Biophysical Chemistry 4 (1976) 181–190

SELF-ASSOCIATION OF β-LACTOGLOBULIN C IN ACETATE BUFFERS*

Jerry L. SARQUIS** and E.T. ADAMS, Jr.***
Chemistry Department, Texas A&M University, College Station, Texas 77843, USA

Received 11 November 1975

The self-association of β-lactoglobulin C at pH 4.65 (23° C) in acetate buffer has been studied at various temperatures, 10, 16, 20 and 25° C, by a series of sedimentation equilibrium experiments. Two different buffers were used. Buffer I with an ionic strength of 0.1 consisted of 0.1M acetic acid and 0.1M sodium acetate; buffer II had 0.1M KCl in addiation so that its ionic strength was 0.2. The variation of the apparent weight average molecular weight, M_{wa}, with the total solute concentration, c, was characteristic of a self-association. In contrast to the behavior of β-lactoglobulin A in acetate buffer, the association of β-lactoglobulin C did not proceed beyond dimer. Furthermore, within the experimental error, the self-association of β-lactoglobulin C was independent of temperature and ionic strength; all experimental data could be put on the same M_{wa}(or M_1/M_{wa}) vs. c plot! Several models were used to test the self-association, and a monomer–dimer association with $K_2 = 2.10 \times 10^3$ dl/g and $BM_1 = -1.2 \times 10^{-2}$ dl/g seemed to give a good description of the M_1/M_{wa} vs. c curve.

1. Introduction

The self-association of the bovine β-lactoglobulins has previously been studied under a variety of solution conditions [1–13]. There are four known genetic variants of the β-lactoglobulins, designated as the A, B, C and D variants [11,13]; hereafter, we shall refer to these β-lactoglobulin variants as βA, βB, βC and βD. There is also a carbohydrate-containing variant of type A found in Droughtmaster cattle [11,13]. These four variants differ from one another by amino acid substitutions at only four of the 162 amino acid residues [11,14]. For example, the amino acid composition of βC differs from βA by only three amino acid residues: βC has a glycine, a histidine and an alanine replacing an aspartic acid, a glutamine and a valine, respectively. The amino acid substitutions for the four variants are summarized in table 1. Thus, the β-lactoglobulins are ideally suited for the study of the effects of amino acid substitution on the self-association behavior.

Here we report on some studies of the self-association of βC by low speed sedimentation equilibrium experiments in 0.2M acetate buffer (ph 4.65 at 23° C). Two buffers were used: buffer I consisted only of sodium acetate and acetic acid and has an ionic strength of 0.1; buffer II had KCl in addition so that its ionic strength was 0.2. Experiments in buffer I were carried out at 10, 16, 20 and 25° C, while the experiments in buffer II were performed at 10 and 25° C only. We will compare the self-association behavior of βC under these solution conditions with the self-association of βA under similar solution conditions [6,15]. We will also contrast the self-association behavior of βC in acetate buffers with that found in glycine buffers [13]. Thus, we will be able to see differences in self-association behavior arising from both genetic variation and changes in solution conditions. We find that a stronger monomer–dimer association is present with βC in the acetate buffers than was encountered in the glycine buffers. Interestingly, the self-association of βC in acetate buffers is independent of both temperature and ionic strength over the ranges studies, whereas βC was reported to undergo a temperature-dependent, monomer–dimer self-association in glycine buffers [13]. In contrast βA was found to undergo a temperature-dependent, monomer–dimer association in glycine buffer

* This paper X in the series: Sedimentation Equilibrium in Researching Systems.

** Present address: Miami University – Middletown, 4200 E. University Blvd., Middletown, Ohio 45042, USA.
*** Please address all correspondence to Dr. E.T. Adams, Jr.

Table 1
Amino acid residue sequence of bovine β-lactoglobulin A, B, C and D [a]

				5					10				15
LEU	ILE	VAL	THR	GLN	THR	MET	LYS	GLY	LEU	ASP	ILE	GLN	LYS	VAL
				20					25				30
ALA	GLY	THR	TRP	TYR	SER	LEU	ALA	MET	ALA	ALA	SER	ASP	ILE	SER
				35					40				45
LEU	LEU	ASP	ALA	GLN	SER	ALA	PRO	LEU	ARG	VAL	TYR	VAL	GLU	GLU
				50					55				60
LEU	LYS	PRO	THR	PRO	GLU	GLY	ASP	LEU	GLU	ILE	LEU	LEU	GLN	LYS
				65					70				75
TRP	GLU	ASN	64	GLU	CYS	ALA	GLN	LYS	LYS	ILE	ILE	ALA	GLU	LYS
				80					85				90
THR	LYS	ILE	PRO	ALA	VAL	PHE	LYS	LEU	ASP	ALA	ILE	ASN	GLU	ASN
				95					100				105	
LYS	VAL	LEU	VAL	LEU	ASP	THR	ASP	TYR	LYS	LYS	TYR	LEU	LEU	PHE
				110									120	
CYS	MET	108	ASN	SER	ALA	GLU	PRO	GLU	115	SER	LEU	118	CYS	GLN
				125					130				135	
CYS	LEU	VAL	ARG	THR	PRO	GLU	VAL	ASP	ASP	GLU	ALA	LEU	GLU	LYS
				140					145				150	
PHE	ASP	LYS	ALA	LEU	LYS	ALA	LEU	PRO	MET	HIS	ILE	ARG	LEU	SER
				155					160					
PHE	ASN	PRO	THR	LEU	GLN	GLU	GLU	GLN	CYS	HIS	ILE			

residue number	A	B	C	D
64	ASP	GLY	GLY	GLY
108	GLU	GLU	GLU	GLN
115	GLN	GLN	HIS	GLN
118	VAL	ALA	ALA	ALA

[a] Based on the data of Braunitzer et al. [14]; see also ref. [11].

[12] and a temperature-dependent self-association in acetate buffer [15].

2. Preparation of protein solutions

The β-lactoglobulin C was kindly provided by Drs. J.J. Basch and Edwin A. Kalan of the Eastern Utilization Research and Development Division Agricultural Research Service, U.S. Department of Agricultural. It was the same βC protein preparation that was used in previously reported studies in glycine buffers [13]. Two different buffer solutions were used. Buffer I contained 0.1M acetic acid and 0.1M sodium acetate, pH 4.65 at 23° C, ionic strength (I) 0.1. This buffer was the same as had been used previously in some studies on the self-association of βA [6,15]. Buffer II contained 0.1M acetic acid, 0.1 sodium acetate, 0.1M KCl, pH 4.66 at 23° C, $I = 0.2$. The preparation and dialysis of the protein solutions is identical to that reported previously [13]. The protein concentrations were measured by differential refractometry in the same manner as before, and the partial specific volume of βC was again assumed to be the same as that for βA, namely $\bar{v} = 0.751$ at 20° C [13,16]. Protein concentrations are reported in terms of fringes, J, at $\lambda = 632.8$ nm for a 12 mm ultracentrifuge cell centerpiece. Values of the concentration and the partial specific volume were corrected for the different temperatures using previously described methods [17]. At $\lambda = 632.8$ nm and 25° C, $J = 33.94c$, where the concentration c is expressed in g/dl.

3. Ultracentrifugation

Beckman Model E analytical ultracentrifuges were used for these experiments. Three different instruments

were used. One ultracentrifuge was equipped with a modulatable, helium–neon laser light source and a multiplexer, which allowed three solutions of different concentrations to be studied at the same time under identical conditions. For the most dilute solutions, an ultracentrifuge with an ultraviolet, photoelectric scanner was used. Both 12 and 30 mm centerpieces were used, but all concentration data are reported in terms of 12 mm fringes at $\lambda = 632.8$ nm, the wavelength of the laser light. In the runs made on the ultracentrifuge equipped with the photoelectric scanner, the absorbance was measured spectrophotometrically during the experiment. The value of the extinction coefficient used to calculate the protein concentration was $E^{1\%}_{278} = 9.2$ dl/gm cm which was the value suggested by Visser et al. for βB [8]. These concentrations were also converted to fringes ($\lambda = 632.8$ nm, $h = 12$ mm). Speeds of 10 000 and 11 000 rpm were used and the usual experimental techniques were followed [12,13]. The cell filling and cleaning was done as reported previously [6].

4. Evaluation of M_{wa}, M_{na} and $\ln f_a$

All protein solutions were dialyzed against the appropriate buffer so that the associating solute would be defined by the Vrij–Overbeek [18,19] and Casassa–Eisenberg [20] conventions. The equilibrium constants and second virial coefficients which are reported refer to an associating solute defined by these conventions. Since the samples were dialyzed, one could also use sedimentation equilibrium equations analogous to those for a two-component system [20].

We have made the usual assumptions concerning the associating species [5,6,8,21], namely, that (i) the partial specific volumes are equal, (ii) the refractive index increments are equal, and (iii) the natural logarithm of the activity coefficients, y_i, for species i is given by

$$\ln y_i = i\mathrm{BM}_1 c\ , \quad i = 1, 2, \ldots , \tag{1}$$

where BM_1 is the second virial coefficient and c is the associating solute concentration. With these assumptions, we can obtain the apparent weight average molecules weight, M_{wa}, from

$$\frac{1}{A}\frac{\mathrm{d}\ln c}{\mathrm{d}(r^2)} = M_{wa}\ . \tag{2}$$

Here c represents the associating solute concentration at radial position r ($r_m \leqslant r \leqslant r_b$) in the solution column of the ultracentrifuge cell. The quantities A and M_{wa} have their usual definitions [5,6,8,21]:

$$A = (1 - \bar{v}\rho)\omega^2/2RT \tag{3}$$

and

$$M_{wa} = M_{wc}/(1 + \mathrm{BM}_{wc}c)\ . \tag{4}$$

Here M_{wc} is the true weight average molecular weight. Since both M_{wa} and M_{wc} are functions of the total solute concentration, c, several experiments with different initial concentrations can give values of M_{wa} over the relatively wide concentration range necessary to successfully analyze self-associations. Fig. 1 shows a plot of M_{wa} vs. J for several experiments at 20° C in buffer I; the increase in M_{wa} and the subsequent levelling off with increasing J is characteristic of a self-association. In fig. 2 we have shown M_{wa} vs. J data obtained at different temperatures in both buffers. Note that within the limits of our experimental precision, there appears to be no dependence on temperature or ionic strength for the self-association of βC in acetate buffers. As can be seen from these plots, the association is quite strong. Thus, we relied upon the monomer molecular weight, M_1, of 18 344 Daltons determined from the amino acid composition [22,23] rather than an extrapolation of the low concentration data to zero concentration.

Fig. 3 shows a plot of M_1/M_{wa} vs. J for the data we collected. To construct fig. 3 we drew a smooth curve through the plot in fig. 2 and calculated values of M_1/M_{wa} (open circles in fig. 3) for various values of J from the smooth curve. Values of M_1/M_{wa} vs. J were used to determine M_{na}, the apparent number average molecular weight, and $\ln f_a$, the natural logarithm of the apparent weight fraction of monomer. The methods for the evaluation of M_{na} and $\ln f_a$ have been reported in detail previously [21]. When eq. (1) applies, M_{na}, M_{wa} and $\ln f_a$ can be combined in such a way as to eliminate the second virial coefficient, BM_1. Two such functions are

$$\xi = \frac{2M_1}{M_{na}} - \frac{M_1}{M_{wa}} = \frac{2M_1}{M_{nc}} - \frac{M_1}{M_{wc}} \tag{5}$$

and

$$\eta = \frac{M_1}{M_{wa}} - \ln f_a = \frac{M_1}{M_{wc}} - \ln f_a\ . \tag{6}$$

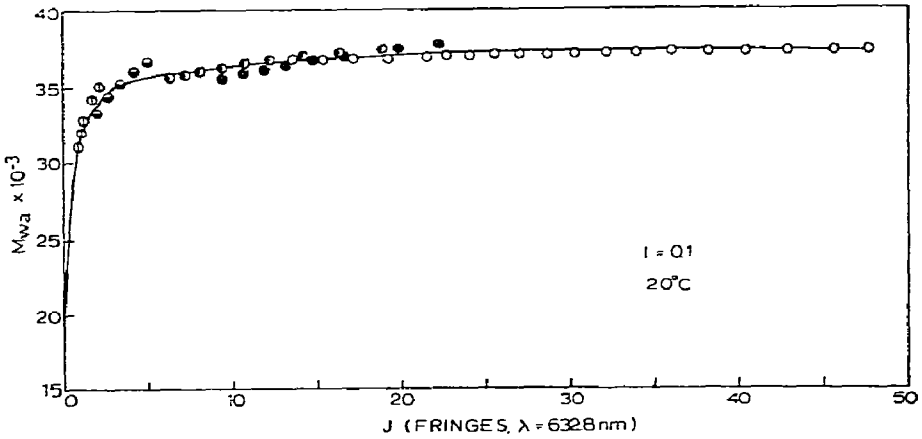

Fig. 1. Plot of M_{wa} vs. J for β-lactoglobulins C in buffer I at 20° C. The increase in M_{wa} with J and subsequent levelling off is characteristic of some self-associations.

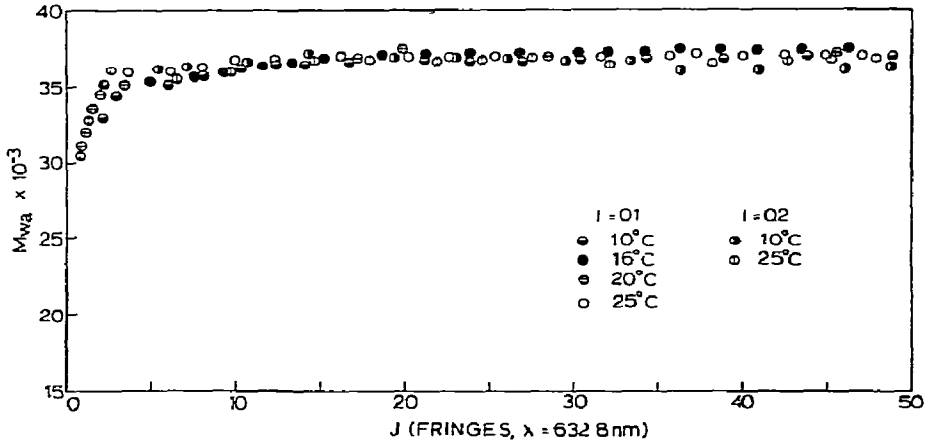

Fig. 2. Plot of M_{wa} vs. J for β-lactoglobulin C in acetate buffers. Note how the data collected under various solutions conditions appear to fall on the same plot. This indicates that the self-association of β-lactoglobulin C is not affected by changes in temperature or ionic strength.

These functions can be used to test for the presence of some self-associations and also for the analysis of these self-associations [12,24,26]. In eqs. (5) and (6) f_1 is the weight fraction of monomer and M_{nc} and M_{wc} are the true number and weight average molecular weights, respectively.

5. Tests for the type of association present

5.1. Monomer–n-mer associations

A monomer–n-mer association is described by

$$nP_1 \rightleftarrows P_n, \quad n = 2,3, \ldots, \tag{7}$$

where P represents the associating solute. With a very strong association as found in the acetate buffers, the usual analysis for a monomer–n-mer association is more difficult due to greater uncertainties in the values of M_{na} and $\ln f_a$, particularly the latter. Both quantities are dependent upon extrapolation of the low concentration data, and as the association becomes stronger, these quantities are subject to more error. However, it seemed clear from the experimental data that the predominant species was dimer. Since values of M_{wa}

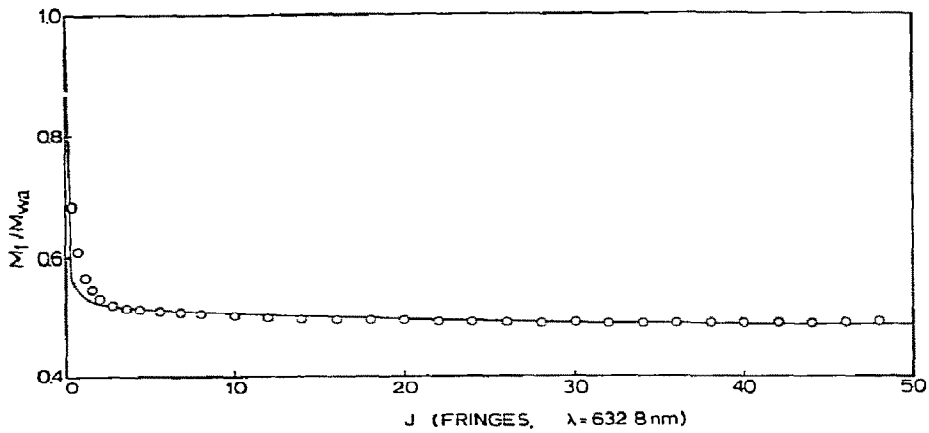

Fig. 3. Plot of M_1/M_{wa} vs. J. A smooth curve was drawn through the plot in fig. 2; the values of M_1/M_{wa} vs. J indicated by the open circles were obtained from fig.2. The solid line shows the curve obtained using $K_2 = 2.10 \times 10^3$ dl/g and $BM_1 = -0.012$ dl/g.

slightly greater than dimer were found, a monomer-dimer model invoking a negative virial coefficient was tried. Trial values of K_2 and BM_1 were chosen, and values of M_1/M_{wa} over the concentration range studied were calculated from these parameters. These values of K_2 and BM_1 were varied until a minimum in the square of the deviations between the calculated and experimental values of M_1/M_{wa} was obtained. This procedure is illustrated in table 2. The values of the association equilibrium constant, K_2, and the second virial coefficient, BM_1, that were chosen were $K_2 = (2.10 \pm 0.20) \times 10^3$ dl/g and $BM_1 = -0.012 \pm 0.002$ dl/g. The solid line in fig. 3 shows the curve of M_1/M_{wa} vs. J calculated using these values.

Other monomer–n-mer self-associations, from $n = 3$ (monomer–trimer) to $n = 8$ (monomer–octamer), were tried. Fig. 4 shows a standard plot of $(1 - f_1)/f_1$ vs. $(cf_1)^{n-1}$ for $n = 3$ and $n = 4$ (monomer–tetramer). As one can see, the plots are not linear as required by the theory. The corresponding plot for $n = 2$ (monomer–dimer) gave scattered points, and thus could not be used for the evaluation of K_2 in the customary manner. Again, this is thought to be a result of the error in the extrapolation of the M_1/M_{wa} curve to zero concentration. The uncertainty of this extrapolation increases as the association increases.

As a further check, we calculated the concentration at various radial positions in two of the cells. Then the calculated concentrations obtained using $K_2 = 2.10 \times 10^3$ dl/g and $BM_1 = -0.012$ dl/g were compared with the experimentally obtained concentrations. Fig. 5 shows the percent deviation of the calculated values. All values are within one percent of the experimentally obtainted values.

Table 2
Trail values of K_2 and BM_1

K_2 (dl/g)	BM_1 (dl/g)	$\sum_i (\delta_i)^2$ a)
1.90×10^3	−0.012	0.455×10^{-3}
2.10	−0.012	0.448
2.30	−0.012	0.451
2.10	−0.011	0.468
2.10	−0.012	0.448
2.10	−0.013	0.467

a) $\delta_i = [(M_1/M_{wa})_{obs} - (M_1/M_{wa})_{calc}]_i$.

5.2. Indefinite self-associations

Several other models were checked to see if the experimental data could be reproduced by these models. A sequential, indefinite self-association is represented by

$$nP_1 \rightleftarrows qP_2 + hP_3 + \ldots . \quad (8)$$

Two types of associations can be represented by eq. (8). In type I (also known as an isodesmic association) all molar equilibrium constants are assumed to be equal.

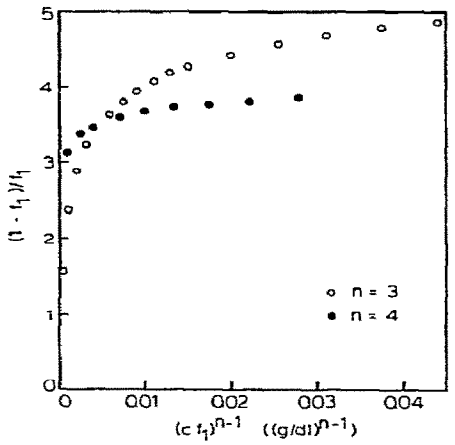

Fig. 4. Test for a monomer–trimer ($n = 3$) and monomer–tetramer ($n = 4$) association. If either model were correct a plot of $(1 - f_1)/f_1$ vs. $(cf_1)^{n-1}$ would give a straight line going through or close to the origin. The curvature in these plots indicates the failure of these models.

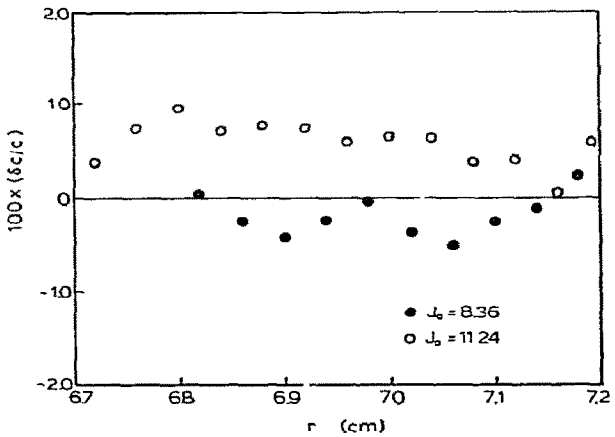

Fig. 5. Deviation plot of 100 ($\delta c/c$) vs. r. Here $\delta c = c_r(\text{calc}) - c_r(\text{obs})$ and $c_r(\text{calc}) = c_{1m}\exp\phi'_{1r} + K_2 c_{1m}^2 \exp 2\phi'_{1r}$. The quantity $\phi'_{1r} = AM_1(r^2 - r_m^2) - BM_1(c_r - c_m)$. Here we used $K_2 = 2.10 \times 10^3$ dl/gm and $BM_1 = -0.012$ dl/g. Note that the deviation plots for two different c_0's is less then 1%.

In type II the molar association constant for dimerization, K_{12}, is assumed to be different from the other association constants, but all other association constants are assumed to be equal. For type I the quantity $\sqrt{f_1}$ can be evaluated from ξ [12,13,24–26]; here f_1 is the weight fraction of monomer. A plot of $1 - \sqrt{f_1}$ vs. Cf_1 is linear and should go through or close to the origin when a type I sequential, indefinite association is present. Such a plot for βC is shown in fig. 6. Since this plot is not linear, it appears that a type I indefinite self-association is not present. The related type II indefinite self-association was also tested and discarded. The even indefinite self-association where trimer, pentamer, heptamer, etc. are absent can be represented by

$$nP_1 \rightleftarrows qP_2 + hP_4 + mP_6 + \ldots . \qquad (9)$$

Again, there are two cases that were tested: one in which all molar equilibrium constants are equal (type III), and one where K_{12} differs from all other molar equilibrium constants (type IV). Neither of these cases adequately described the experimental data.

5.3. Monomer–dimer–trimer and monomer–dimer–tetramer

In addition, two discrete self-associations involving

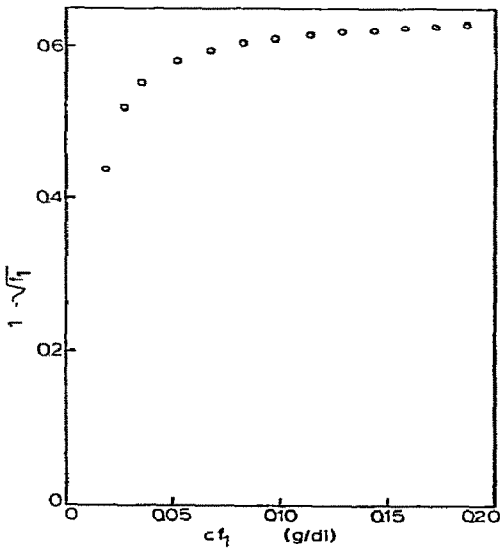

Fig. 6. Test for a type I sequential, indefinite self-association with all molar equilibrium constants equal (an isodesmic self-association). If this model were correct, then a plot of $(1 - \sqrt{f_1})$ vs. Cf_1 would give a straight line passing through or close to the origin. The curvature in this plot indicates the failure of this model.

three species were tried; these were the monomer–dimer–trimer and the monomer–dimer–tetramer associations. Here we used a variation [26] of the methods used by Ferguson et al. [25] to analyze these associations. Again, neither of these models described the data as well as the monomer–dimer model did. A plot similar to that shown in fig. 6 of the paper by Sarquis and Adams [13] gave deviations of less than 2% except at very low concentrations. This we believe is due to the deviation between the calculated and observed M_1/M_{wa} vs. J curve (see fig. 3). If we tried to fit the M_1/M_{wa} vs. J curve in fig. 3 better in this low concentration region, we got poorer fits in the higher concentration region and vice versa. Some details of the tests for various self-associations are summarized in the paper by Adams et al. [27].

5.4. A new check for a monomer–dimer association

We also checked the adequacy of the monomer–dimer model by using a graphical method of analysis which is based on the concentration of solute, c_r, as a function of radial distance, r, in the solution column of an ultracentrifuge cell. This method of analysis, based on c_r, has been recently developed by Chun and Kim [28]. Plots of $c_r - c_{r_0}$ vs. $r^2 - r_0^2$ ($r_m \leqslant r_0$ or $r \leqslant r_b$) were made, where c_{r_0} was the concentration at an arbitrary radial position r_0. For the analysis of a monomer–dimer association, the quantities c_{r_0}, c_{r_1}, and c_{r_2} are required; these latter two concentrations are related to c_{r_0} by

$$c_{r_1} = c_{r_0} + \Delta c\,, \quad c_{r_2} = c_{r_0} + 2\Delta c\,. \tag{10}$$

Here Δc is an increment of c_r chosen such that c_{r_2} will be a concentration occuring near the bottom of the cell. In addition, the quantities ϕ_{r_1} and ϕ_{r_2} are evaluated using the equation

$$\phi_r = \exp[\mathrm{AM}_1(r^2 - r_0^2)]\,. \tag{11}$$

Here r_1 and r_2 are the radial positions at which the concentrations are c_{r_1} and c_{r_2}, respectively. The values of $r_1^2 - r_0^2$ and $r_2^2 - r_0^2$ are chosen from a plot of $c_r - c_{r_0}$ vs. $(r^2 - r_0^2)$. For a monomer–dimer association, the Chun–Kim method [28] gives

$$\beta^4(-c_{r_0}\phi_{r_1}\phi_{r_2}^2) + \beta^3(c_{r_1}\phi_{r_2}^2 + c_{r_0}\phi_{r_1}^2\phi_{r_2}) - \beta(c_{r_2}\phi_{r_1}^2 + c_{r_1}\phi_{r_2}) + c_{r_2}\phi_{r_1} = 0\,. \tag{12}$$

Here β is defined by

$$\beta = \exp[-\mathrm{BM}_1\Delta c]\,, \tag{13}$$

and BM_1 can be obtained from

$$\mathrm{BM}_1 = -(\ln\beta)/(\Delta c)\,. \tag{14}$$

Values of BM_1 were tried until a solution of eq. (12) was found. Several values of BM_1 may satisfy eq. (12), but only one will be meaningful, i.e., a value of BM_1 which leads to a negative value of K_2 is physically meaningless. Once the value of BM_1 is determined, then the concentrations of monomer, c_{1r_0}, dimer, c_{2r_0}, are found. Then K_2 can be evaluated from the usual relation

$$K_2 = c_{2r_0}/c_{1r_0}^2\,. \tag{15}$$

The reader is referred to eqs. (18) and (19) in Chun and Kim's paper [28] for further details.

We assumed that a monomer–dimer self-association was present, and we analyzed the data from two different experiments as defined above. In both cases, the only physically meaningful solutions required negative values of BM_1, which is consistent with the results of the analysis discussed earlier. Presumably, the choice of Δc is arbitrary, so several different choices of Δc can be made for one experiment. We chose a large value of Δc in order to minimize error. Still, we found that the values of BM_1 and K_2 were not the same for each experiment. The effect of uncertainty in the graphical interpolation of $r^2 - r_0^2$ values was analyzed. It was found that the error involved in reading the graph did cause very significant variations in the values of BM_1 and K_2 that were determined. This is illustrated in table 3. We also tried using the approach of Chum and Kim [28] to analyze the monomer–dimer self-association of βC in glycine buffers which we have previously reported [13]. Here we found much better agreement. We applied the Chun–Kim method to three different experiments with βC in 0.2M glycine buffer (pH 2.46, $I = 0.1$) at 10° C. The average values obtained were $K_2 = 27.4$ dl/g (range 19.1 – 38.7) and $\mathrm{BM}_1 = 0.144$ dl/g (range 0.135 – 0.154). The previously reported [13] values were $K_2 = 27.2$ dl/g and $\mathrm{BM}_1 = 0.146$ dl/g. The difficulty with the analysis in the acetate buffer is most likely due to difficulties inherent with extremely strong associating systems.

Table 3
Effect of error in radial position on the analysis of β-lactoglobulin C by the method of Chun and Kim [28]

$r_1^2 - r_0^2$ (cm^2)	$r_2^2 - r_0^2$ (cm^2)	BM_1 (dl/g)	K_2 (dl/g)
3.040	4.850	−0.021	960
3.045	4.850	−0.028	645
3.040	4.855	−0.016	1220

ΔJ = 7.5 fringes J_0 = 8.36 fringes
T = 283.15 K Run No. 92-187-5

6. Thermodynamic functions for the self-association of βC

Generally, from values of K_2 at different temperatures, one can make a plot of ln K_2 vs. $1/T$, the van't Hoff plot, and evaluate ΔH^0, the standard enthalpy change, from the slope of this plot. Having ΔH^0, one can determine ΔS^0, the standard entropy change, from the values of ΔG^0 and ΔH^0, since

$$\Delta S^0 = (\Delta H^0 - \Delta G^0)/T . \quad (16)$$

However, since there appears to be no significant temperature dependence of the self-association of βC in the acetate buffers, this implies that $\Delta H^0 \approx 0$ within the limits of our experimental measurements. Since $\Delta H^0 \approx 0$, the association reaction is athermic and

$$\Delta S^0 \approx -\Delta G^0/T \quad (\text{for } \Delta H^0 \approx 0). \quad (17)$$

The value K_2 in dl/g can be converted to a molar equilibrium constant, k_2 (ℓ/mol), by the relation

$$k_2 = M_1 K_2/20 . \quad (18)$$

Table 4 lists the values of K_2, k_2, BM_1, ΔG^0 and the other thermodynamic functions.

7. Discussion

The self-association behavior of βC in 0.2M acetate buffer (pH ≈ 4.7) is best described as a nonideal, monomer–dimer association. This is the same model that described the self-association of βC in 0.2M glycine buffer (pH ≈ 2.5) [13], but the strength of the association, as judged by values of $M_1/M_{w\bar{a}}$ vs. J, is much stronger in the acetate buffer. For βC in the acetate buffer, $K_2 = 2.10 \times 10^3$ dl/g, while in the glycine buffer, the strongest monomer–dimer association (observed at 10° C, pH ≈ 2.5, I = 0.2) had K_2 = 60 dl/g. The increased strength of the association in the acetate buffers may be a reflection of weaker electrostatic repulsions between subunits, since the solution conditions are nearer to the isoionic points of the protein. The values of BM_1 in glycine and in acetate buffers indicate a decrease in nonideal behavior in acetate buffers. This, too, may be due to smaller electrostatic repulsions present when the protein has a smaller net charge in the acetate buffer. Negative virial coefficients usually indicate solute–solute attractions [29,30]. In the glycine buffers, values of BM_1 range from 0.07 to 0.08 dl/g at I = 0.2 and from 0.123 to 0.146 dl/g at I = 0.1. In the acetate buffer, BM_1 was −0.012 dl/g.

Table 4
Thermodynamic functions for the self-association of β-lactoglobulin C in acetate

T (K)	ΔG^0 (kcal/mol)
283.2	−8.14 ± 0.06
289.2	−8.32 ± 0.06
293.2	−8.43 ± 0.06
298.2	−8.57 ± 0.06

$K_2 = (2.10 \pm 0.20) \times 10^3$ dl/g $BM_1 = 0.012 \pm 0.002$ dl/g
$k_2 = (1.93 \pm 0.18) \times 10^6$ ℓ/mol $\Delta H^0 \approx 0$
$k_2 = K_2 M_1/20$ M_1 = 18 344 Daltons
$\Delta S^0 = 0.29 \times 10^2$ cal/deg mol R = 1.987 cal/deg mol

In the acetate buffers, the monomer–dimer self-association did not exhibit the temperature dependence observed in the glycine buffers. As shown in fig. 2, over the 10–25° C range studied there is no temperature dependence of the self-association within the precision of the experimental data. This implies that ΔH^0 is approximately zero (an athermic reaction) under these experimental conditions. A similar result has been reported for the self-association of chymotrypsinogen A at low ionic strength [31]. Furthermore, as is illustrated in fig. 2, increasing the ionic strength from 0.1 to 0.2 did not change the association behavior. This suggests that electrostatic interactions are very weak or nonexistent under these conditions. Herskovits et al. [32] reported similar behavior for βA around pH 4.6; here the association of βA was found to be independent of ionic strength for $I < 0.3$.

Since the βC and βA variants differ by only 3 amino acid residues per monomer unit (see table 1), it is informative to compare and contrast the behavior of these variants. Previously, both βA and βC were found to undergo a monomer–dimer self-association in glycine buffers (pH ≈ 2.5) [12,13,15]. The βC association was found to be slightly stronger [13]. This difference in the strength of the association at two temperatures is illustrated in fig. 7. With the present study, both variants have been studied in acetate buffer (pH 4.7). βA has been reported to undergo an indefinite type self-association in acetate buffers. Adams and Lewis [6] report that a type I sequential, indefinite (isodesmic) self-association fits their data best at 16° C. Tang, using a different βA preparation, has studied the self-association in 0.2M acetate buffer (buffer I) at various temperatures [15]. At 30° C a type II sequential, indefinite self-association with k_{12} different from k, the other intrinsic equilibrium constant, seemed to describe his data best. However, at the other temperatures (11, 16 and 20° C) a type IV even indefinite self-association (see eq. (9)) with $k_{12} \neq k$ seemed to give a better description of the M_1/M_{wa} vs. J data. Both models required two equilibrium constants and one virial coefficient. Tang [15] found that the indefinite self-association of βA in 0.2M acetate buffer (buffer I) to be temperature dependent, whereas, βC shows no temperature-dependence for the

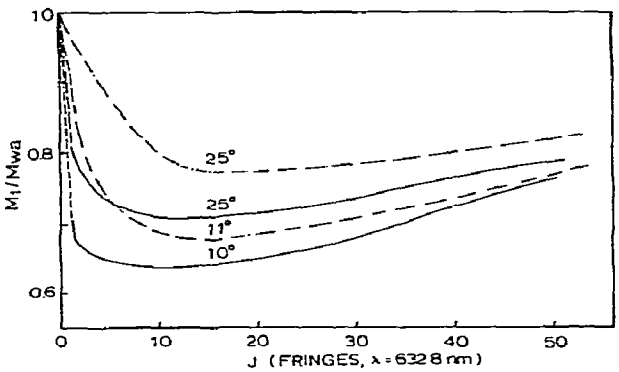

Fig. 7. Comparison of the self-association of β-lactoglobulin A and β-lactoglobulin C in 0.2M glycine buffer (0.2M glycine, 0.1M HCl, pH 2.47 at 23° C, $I = 0.1$). Note how the self-association, judged by the plots of M_1/M_{wa} vs. J, of βC appears to be stronger than that of βA at both temperatures; also note the temperature-dependence of the self-association for both variants. βA (–·–·–), βC (——).

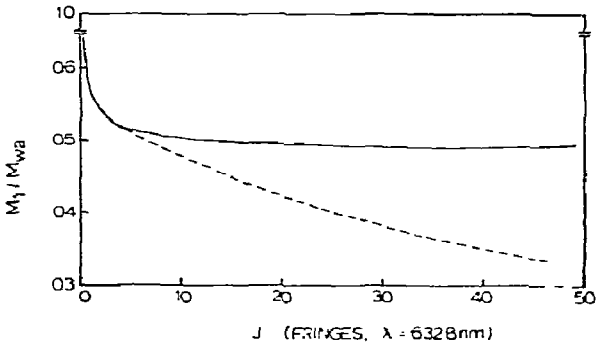

Fig. 8. Comparison of the self-association of β-lactoglobulin A and β-lactoglobulin C in 0.2M acetate buffer (buffer I). Note how much stronger the self-association of βA is. βA (– – –), βC(——).

self-association under these conditions. Fig. 8 illustrates the very different behavior of the βA and βC variants in acetate buffer (buffer I).

Timasheff and his associates [1,2,4,33] have studied the self-association of βA, βB and mixtures of βA and βB around pH 4.6 using light scattering and sedimentation velocity techniques. A higher degree of association, compared to that of βB, was observed with the βA or the mixture of βA and βB. Armstrong and McKenzie [11,34] report that the association of βA is anomalously high when compared to the association of βB or βC in the same pH range (3.5–5.2).

Timasheff and Townend [3] suggested that the anomalous behavior of βA is due to carboxyl groups. βA has one more aspartic acid and one less glycine than the other variants (see table 1). Armstrong and McKenzie [34] have chemically modified the carboxyl groups of βA and found that the modified protein showed little tendency to associate beyond dimer. They also found that the chemical modification had little effect on optical rotatory dispersion measurements, which indicates conformational changes as a result of modification were minimal. The carbohydrate containing variant of βA does not exhibit the anomalous, high degree of association; this has been attributed to steric factors [11]. Our results, together with the observations of Timasheff and associates [1,2,4,33] on βA and βB plus the work of Armstrong and McKenzie [34], indicate that the aspartic acid residue at position number 64 plays a very important role in the anomalous association (association beyond dimer) of βA. Without this aspartic acid residue the extent of associa-

tion is considerably reduced. A clear advantage of the studies with genetic variants of β-lactoglobulin is that one does not have to chemically modify the protein and worry about denaturation and other problems arising from the chemical modification.

Acknowledgement

Part of this material is taken from the dissertation submitted by J.L. Sarquis to the Graduate College, Texas A&M University in partial fulfillment for the requirements for the Ph.D. degree (August 1974). Jerry L. Sarquis was a Texas A&M University Graduate College Fellow, 1971–1973, and a Robert A. Welch Foundation Postdoctoral Fellow, 1974–1975. This work was supported by grants from the National Science Foundation (BMS 72-01873) and the Robert A. Welch Foundation (A 485). We are very grateful to Ms. Barnee M. Escott, Dr. C.N. Pace and Dr. Peter J. Wan for their interest and comments on this work. Many thanks are due to Drs. J.J. Basch and Edwin A. Kalan for providing us with the β-lactoglobulin C. We are very grateful to Dr. James O. Norman for letting us use his ultracentrifuge equipped with a photoelectric scanner.

References

[1] R. Townend and S.N. Timasheff, J. Amer. Chem. Soc. 82 (1960) 3168.
[2] S.N. Timasheff and R. Townend, J. Amer. Chem. Soc. 83 (1961) 464.
[3] S.N. Timasheff and R. Townend, J. Amer. Chem. Soc. 83 (1961) 470.
[4] T.F. Kumosinski and S.N. Timasheff, J. Amer. Chem. Soc. 88 (1966) 5635.
[5] D.A. Albright and J.W. Williams, Biochemistry 7 (1968) 67.
[6] E.T. Adams, Jr. and M.S. Lewis, Biochemistry 7 (1968) 1044.
[7] J.K. Zimmerman, G.H. Barlow and I.M. Klotz, Arch. Biochem. Biophys. 138 (1970) 101.
[8] J. Visser, R.C. Deonier, E.T. Adams, Jr. and J.W. Williams, Biochemistry 11 (1972) 2634.
[9] M.J. Kelly and F.J. Reithel, Biochemistry 10 (1971) 2639.
[10] H.A. McKenzie and W.H. Sawyer, Austr. J. Biol. Sci. 25 (1972) 949.
[11] H.A. McKenzie, in: Milk proteins – chemistry and molecular biology, Vol. II, ed. H.A. McKenzie (Academic Press, New York, 1971) pp. 257–325.
[12] L.-H. Tang and E.T. Adams, Jr., Arch. Biochem. Biophys. 157 (1973) 520.
[13] J.L. Sarquis and E.T. Adams, Jr., Arch. Biochem. Biophys. 163 (1974) 442.
[14] G. Braunitzer, R. Chen, B. Schrank and A. Stangl, Hoppe–Seyler Z. Physiol. Chem. 353 (1972) 832.
[15] L.-H. Tang, Ph.D. Dissertation, Illinois Inst. of Tech., Chicago (1971).
[16] O. Lamm and A. Polsen, Biochem. J. 30 (1936) 253.
[17] E.T. Adams, Jr., and D.L. Filmer, Biochemistry 5 (1966) 2971.
[18] A. Vrij, Ph.D. Dissertation, University of Utrecht (1959).
[19] A. Vrij and J.Th.G. Overbeek, J. Colloid Sci. 17 (1962) 570.
[20] E.F. Casassa and H. Eisenberg, Advan. Protein Chem. 19 (1964) 287.
[21] E.T. Adams, Jr., Fractions N0. 3 (1967).
[22] E.B. Kalan, R. Greenberg and M. Walter, Biochemistry 4 (1965) 991.
[23] V.G. Frank and G. Braunitzer, Hoppe–Seyler Z. Physiol. Chem. 348 (1967) 1691.
[24] P.W. Chun, S.J. Kim, J.D. Williams, W.T. Cope, L.-H. Tang and E.T. Adams, Jr., Biopolymers 11 (1972) 197.
[25] W.E. Ferguson, C.M. Smith, E.T. Adams, Jr., and G.M. Barlow, Biophys. Chem. 1 (1974) 325.
[26] F.Y.-F. Lo, B.M. Escott, E.J. Fendler, E.T. Adams, Jr., R. D. Larsen and P.W. Smith, J. Phys. Chem. 79 (1975) 2609.
[27] E.T. Adams, Jr., E.E. Ferguson, P.J. Wan, J.L. Sarquis and B.M. Escott, Separation Sci. 10(2) (1975) 175.
[28] P.W. Chun and S.J. Kim, Biophys. Chem. 3 (1975) 46.
[29] C. Tanford, Physical chemistry of macromolecules (Wiley, New York, 1961) pp. 202–204, 236.
[30] K.E. Van Holde, Physical biochemistry (Prentice–Hall, Englewood Cliffs, N.J., 1971) pp. 33–37.
[31] J. C. Nichol, J. Biol. Chem. 243 (1968) 4065.
[32] T.T. Herskovits, R. Townend and S.N. Timasheff, J. Amer. Chem. Soc. 86 (1964) 4445.
[33] R. Townend, R.J. Winterbottom and S.N. Timasheff, J. Amer. Chem. Soc. 82 (1960) 3161.
[34] J. McD. Armstrong and H.A. McKenzie, Biochem. Biophys. Acta. 147 (1967) 93.