

Solution Physicochemical Properties of Bovine β_2 -Microglobulin

AGGREGATION STATES*



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Bovine β_2 -microglobulin (β_2 -m), the light chain of the histocompatibility antigen, was isolated in crystalline form from colostrum. Previous studies from this laboratory on the solution properties of this protein suggest the existence of a time-dependent multiple aggregation phenomenon. To clarify the molecular states of β_2 -m, its solution properties were studied by ultracentrifugation and spectropolarimetry. Sedimentation equilibrium experiments at pH 5.0 (0.08 M NaCl, 0.02 M sodium phosphate) at concentrations less than 0.3 mg/ml give $M_r = 11,800$. From sedimentation velocity results, we conclude that bovine β_2 -m is a much more symmetrical and compact molecule than either guinea pig or human β_2 -m. At concentrations above 0.4 mg/ml under the same conditions, sedimentation equilibrium experiments show that a monomer to tetramer reversible self-association occurs. Also, the tetramerization increases with decreasing temperature. β_2 -Microglobulin undergoes an irreversible temperature-dependent association to a much larger aggregate over a period of 7 days, as evidenced by sedimentation equilibrium and velocity results. The rate of this aggregation decreases as the pH approaches the isoelectric point (pH 7) from either side. Furthermore, circular dichroism measured at pH 5.0 under time-dependent aggregating conditions showed a marked increase in the percentage of disordered structure, leading to the conclusion that this effect is a denaturation phenomenon.

β_2 -Microglobulin is structurally related to the immunoglobulins and is homologous to the constant domains of these proteins. More importantly, it occurs both noncovalently bound to cell surfaces and in free form in various body fluids. In its bound state, β_2 -m¹ is a subunit of histocompatibility antigens; for a review, see Peterson *et al.* (1). Elevated levels of free β_2 -m in body fluids have been related to certain pathological conditions. Although it forms complexes with and is related to proteins of the immune system, the precise biological function of bound β_2 -m and the reasons for elevated levels of the uncomplexed form in pathological body fluids are still unknown.

Previous work at this laboratory has shown that lactollin, a minor whey protein in milk, is bovine β_2 -m (2). Preliminary sedimentation velocity measurements on bovine β_2 -m suggested $M_r = 43,000$ (3). More recent data by sedimentation

equilibrium gave a value of 12,000 (4), which agrees with the monomer $M_r = 11,600$ by sedimentation equilibrium for human β_2 -m (5). Also, earlier studies by Timasheff (6) indicate that bovine β_2 -m (lactollin) undergoes a complicated time-dependent aggregation phenomenon which had not been reported for β_2 -m from other species. Due to these discrepancies, clarification of the molecular states of bovine β_2 -m was initiated through studies of its solution properties by ultracentrifugation and spectropolarimetry; the results are compared here to those found for β_2 -m from other species.

MATERIALS AND METHODS

Bovine β_2 -m was isolated from colostrum casein as described (2). All solutions of β_2 -m for ultracentrifugation and circular dichroism spectral measurements were dialyzed in 3500 molecular weight cutoff tubing for 16 h, 4 °C, against appropriate buffers. Most experiments used a 0.02 M sodium phosphate, 0.08 M NaCl, pH 5.0, buffer. β_2 -Microglobulin solutions were prepared at concentrations slightly higher than needed. After dialysis, the pH of each protein solution was checked and its concentration determined spectrophotometrically at 280 nm with $E_{1\text{mg/ml}} = 1.65$ (6). Dilutions were made with the dialysis buffer. The protein solutions were finally filtered through a 0.45- μm Millipore² filter.

In one experiment, 0.04 M sodium phosphate, 0.08 M NaCl buffers at pH 5.0, 6.5, 9.0, and 9.5 were used. Since β_2 -m readily crystallizes at neutral pH, solutions at pH 6.5 and 9.0 required special treatment. For the pH 6.5 buffer, 1 mg of β_2 -m suspended in 0.7 ml of buffer showed strong birefringence, and a small amount of acetic acid was added to dissolve the protein at this pH. For the pH 9.0 buffer, 1 mg of β_2 -m with 0.6 ml of buffer showed strong birefringence, and NaOH was added to pH 9.5; the resulting solution was then dialyzed to pH 9.0 with a large excess of pH 9.0 buffer.

Sedimentation equilibrium studies (7) were performed at 40,000 rpm on a Beckman Model E analytical ultracentrifuge equipped with electronic speed control and photometric scanner. The output of the scanner was interfaced with a Mod Comp III computer equipped with a real-time analog-to-digital voltmeter set to take in readings at 40-ms intervals. Hence, for a medium speed scan of an equilibrium boundary with a 0.2-cm column height, at least 60 data points were obtained. A minimum of 25 separate scans was averaged for each solution and its corresponding solvent blank to maximize the precision to a theoretical value of ± 0.002 absorbance unit. The averaging technique was performed by specially developed computer programs.³

Preliminary data analysis was via the Roark and Yphantis program (8). Actual weight average molecular weights were obtained by use of the exponential function (9) derived by integration of the usual equilibrium expression, *i.e.*

$$\int_{c_0}^c dc/c = \int_{r_0}^r \sigma_w r dr \quad (1)$$

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¹ The abbreviation used is: β_2 -m, β_2 -microglobulin.

² Reference to brand or firm name does not constitute endorsement by the United States Department of Agriculture over others of a similar nature not mentioned.

³ T. F. Kumosinski, J. J. Hunter, and W. C. Damert, manuscript in preparation.

which results in

$$c = c_0 e^{(\sigma_w/2)(r^2 - r_0^2)} \quad (2)$$

where $\sigma_w = M_w \omega^2 (1 - \bar{v}\rho)/RT$, M_w is the weight average molecular weight, \bar{v} is the partial specific volume and is equal to 0.727 for bovine β_2 -m as calculated from the amino acid composition (2), ρ is the density of solvent, R is the ideal gas constant, T is the temperature in K, c_0 is the concentration in milligrams/ml at the meniscus positioned at a distance r_0 from the center of rotation, and ω is the rotor speed in radians/s. Here, all data points were fitted with this exponential function by a specially developed computer program based on a Gauss-Newton nonlinear iterative regression analysis. Molecular weights thus determined are not only precise but also reflect the homogeneity of the sample.

For associating systems, determination of the degree of association as well as the corresponding equilibrium constant was accomplished by use of an exponential fit in the following manner

$$c = c_0 e^{(\sigma_w/2)(r^2 - r_0^2)} + nKc_0^n e^{(n\sigma_w/2)(r^2 - r_0^2)} \quad (3)$$

where c_0 is the concentration of the monomer at the meniscus, σ_w is proportional to the molecular weight of the monomer, K is the equilibrium constant for the association of degree n , r is the distance from the center of rotation in centimeters, and r_0 is the position of the meniscus.

Sedimentation velocity experiments by use of the same scanner-computer interface system resulted in a minimum of 200 points/boundary. Each boundary was analyzed by a fit with an integral of a Gaussian function, again by the Gauss-Newton procedure. Analysis by linear regression of the log of the boundary position and the square of its spreading factor, each as a function of time for a minimum of 15 scans, resulted in $s_{20,w}$ and D values with a precision within $\pm 0.1\%$. The above method is analogous to performing a second moment calculation for Schlieren data. Heterogeneity of the sample can be verified easily by the nonlinearity of either the log of the position or the square of the spreading factor versus time plots (10). A final benefit of use of the scanner over the Schlieren or fringe optical system is the virtual elimination of nonideal effects. For example, in velocity experiments, concentration dependency studies of $s_{20,w}$ are not necessary, since the relatively large extinction coefficients of most proteins at 280 nm mean that precise experiments can be performed at concentrations of less than 1 mg/ml; hydrodynamic drag is essentially nonexistent and $s_{20,w}$ values at these concentrations are essentially equal to those at infinite dilution. Furthermore, under these conditions, sedimentation equilibrium results would have negligible contribution from virial coefficients (10).

Circular dichroism spectra were obtained at ambient temperature by use of a Jasco Model J41C spectropolarimeter. Samples were filtered through a 0.45- μ m Millipore filter into a 0.05-cm path length cell. A mean amino acid residue weight of 118 was used to calculate ellipticities.

RESULTS

Analysis by the Roark and Yphantis program (8) of time-averaged sedimentation equilibrium results for β_2 -m at pH 5.0 in 0.08 M NaCl, 0.02 M sodium phosphate, 25 °C, and at concentrations less than 0.3 mg/ml is shown in Fig. 1. The σ_n , σ_w , and σ_{Y1} values, which are proportional to the number average, weight average, and charge-independent moment, respectively, are all fairly constant with increasing concentration across the cell as expressed in absorbance at 280 nm (11). Hence, under these conditions β_2 -m exists as a monomer. Moreover, fitting the above data with an exponential function (9) described under "Materials and Methods" results in a weight average molecular weight of 11,800, in agreement with a value of 11,537 calculated from amino acid composition (2).

Sedimentation velocity experiments performed under similar conditions of buffer, temperature, and concentration gave values of $s_{20,w} = 1.86$ and $D = 13.72 \times 10^{-7}$ cm²/s, resulting in $M = 11,900$. These were used to calculate the frictional ratio (f/f_0) and hydration (h) in grams of H₂O/g of protein (Table I). A typical velocity scan is shown in Fig. 2A. The 200 data points obtained gave an excellent fit with an integral of a Gaussian function. The linearity in plots of either the log of

the position or the square of the spreading factor versus time gave further evidence of sample homogeneity (10).

Since β_2 -m was found to undergo aggregation at concentrations above 0.3 mg/ml, gel filtration experiments were performed on β_2 -m as a function of concentration at 3 °C. Fig. 3 shows a marked decrease in the elution peak volume for β_2 -m with increasing concentrations of 4.0, 10.0, and 20.0 mg/ml, respectively. This is unexpected for a presumed time-dependent aggregating system because irreversible aggregation should show two or more separable peaks. These results suggest a rapidly re-equilibrating reversible associating system (13). Gel filtration of guinea pig β_2 -m shows similar concentration dependence of the elution peak volume (12).

Sedimentation equilibrium experiments were performed at higher concentrations and at 2 and 25 °C by use of 0.6- and 0.3-cm path length cells. These were used instead of the usual 1.2-cm cells to keep absorbance values within the range specified by Beer's law. The results of Roark and Yphantis (8) calculations for β_2 -m at concentrations from 0.2 to 0.5 mg/ml, 2 °C (Fig. 4A) are clearly different from Fig. 1 in which β_2 -m concentrations were less than 0.3 mg/ml, 25 °C. Here, not only the σ_n and σ_w values increase with increasing absorbance at 280 nm, but also σ_{Y1} , the charge-independent moment which eliminates the second virial coefficient, increases monotonically with concentration, a clear indication of self-association. The degree of association as well as corresponding association equilibrium constant were obtained by fitting all results with a sum-of-two exponential relationship as described under "Materials and Methods" (Equation 3). Use of the Gauss-Newton nonlinear regression analysis program with Equation 3 gave a best fit for a degree of association of four, with an association constant of 6.62×10^{13} ml³/mg³ at 2 °C (Fig. 4B). Furthermore, similar experiments at 25 °C resulted

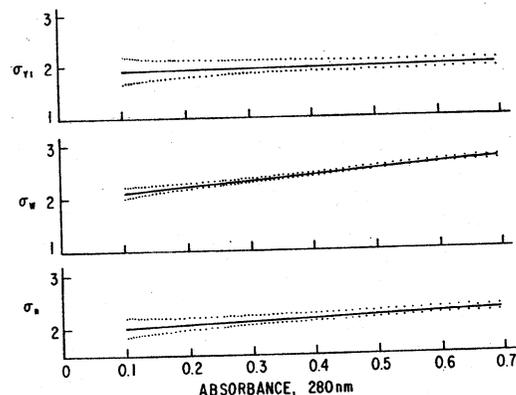


Fig. 1. Roark and Yphantis plots for sedimentation equilibrium data for β_2 -m in 0.08 M NaCl, 0.02 M sodium phosphate, pH 5.0, 25 °C, at 40,000 rpm, 1.2-cm cell, $c < 0.3$ mg/ml. Solid lines are actual σ values; dots on either side of solid line represent the average error in that parameter, $\sigma_n = M_n \omega^2 (1 - \bar{v}\rho)/RT$, $\sigma_w = M_w \omega^2 (1 - \bar{v}\rho)/RT$, and $(\sigma_{Y1})^{-1} = (2/\sigma_n) - (\sigma_w)^{-1}$.

TABLE I
Structural properties of bovine β_2 -m from $s_{20,w}$, D , and M

	f/f_0	h^a
	g H ₂ O/g protein	
$s_{20,w}$ and M^b	1.0	0.0
D and M^c	1.031	0.063
$s_{20,w}$ and D^d	1.021	0.051

^a $h = [(f/f_0)^3 - 1]\bar{v}\rho$, where $f/f_0 = (f_e/f_0) (f/f_e)$, assuming $(f_e/f_0) = 1$ for a sphere.

^b $f/f_0 = 1.19 \times 10^{-15} M^{2/3} (1 - \bar{v}\rho_0)/s_{20,w} (\bar{v})^{1/3}$.

^c $f/f_0 = 2.89 \times 10^{-5}/D(M\bar{v})^{1/3}$.

^d $f/f_0 = 10^{-8} [(1 - \bar{v}\rho)/(D^2 s_{20,w} \bar{v})]^{1/3}$.

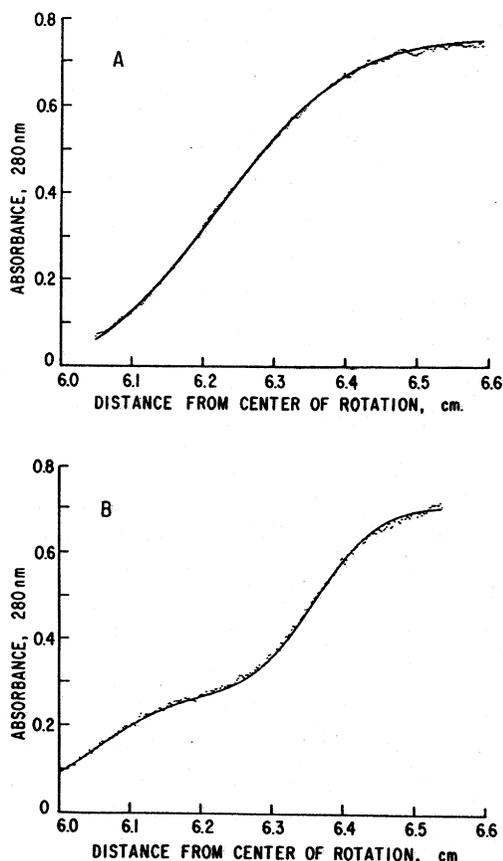


FIG. 2. A, sedimentation velocity pattern of β_2 -m in 0.08 M NaCl, 0.02 M sodium phosphate at pH 5.0, 25 °C, at 52,000 rpm for 96 min, 1.2-cm cell. Dots are actual data points; solid line is best fit for an integral of Gaussian. B, sedimentation velocity pattern of β_2 -m after 7 days at 25 °C, 60,000 rpm for 40 min, 1.2-cm cell. Dots are actual data points; solid line is best fit for sum of two integrals of a Gaussian.

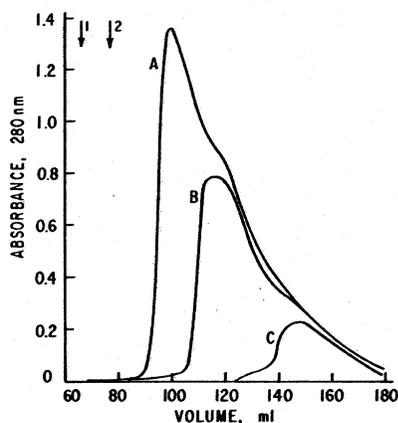


FIG. 3. Gel filtration on Bio-Gel P-60 of β_2 -m in 0.025 M sodium acetate, 0.1 M NaCl, pH 5.0, at 3 °C. 1, blue dextran; 2, ovalbumin. Loading concentrations of bovine β_2 -m: A, 20, B, 10, and C, 4 mg/ml.

in the same degree of association, namely four, with a corresponding association constant of $1.95 \times 10^{11} \text{ ml}^3/\text{mg}^3$. All equilibrium experiments gave a value of monomer $M_r = 12,000 \pm 800$.

For determining whether time-dependent aggregation exists in bovine β_2 -m, hydrodynamic and circular dichroism experiments were performed on samples incubated 1 week at 25 and 4 °C. Sedimentation velocity (Fig. 2B) performed on the samples incubated at 25 °C showed bimodality with $s_{20,w}$

values of 1.21 and 5.10, respectively (Table II). Moreover, the relative heights of the two components were invariant to loading concentration, which is clearly indicative of irreversible heterogeneity (14); samples incubated at 4 °C showed little bimodality with a $s_{20,w}$ value in agreement with the native protein.

Circular dichroism experiments in the far-UV region for samples incubated at 25 °C showed changes in secondary structure as a function of time of incubation (Fig. 5). The spectrum of a freshly dissolved sample of bovine β_2 -m showed a minimum of $-2200^\circ \text{ cm}^2/\text{dmol}$ at 218 nm, in agreement with Karlsson's value (15) of -1900 at 218 nm for human β_2 -m. With incubation time, the minimum shifted to lower wavelength accompanied by marked increases in negative ellipticity. After a week at 25 °C, the minimum had shifted to 212 nm with an ellipticity of -4700 , qualitatively this suggests an

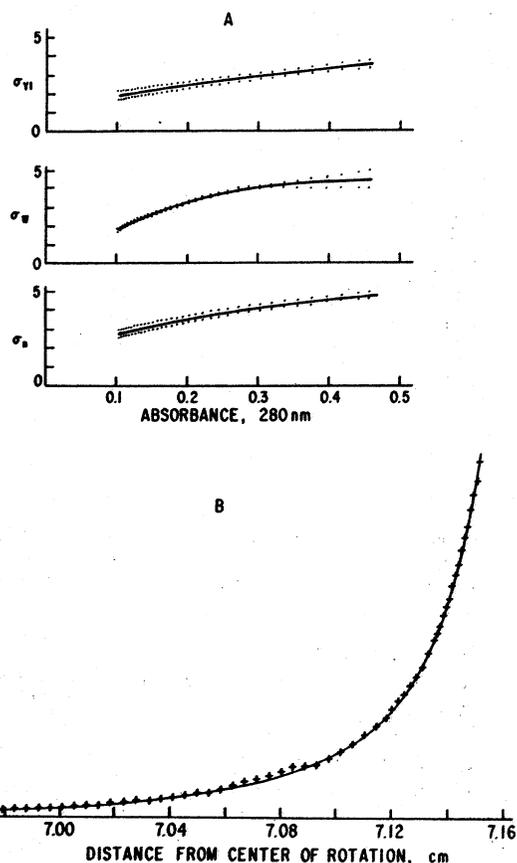


FIG. 4. A, Roark and Yphantis plots of sedimentation equilibrium at 40,000 rpm of β_2 -m in 0.08 M NaCl, 0.02 M sodium phosphate, pH 5.0, at 2 °C, 0.6-cm cell; symbols are described in Fig. 1. B, sedimentation equilibrium at 40,000 rpm of β_2 -m in 0.08 M NaCl, 0.02 M sodium phosphate, pH 5.0, 2 °C, 0.6-cm cell; +, actual time-averaged data; —, best fit for monomer-tetramer sum of two exponential relationship.

TABLE II
Hydrodynamic results of time-dependent irreversible aggregation of bovine β_2 -m

	$s_{20,w}$	$(f/f_0)^a$	Stokes radius, R^b
			A
Slow peak	1.21	1.56	17.0
Fast peak	5.10	1.48	34.0

^a Assuming an octamer as a limiting polymer of degree n , from $s_{fast}/s_{slow} = n^{2/3}$ (18).

^b From $R = M(1 - \bar{v}\rho)/s_{20,w} 6\pi\eta (f/f_0)$, where η is the viscosity of the solvent (10).

DISCUSSION

Sedimentation equilibrium experiments (Fig. 1) show that β_2 -m exists essentially as a monomer with $M_r = 11,800$ at 25 °C and at concentrations less than 0.3 mg/ml. Size and shape can be determined from sedimentation velocity experiments performed under these conditions (Fig. 2A). These experiments give $M_r = 11,900$, in agreement with both the sedimentation equilibrium value, 11,800, and that calculated from the amino acid composition, 11,537 (2), adding confidence to the accuracy of these experimentally determined constants. Frictional ratios (f/f_o), calculated from molecular weight and $s_{20,w}$, molecular weight and D , as well as $s_{20,w}$ and D (18), show close agreement with one another (Table I). The average value of these derived frictional ratios, 1.02, which is close to unity, indicates a very symmetrical molecule.

Human (12) and guinea pig (15) β_2 -microglobulins, both with (f/f_o) = 1.12, are more asymmetric than bovine β_2 -m. If spherical symmetry, (f_e/f_o) = 1, is assumed, an average calculated hydration (h) gives a low value of 0.038 g of H₂O/g of protein (19, 20). Using this value of h , the Stokes radius, R , can be calculated directly from \bar{v} and Avogadro's number N by the relationship

$$R = [(\bar{v} M/N) (3/4\pi)]^{1/3} [1 + h/\bar{v}\rho]^{1/3} \quad (4)$$

resulting in a value of 15.3 Å. This compares with 16.0 Å for the corresponding human (12) and guinea pig proteins (15) and is further evidence that bovine β_2 -m is more compact. One possible explanation for this result is that the bovine protein contains Pro-Pro sequences at residues 4-5 and 14-15 (21) which would increase the probability of β -turns and a more rigid secondary structure (22), resulting in a more compact, less hydrated molecule.

Higher concentration studies by gel filtration (Fig. 3) and sedimentation equilibrium (Fig. 4, A and B) show that bovine β_2 -m exhibits a reversible, rapidly re-equilibrating monomer to tetramer association. Guinea pig β_2 -m shows a concentration-dependent association (12) which does not occur in human β_2 -m (5). Association constants and their temperature dependence have not been determined for the guinea pig protein. Exponential fits of equilibrium data for bovine β_2 -m obtained by use of both 0.6- and 0.3-cm path length ultracentrifuge cells, which effectively increases the concentration range of protein solutions, give values of 6.62×10^{13} and 1.95×10^{11} ml³/mg³ for the tetramerization constant at 2 and 25 °C, respectively. Assuming that the enthalpy of association does not have a nonlinear heat capacity at constant pressure, an enthalpy of approximately -40 kcal may be calculated from the usual van't Hoff relationship. This value indicates that the association is controlled primarily by hydrophilic rather than hydrophobic interactions. It is unlikely that the association is controlled solely by electrostatic, *i.e.* ion-pair, interactions because of the high negative enthalpy; however, interactions involving internal isomerization to control the reaction are possible.

The time-dependent aggregation previously reported (6) was investigated by several methods with samples that were incubated at 25 and 4 °C for up to 7 days. Sedimentation velocity experiments (Fig. 2B) on samples incubated at 25 °C gave a diffuse bimodal pattern; analysis of these boundaries resulted in $s_{20,w}$ values of 1.2 and 5.1 for the slow and rapid component, respectively. An approximate value of eight was determined for the degree of irreversible aggregation (n) using the relationship $s_{fast}/s_{slow} = n^{2/3}$. This simple expression is easily derived from a combination of Svedberg's and Stokes' equations assuming spherical symmetry. Frictional ratios (f/f_o), calculated for both the slow and the fast component by

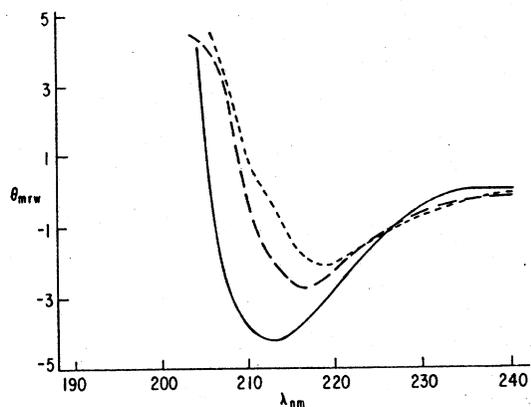


FIG. 5. Far ultraviolet circular dichroism of β_2 -m in 0.08 M NaCl, 0.02 M sodium phosphate, pH 5.0, 25 °C. ----, native protein; ---, incubated for 7 days at 4 °C; —, incubated for 7 days at 25 °C.

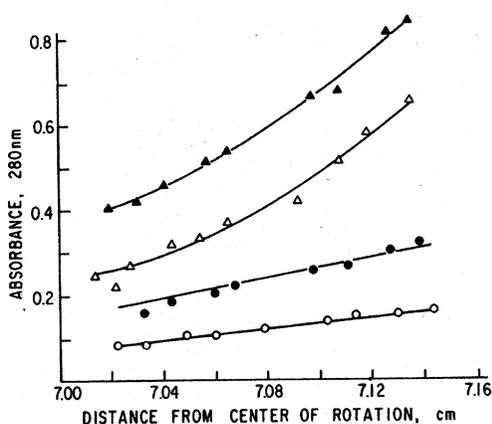


FIG. 6. Sedimentation equilibrium data of β_2 -m in 0.04 M sodium phosphate, 0.08 M NaCl at 40,000 rpm, 1.2-cm cell, at 25 °C after 7 days. O, pH 5.0; ●, pH 9.5; Δ, pH 6.5; ▲, pH 9.0.

increase in unordered and a decrease in β structure. Interpretation of the CD spectrum of native β_2 -m by use of the method of Chen *et al.* (16) shows less than 5% helical structure, with 60-65% β structure and 30-35% unordered structure. Although this method gives poor fits for proteins which lack significant helical structure, the estimates are reasonable considering the predicted secondary structure of human β_2 -m (17) and the partial sequence of the bovine protein (2). When the sample was held at 4 °C rather than 25 °C, the spectrum was more like that of the native protein with a minimum of -3200 at 217 nm. Thus, denaturation seems to have occurred on standing, with the rate increasing with increasing temperature.

For ascertaining the pH dependence of the irreversible aggregation and minimizing the possibility that this effect was due to microbial contamination, long time sedimentation equilibrium experiments were performed on fresh samples of β_2 -m at pH values of 5.0, 6.5, 9.0, and 9.5. After a week at equilibrium, boundaries at pH 5.0 and 9.5 were almost depleted, while at pH 6.5 and 9.0, only small decreases in the absorbance at 280 nm were observed (Fig. 6). Moreover, analysis of all the boundaries gave values for the monomer $M_r = 12,000 \pm 1,000$. Under these conditions, all irreversible aggregates as well as any microbial contamination that escaped Millipore filtration would sediment to the bottom of the cell at 40,000 rpm; only the monomer would be left. The amount of irreversible aggregation is directly related to the decrease in absorbance at a fixed position from the center of rotation.

assumption of an octamer and use of the equation (Footnote a) in Table II gave values of 1.56 and 1.48, respectively; these are much larger than those for the native protein (Table I). The Stokes' radius, R , calculated from the $s_{20,w}$ of the slow component was 17 Å, which is larger than the native protein and those of human (15) and guinea pig (12) β_2 -m. This large increase in (f/f_0) and R upon incubation, probably reflects an increase in hydration, but primarily indicates a large increase in the asymmetry of the molecule. In fact, if it is assumed that the increase in hydration can be accounted for by the addition of a layer of water about the β_2 -m native monomer ($R = 15.3$ Å) that is an h of 0.64 g of H₂O/g of protein, assuming a unit density of water which yields a molecular volume of water of 30 Å³/H₂O molecule, a buoyancy frictional coefficient (f/f_0) of 1.24 can be calculated. Using the total frictional coefficient (f/f_0) of the slow peak of 1.56 (Table II) and the (f/f_0) of 1.24, a structural frictional coefficient (f_s/f_0) of 1.26 can be determined for the denatured monomer. This value together with Perrin's equation (23) yields an axial ratio of 5.2 for an assumed prolate ellipsoid of revolution model and 6.0 for the oblate model. Hence, it is noted that even an equal contribution of the structural and buoyancy terms to the total frictional coefficient can result in a large change in the asymmetry of the molecule. This asymmetric change upon denaturation may be due to one or more of the Pro-Pro sequences unfolding from the *cis* (β turn) to the *trans* configuration. Brandts *et al.* (24) have shown that such an isomerism can occur during a denaturation process. Also sedimentation equilibrium experiments as a function of pH (Fig. 6) show that the amount of irreversible aggregation decreases as the pH approaches the isoelectric point of bovine β_2 -m from either the acidic or basic side. Qualitative interpretation of the circular dichroism spectra of bovine β_2 -m suggests that the protein's conformation consists primarily of β and unordered structure. The blue shift of the minimum combined with a more negative ellipticity when samples were incubated for 7 days suggests an increase in unordered structure which is greater at room temperature than in the cold. The difference between incubation at 4 and at 25 °C is less dramatic in these data than those in the ultracentrifuge experiments, suggesting that a degree of denaturation may be necessary before the irreversible aggregation can occur.

In conclusion, native β_2 -m, with a monomer $M_r = 11,800$, exists as a more compact symmetrical molecule than either guinea pig or human β_2 -m. It undergoes a concentration-dependent reversible tetramerization with a large negative

enthalpy. Such an enthalpy is indicative of an interaction site involving hydrophilic groups. However, when solutions are kept for long periods of time at 25 °C, a time-dependent denaturation leading to an irreversible octamerization occurs. For this reason, the isolation of bovine β_2 -m should be carried out at 4 °C to minimize denaturation.

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