T. (1978) *J. Magn. Resonance* 31, 207-229
- 39 Lee, J.C. and Timasheff, S.N. (1974) *Biochemistry* 13, 257-265
- 40 Pessen, H., Kumosinski, T.F. and Timasheff, S.N. (1973) *Methods Enzymol.* 27, 151-209
- 41 Na, G.C. and Timasheff, S.N. (1981) *J. Mol. Biol.* 151, 165-178
- 42 Walmsley, R.H. and Shporer, M. (1978) *J. Chem. Phys.* 68, 2584-2590
- 43 Halle, B., Andersson, T., Forsén, S. and Lindman, B. (1981) *J. Am. Chem. Soc.* 103, 500-508
- 44 Koenig, S.H., Hallenga, K. and Shporer, M. (1975) *Proc. Natl. Acad. Sci. USA* 72, 2667-2671
- 45 Tanford, C., Bunville, L.G. and Nozaki, Y. (1959) *J. Am. Chem. Soc.* 81, 4032-4035