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Interactions of Vitamin D_3 with Bovine β-Lactoglobulin A and β-Casein

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It is of nutritional significance to fortify processed dairy products (e.g., cheese, yogurt, and ice cream) with vitamin D_3; however, the inherent complexity of these foods may influence the stability and bioavailability of this nutrient. In the present study, the interactions of vitamin D_3 with β-lactoglobulin A and β-casein were investigated under various environmental conditions (i.e., pH and ionic strength) using fluorescence and circular dichroism spectroscopic techniques. The results indicated that vitamin D_3 was bound to both β-lactoglobulin A and β-casein depending on the solution conditions. The apparent dissociation constants ranged from 0.02 to 0.29 μM for β-lactoglobulin A, whereas the β-casein apparent dissociation constants ranged from 0.06 to 0.26 μM. The apparent mole ratios were also comparable, i.e., 0.51−2.04 and 1.16−2.05 mol of vitamin D_3 were bound per mole of β-lactoglobulin A and β-casein, respectively. It was concluded that these interactions may strongly influence vitamin D_3 stability and, hence, bioavailability in processed dairy products.

KEYWORDS: Vitamin D_3 binding; β-lactoglobulin A; β-casein

INTRODUCTION

The primary biological function of vitamin D_3 is to maintain serum calcium and phosphorus concentrations within the normal range (*1*). In addition to its classic role in mineral metabolism, other positive attributes include roles in osteoblast formation, fetus development, pancreatic function, neural function, and immunity (*2*). Several studies also suggest that higher serum vitamin D_3 concentrations are associated with lower rates of breast, ovarian, prostate, and colorectal cancers, as well as a decreased risk of developing multiple sclerosis (*3*).

Vitamin D is naturally found in very few foods (*4*). The richest source is cod liver oil (210 μg/100 g), while smaller amounts are found in the edible portion of fatty fish (∼6−22 μg/100 g), mammalian liver (0.75 μg/100 g), eggs (1.75 μg/100 g), and dairy products, including unfortified milk (0.03 μg/100 g) (*5*). The primary source of this essential nutrient is produced in the skin via exposure to sunlight; however, living in northern latitudes minimizes skin exposure to ultraviolet radiation, particularly during the winter months (*6*, *7*). Thus, populations in North America depend largely on fortified foods and dietary supplements to meet their vitamin D needs, given that foods naturally rich in vitamin D are rather limited.

In the United States, fluid milk and breakfast cereals are the chief vehicles for vitamin D consumption, whereas in Canada the main sources are found in fluid milk and margarine. Through cross-sectional studies, Calvo et al. (*8*) found that current North American fortification procedures do not provide sufficient vitamin D in the human diet. Part of this inadequacy is also because a growing section of the population does not drink milk at all (*7*). As well, the current legislation assumes that half of our vitamin D_3 daily requirements are met through exposure to sunlight, even though sufficient evidence indicates otherwise (*3*, *7*).

Therefore, it is of nutritional significance to fortify processed dairy products (e.g., cheese, yogurt, and ice cream) with vitamin D_3. Such fortification would benefit the general population by offering an additional source of this essential nutrient. However, very little has been reported on the stability of vitamin D_3 in processed dairy products with the exception of two cheese-related studies (*6*, *9*). The compositional complexity of dairy products may influence the stability and availability of this nutrient, especially in low-fat or nonfat products where vitamin D_3 would be void of its protective matrix. It was the objective of this study to characterize the interactions of vitamin D_3 with dairy proteins. As such, β-lactoglobulin A (β-LG A) and β-casein (β-CN) were chosen because they are major whey and casein proteins, respectively.

***β*-Lactoglobulin A.** The structure of β-LG A is composed of eight up and down antiparallel β-strands (labeled A−H) folded into a flattened cone or a calyx, which is closed at one end by Trp_{19} while the EF loop (residues 85−90) acts as a door at the open end (*10*).

In vitro research has shown that β-LG A can bind a variety of hydrophobic ligands (*11*−*20*); however, results from investigations aimed at identifying the precise binding site(s) remain controversial. It is unclear whether ligands bind within the central calyx or to an external hydrophobic surface patch found

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between the α-helix and the surface of the β-barrel; however, prevailing evidence points toward the inner cavity (*11*, *17*−*20*). On the other hand, it is clear that binding to either of these sites is strongly dependent on pH (*21*). Access to the internal binding site is moderated by the EF loop region that moves from a closed to an open position between ∼pH 6.2 and 8.2 (*22*, *23*).

β-Casein. The N-terminal portion (residues 1−25) of β-CN contains four out of the five anionic phosphoserine residues and carries the bulk of the protein's net charge, whereas the C-terminal essentially carries no net charge and is primarily composed of hydrophobic residues. As such, β-CN is the most hydrophobic, and more importantly, the most amphipathic of all caseins (*24*, *25*). β-CN can exist in solution as a monomer; however, its distinctly amphiphatic structure is responsible for micelle formation, where hydrophobic interactions are the principal attractive force (*26*). Recent evidence indicates that micellization is dependent on temperature, ionic strength, and pH when the critical micellar concentration (CMC) (i.e., 0.5−2 mg/mL) is exceeded (*25*, *27*). Hydrophobic ligands have also been shown to induce micelle formation (*28*).

The few reported studies investigating the binding of lipophilic molecules to β-CN suggest that hydrophobic forces are largely responsible and ∼1 mol of ligand interacted with each β-CN monomer (*24*, *29*).

The objective of the present study was to investigate the interactions of vitamin D_3 with β-LG A and β-CN under various environmental conditions (i.e., pH and ionic strength) using fluorescence and circular dichroism spectroscopic techniques.

MATERIALS AND METHODS

Vitamin D_3 (C-9756, crystalline cholecalciferol, purity >99%), bovine β-lactoglobulin A (L-7880, purity 91%), and bovine β-casein (C-6905, purity 95%) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada.) and used without further purification. To prevent oxidation and isomerization, vitamin D_3 stock solutions of the appropriate concentrations (w/v) were prepared in reagent alcohol, purged with nitrogen gas, and stored in the dark at ∼4 °C. The vitamin D_3 concentrations were confirmed by measuring the absorbance at 265 nm and dividing by the mol extinction coefficient of 18 300 AU (absorbance units)/mol/L (*3*). Protein solutions of the appropriate concentrations (w/v) were prepared fresh daily in a 25 mM phosphate (pH 2.5, 6.6, and 8.0) or 25 mM acetate (pH 4.6) buffer. Each buffer was prepared using deionized water and carried a final ionic strength of 0.08 M or 0.15 M through the addition of NaCl while taking into consideration the initial ionic strength of the buffer. The concentration of β-lactoglobulin A was determined by measuring the absorbance at 280 nm with an extinction coefficient E (1%/1 cm) of 9.6 (*30*). The concentration of β-casein was determined by measuring the absorbance at 280 nm with an extinction coefficient E (1%/1 cm) of 4.6 (*27*).

Fluorescence Spectroscopy. Fluorescence spectra were recorded at room temperature (from ∼22 to 25 °C) on a Perkin-Elmer luminescence spectrometer LS50B using an excitation wavelength of 287 nm and an emission wavelength of 332 nm (*13*). The excitation and emission slit widths were set at 6 and 4 nm, respectively. The binding of vitamin D_3 was followed by measuring the fluorescence quenching of protein tryptophanyl residues. The procedures of Cogan et al. (*31*) and Dufour and Haertlé (*32*) were used to titrate the proteins with vitamin D_3. Accordingly, 3 mL of 8 μM protein (filtered with a 0.22 μm syringe filter) solutions were placed in a cuvette and small increments (1 to 5 μL) of a 3 mM vitamin D_3 stock solution (filtered with a 0.22 μm syringe filter) were added until saturation was reached. At the end of the titration, the alcohol added with the vitamin D_3 did not exceed 3% (v/v). The protein/vitamin solutions were vortexed for ∼15 s and allowed to equilibrate for 5 min prior to fluorescence measurement. All titration points were run in triplicate. For each titration point, a buffer/ethanol blank was prepared and treated in the same manner as the sample. This blank was subtracted from a second blank containing *N*-acetyl-L-tryptophanamide (NATA). NATA displays a fluorescence spectrum typical of tryptophan yet is unable to interact with vitamin D_3. NATA was used to exclude the possibility of unspecific interactions of the vitamin with the protein's tryptophan indoles, where at increasing vitamin D_3 concentrations the vitamin may absorb light, which would otherwise excite the indole groups, and thus fluorescence would decrease for this reason (*32*, *33*). The NATA solution had an absorbance at 287 nm equal to that of the protein solution and was titrated in a manner similar to all samples (*32*).

After correcting for the NATA blank according to the method of Birdsall et al. (*34*), differences in fluorescence intensity at 332 nm between the complex and free protein were monitored in order to measure the apparent dissociation constant (K'_d) and the apparent mole ratio of ligand to protein at saturation (n), according to the methods of Eisenthal and Cornish-Bowden (*35*) and MacLeod et al. (*36*). It should be noted that prior to using the direct linear plotting method, binding constants were determined using Cogan's method by constructing a plot of $P_{T\alpha}$ versus R_T [α/(1 − α)]. In theory, this plot was supposed to yield a straight line with an intercept of K'_d/n and a slope of $1/n$ according to the eq (*31*):

$$P_{T\alpha} = (1/n)\,[L_{T\alpha}/(1-\alpha)] - (K'_d/n) \quad (1)$$

where P_T = total protein concentration, L_T = total vitamin D_3 concentration, n = apparent mole ratio of ligand to protein at saturation, K'_d = apparent dissociation constant, and α = fraction of unoccupied binding sites on the protein molecule.

However, a plot of the data points was not linear. MacLeod et al. (*36*) made similar observations and noted that the linearization of the curvilinear data misrepresented K'_d and n. On the basis of that report, it was determined that the method of Eisenthal and Cornish-Bowden (*35*) was more appropriate as it eliminates transformation errors.

The direct linear plotting method of Eisenthal and Cornish-Bowden (*35*), where the corrected fluorescence is plotted directly against vitamin D_3 concentrations, was used to obtain K'_d directly from the median of intersecting regression lines representing individual observations on the abscissa. The method of MacLeod et al. (*36*) was used to obtain n from the direct linear plot by extracting the vitamin D_3 concentration using predicted curve values correlating to the saturation point.

Circular Dichroism (CD). The CD spectra were measured at room temperature (from ∼22 to 25 °C) on a Jasco J-600 spectropolarimeter, using a quartz cell with a path length of 0.1 cm in the far-UV spectra (186−260 nm) and 1 cm in the near-UV spectra (250−350 nm). The volume of the (3 mM for far-UV or 30 mM for near-UV) vitamin D_3 stock solution mixed with the (8 μM far-UV (for both proteins) or 65 μM for β-LG A and 55 μM for β-CN in the near-UV) protein solution varied as a function of pH and ionic strength. Added volumes were calculated to correspond to the n values previously obtained from fluorescence spectroscopy results. Prior to measurement, the freshly prepared samples were vortexed for ∼15 s, filtered with a 0.22 μm syringe filter, and degassed for 15 min. Triplicate measurements were made in the presence or absence of vitamin D_3. Baseline spectra of the buffer and alcohol or vitamin D_3 solution were subtracted from the sample spectra. Spectra were taken with a step resolution of 0.2 nm, a time constant of 2 s, and the mean of three measurements. The secondary structure was estimated by fitting the far-UV CD spectra to a combination of reference spectra using the methods of Chang et al. (*37*) and Yada and Nakai (*38*).

Statistical Analyses. Results were analyzed with SPSS 11.0 statistical software. *t*-Tests or one-way ANOVAs were used to assess statistically significant differences between means. Following the one-way ANOVA, a Duncan's post hoc test was used to examine the differences between the means. Statistical analyses were made with a level of significance of $P \leq 0.05$.

RESULTS

Fluorescence Spectroscopy and β-Lactoglobulin A. Two tryptophanyl residues (Trp_{19} and Trp_{61}) are found in β-LG A; however, it has been suggested that β-LG A's intrinsic

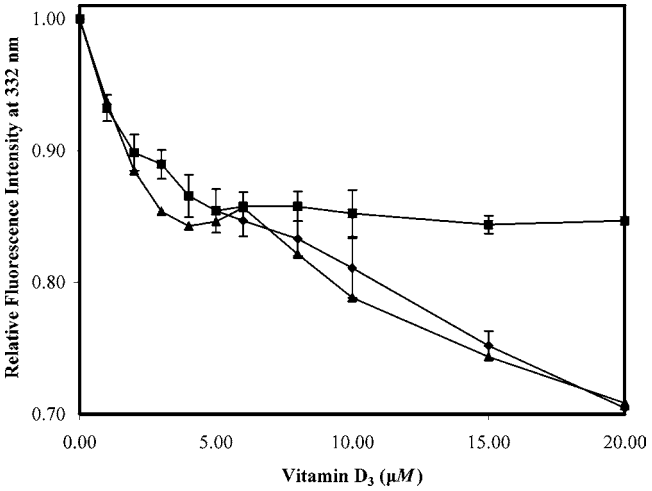

Figure 1. Mean fluorescence titration curve of 7.38 μM β-lactoglobulin A in a 25 mM phosphate buffer, pH 8.0 (I = 0.08 M), as a function of vitamin D_3 concentration. Data are shown as observed fluorescence intensity (◆); fluorescence intensity corrected for fluorescence contribution of free vitamin D_3 (■); and fluorescence intensity of free vitamin D_3 in *N*-acetyl-L-tryptophanamide solution (▲). Reported data are means ± SDs of triplicate analyses (except *N*-acetyl-L-tryptophanamide blank).

Table 1. Apparent Mole Ratios (n) of β-Lactoglobulin A and Vitamin D_3[a]

	n (Mol Vitamin/Mol Protein)	
	ionic strength	
pH	0.08 M	0.15 M
2.5	2.04 ± 0.27 A,a	1.11 ± 0.12 A,b
4.6	0.84 ± 0.11 B,a	0.51 ± 0.10 B,b
6.6	1.27 ± 0.31 C,a	1.36 ± 0.12 C,a
8.0	1.47 ± 0.15 C,a	1.54 ± 0.14 C,a

[a] Reported data are means ± SDs of triplicate analyses; A, B, C means results within the same column without a common letter are significantly different ($P \leq 0.05$); a, b means results within the same row without a common letter are significantly different ($P \leq 0.05$).

fluorescence is almost exclusively attributed to Trp_{19} (*11*, *12*). This residue is found at the bottom of the calyx, whereas Trp_{61} is more accessible to solvent and thus only able to make a minor contribution to fluorescence emission (*39*). Thus, tryptophan quenching in response to proximate ligand binding is a useful technique to study the dynamics of ligand interactions (*13*).

A typical fluorescence titration curve following β-LG A's tryptophan quenching as a function of vitamin D_3 concentration is shown in **Figure 1**. Tryptophan quenching leveled off at ~11 μM vitamin D_3, suggesting that the saturation point of the corrected curve was reached at a vitamin/protein ratio of ~1.5:1. A direct comparison of all corrected β-LG A titration curves clearly indicates variability among saturation points, supporting the hypothesis that changes in pH and ionic strength affect β-LG A's binding properties.

Binding constants, derived using the direct linear plotting method, are presented in **Tables 1** and **2**. The listed values indicate that binding occurred under all conditions but varied as a function of pH and ionic strength.

Irrespective of ionic strength, samples at pH 6.6 and 8.0 displayed similar K'_d and n values. However, values measured below the pI (pH 5.2) significantly differed whereby an increase in ionic strength led to stronger K'_d and reduced binding ($P \leq 0.05$). Among all the samples investigated, β-LG A at pH 4.6 had the lowest number of binding sites but the strongest affinity ($P \leq 0.05$). An opposite trend was observed at pH 2.5 (I = 0.08 M), where the weakest observed affinity was associated with the highest n value ($P \leq 0.05$).

Table 2. Apparent Dissociation Constants (K'_d) of β-Lactoglobulin A and Vitamin D_3[a]

	K_d (μM)	
	ionic strength	
pH	0.08 M	0.15 M
2.5	0.29 ± 0.05 A,a	0.20 ± 0.01 A,b
4.6	0.06 ± 0.01 B,a	0.02 ± 0.02 B,b
6.6	0.14 ± 0.06 B,a	0.16 ± 0.01 C,a
8.0	0.12 ± 0.02 B,a	0.18 ± 0.02 C,b

[a] Reported data are means ± SDs of triplicate analyses; A, B, C means results within the same column without a common letter are significantly different ($P \leq 0.05$); a, b means results within the same row without a common letter are significantly different ($P \leq 0.05$).

Table 3. Secondary Structure Content of β-Lactoglobulin A in the Absence and Presence[a] of Vitamin D_3 as Calculated from the Far-UV Circular Dichroism Spectra[b]

		secondary structures (%)							
		α-helix		β-sheet		β-turn		random	
pH	ionic strength (M)	[P][c]	[PL][d]	[P]	[PL]	[P]	[PL]	[P]	[PL]
2.5	0.08	18.2 A	17.6 A	32.1 A	34.1 A	25.2 A	25.9 A	24.6 A	22.5 A
	0.15	19.3 A	19.2 A	25.8 A	23.4 A	24.6 A	26.5 A	30.7 A	31.0 A
4.6	0.08	7.3 A	5.7 A	46.7 A	50.3 A	17.9 A	19.1 A	28.2 A	24.9 A
	0.15	6.8 A	4.5 A	71.0 A	49.9 B	2.9 A	14.2 B	19.2 A	31.5 B
6.6	0.08	12.9 A	13.1 A	43.3 A	43.4 A	16.7 A	18.0 A	27.1 A	25.6 A
	0.15	12.6 A	17.3 B	39.0 A	26.5 B	17.3 A	22.1 A	31.1 A	34.1 A
8.0	0.08	10.2 A	13.4 A	44.5 A	35.9 A	16.3 A	16.6 A	29.2 A	34.1 A
	0.15	18.0 A	18.7 A	26.1 A	23.9 A	22.7 A	23.7 A	33.3 A	33.6 A

[a] Vitamin D_3 concentrations added to individual samples correspond to n values obtained from fluorescence spectroscopy results. [b] Reported data are means of triplicate analyses. SDs were omitted to facilitate table interpretation. A, B means comparing the absence and presence of vitamin D_3 at constant pH and ionic strength without a common letter are significantly different ($P \leq 0.05$). [c] β-Lactoglobulin A. [d] β-Lactoglobulin A and vitamin D_3.

Circular Dichroism and β-Lactoglobulin A. Secondary structure results obtained with far-UV CD are presented in **Table 3**. Few ligand-induced changes were observed, except at pH 4.6 (I = 0.15 M) where a notable change occurred as evidenced by an increase in β-turn and random structures at the expense of β-sheets ($P \leq 0.05$). Other ligand-induced changes were found at pH 6.6 (I = 0.15 M) and were characterized by an increase in α-helical structures with a concomitant decrease in β-sheets ($P \leq 0.05$).

Near-UV CD results showed that the addition of vitamin D_3 elicited changes in tertiary structure only at pH 8.0 (**Figure 2**). The deepened troughs observed near 258 and 266 nm upon ligand addition were attributed to phenylalanine residues (Phe 82, 106, 136, and 151) that dominate at those wavelengths.

Fluorescence Spectroscopy and β-Casein. Unlike β-LG A, β-CN is not regarded as a "classic" ligand-binding protein due to its rheomorphic conformation, which may explain why its ligand-binding properties have remained largely unexplored (*24*). The authors are aware of only two fluorometric titration studies investigating the binding of ligands to β-CN (*24*, *29*). By assuming the lipophilic vitamin D_3 would bind near the hydrophobic cluster containing a single tryptophanyl residue, it was in theory possible to follow the fluorescence quenching of Trp_{143} in a fashion similar to that of β-LG A.

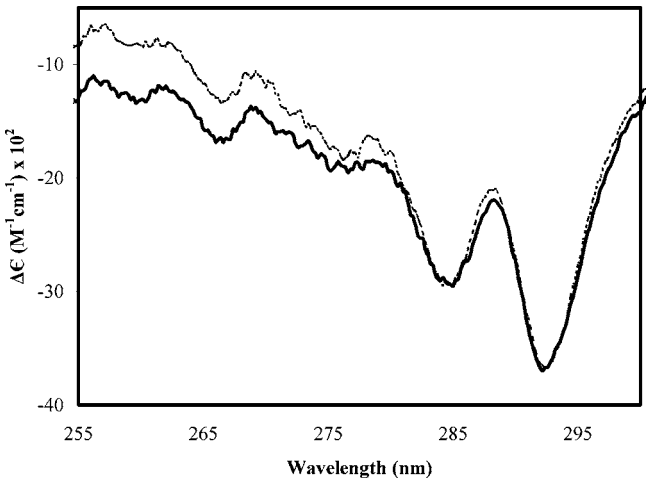

Figure 2. Near-UV circular dichroism spectra of 57.6 μM β-lactoglobulin A in a 25 mM phosphate buffer at pH 8.0 (I = 0.15 M) in the absence (- - -) and presence (—) of 88.7 μM vitamin D_3. Reported spectra are means of triplicate analyses.

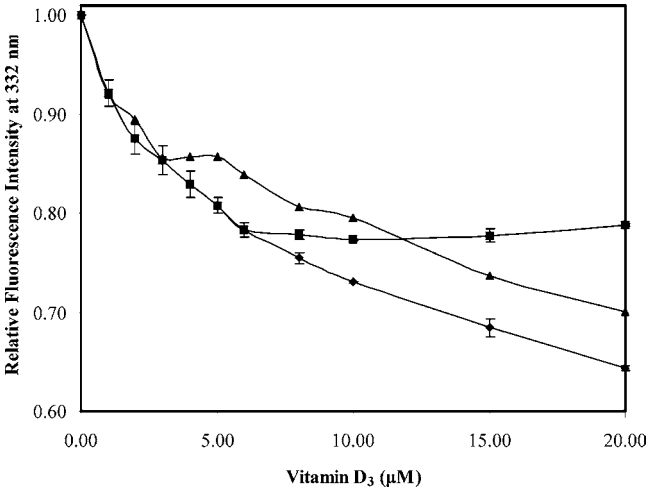

Figure 3. Mean fluorescence titration curve of 7.07 μM β-casein in a 25 mM phosphate buffer at pH 8.0 (I = 0.08 M) as a function of vitamin D_3 concentration. Data are shown as observed fluorescence intensity (◆); fluorescence intensity corrected for fluorescence contribution of free vitamin D_3 (■); and fluorescence intensity of free vitamin D_3 in *N*-acetyl-L-tryptophanamide solution (▲). Reported data are means ± SDs of triplicate analyses (except *N*-acetyl-L-tryptophanamide blank).

The corrected fluorescence titration curves suggested that vitamin D_3 was bound in proximity to the indole chromophore as evidenced by Trp_{143} quenching. A typical fluorescence titration curve presented in **Figure 3** indicates that tryptophan quenching leveled off at ~15 μM vitamin D_3 suggesting that saturation was reached at a vitamin/protein ratio of approximately 2:1. Furthermore, a direct comparison of the saturation points indicated that binding constants differed as a function of measured experimental conditions. The derived binding constants are presented in **Tables 4** and **5**. As previously pointed out from the titration curves, results differed as a function of pH and ionic strength ($P \leq 0.05$).

At pH 8.0 (I = 0.15 M), the strongest affinity was observed in addition to the lowest number of binding sites; conversely, a decrease in ionic strength led to the highest n value and the weakest affinity ($P \leq 0.05$). A reduction in pH led to the reversal of this trend. As the ionic strength increased, so did the number of binding sites ($P \leq 0.05$ M).

Table 4. Apparent Mole Ratios (n) of β-Casein and Vitamin D_3[a]

	n (Mol Vitamin/Mol Protein)	
	ionic strength	
pH	0.08 M	0.15 M
6.6	1.33 ± 0.03	1.94 ± 0.24
8.0	2.05 ± 0.16	1.16 ± 0.03

[a] Reported data are significantly different means ± SDs of triplicate analyses ($P \leq 0.05$).

Table 5. Apparent Dissociation Constants (K'_d) of β-Casein and Vitamin D_3[a]

	K_d (μM)	
	ionic strength	
pH	0.08 M	0.15 M
6.6	0.13 ± 0.01	0.26 ± 0.03
8.0	0.24 ± 0.01	0.06 ± 0.02

[a] Reported data are significantly different means ± SDs of triplicate analyses ($P \leq 0.05$).

Table 6. Secondary Structure Content of β-Casein in the Absence and Presence[a] of Vitamin D_3 as Calculated from the Far-UV Circular Dichroism Spectra[b]

		secondary structures (%)							
		α-helix		β-sheet		β-turn		random	
pH	ionic strength (M)	[P][c]	[PL][d]	[P]	[PL]	[P]	[PL]	[P]	[PL]
6.6	0.08	10.1 A	6.4 A	25.4 A	35.4 A	16.7 A	14.9 A	47.8 A	43.3 B
	0.15	16.2 A	17.9 B	13.4 A	7.2 B	19.8 A	23.1 A	50.7 A	51.8 A
8.0	0.08	18.2 A	18.7 A	4.1 A	5.9 A	26.4 A	24.9 A	51.3 A	50.4 A
	0.15	19.2 A	16.4 A	7.3 A	13.0 A	22.6 A	20.9 A	50.9 A	49.8 A

[a] Vitamin D_3 concentrations added to individual samples correspond to n values obtained from fluorescence spectroscopy results. [b] Reported data are means of triplicate analyses. SDs were omitted to facilitate table interpretation. A, B means comparing the absence and presence of vitamin D_3 at constant pH and ionic strength without a common letter are significantly different ($P \leq 0.05$). [c] β-Casein. [d] β-Casein and vitamin D_3.

Circular Dichroism and *β*-Casein. Calculated secondary structural contents determined from far-UV CD spectra of β-CN, with and without, vitamin D_3 are presented in **Table 6**. At pH 8.0, no large differences were observed ($P > 0.05$); however, at pH 6.6 (I = 0.08 M), there was a significant reduction in random structures ($P \leq 0.05$). Increasing the ionic strength at this pH led to an increase in α-helical formations with a concurrent decrease in β-sheets ($P \leq 0.05$).

Although caseins are known not to have any stable tertiary structure, the near-UV CD spectra were recorded to observe any qualitative changes induced by ligand addition. Although rather noisy, the addition of vitamin D_3 appeared to elicit a slight shift in the phenylalanine peaks centered near 260 and 267 nm (**Figure 4**), which was observed in all samples except at pH 8.0 (I = 0.15 M).

DISCUSSION

***β*-Lactoglobulin A.** Binding capacities mediated by pH were evident in the present study. At pH 2.5 (I = 0.15 M), approximately one molecule of vitamin D_3 was bound per monomer with a relatively weak affinity (K'_d = 0.20 μM). Since the EF loop is in a closed position at this pH, the results suggest that loose binding occurred at the external hydrophobic surface

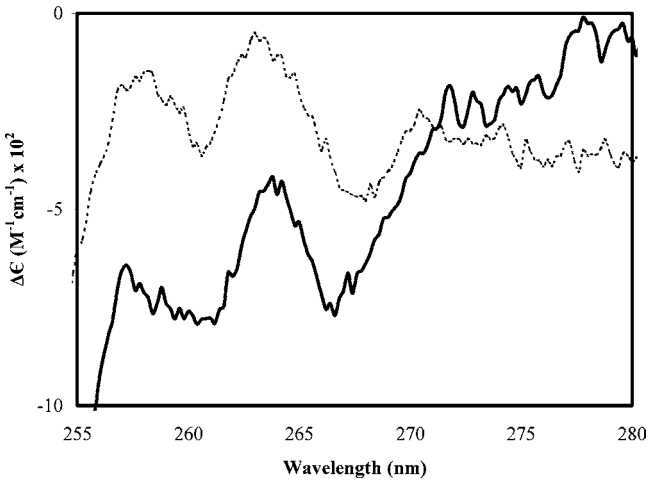

Figure 4. Near-UV circular dichroism spectra of 54.0 μM β-casein in a 25 mM phosphate buffer at pH 6.6 (I = 0.08 M) in the absence (- - -) and presence (—) of 71.8 μM vitamin D_3. For clarity, only a selected portion is shown. The reported spectra are means of triplicate analyses.

patch. Upon lowering the ionic strength (I = 0.08 M), binding increased to approximately two molecules of vitamin D_3 per monomer. Since solubility is increased after lowering the NaCl concentration, it is believed that the increased available surface area permitted greater ligand access (*30*). Furthermore, the increased capacity of the external hydrophobic surface patch was accompanied by relatively loose binding as evidenced by the lowest observed affinity (K'_d = 0.29 μM). Similarly, Wang et al. (*15*) found that monomeric β-LG A was capable of weakly binding a large number of palmitate molecules at the surface site. Moreover, they noted that dimer formation decreased the surface area responsible for palmitate binding, but a higher affinity site was created allowing two molecules of palmitate to bind per dimer at an order of magnitude greater than that for the monomer. Observations consistent with these researchers were found in the present study at pH 4.6 (I = 0.15 M) where β-LG A has been found to form octamers in solution (*40*). Since the EF loop is blocking ligand access, it is believed that a single vitamin D_3 molecule was tightly bound between the monomers found within the octamers as evidenced by the strongest observed affinity (K'_d = 0.02 μM). Lowering the NaCl concentration was followed by an increase in binding capacity (n = 0.84). Since intermolecular repulsive forces are increased after lowering the NaCl concentration, some monomers may have existed in solution. As such, one vitamin D_3 molecule may have been bound to the monomer's external hydrophobic surface patch, while most of the vitamin D_3 molecules were shared between dimeric β-LG A monomers. This latter phenomenon would account for the increased apparent mole ratio and lower affinity (K'_d = 0.06 μM) found at this ionic strength. Irrespective of ionic strength, it is assumed that vitamin D_3's polar hydroxyl group is pointed outward, since this orientation would permit the greatest amount of hydrophobic interactions.

At pH 6.6 and 8.0, few differences were observed between samples, likely because they share similar characteristics; dimers predominate, and the EF loop is flipped open allowing internal ligand access. Since $n \approx 1.5$ and $K'_d \approx 0.15$ μM, the results suggest that one vitamin D_3 molecule was bound within the central cavity and another molecule was tightly shared between monomers. Strong, simultaneous binding inside the calyx and hydrophobic surface patch was also observed by Narayan and Berliner (*16*), and Wang et al. (*15*). Furthermore, the current results support the findings of Wang et al. (*13*) outlining vitamin D_3's ability to simultaneously occupy both binding sites with a strong affinity (K'_d = 0.04 μM).

The far-UV CD spectra reflecting β-LG A's secondary structural changes upon vitamin D_3 addition showed no significant differences indicating that the structure was preserved ($P > 0.05$). However, differences were noted at pH 4.6 and 6.6 at the higher ionic strength (I = 0.15 M). Since secondary structural changes were not observed at lower ionic strengths, the results suggest that electrostatic interactions affected the surface area available for binding in such a way that vitamin D_3 disrupted the secondary structure. Far-UV CD results at pH 6.6 indicated that the proportion of β-sheets decreased with an increase in α-helical structures. Since the EF loop is only partially open at this pH, introduction of vitamin D_3 likely resulted in some change in secondary structural elements that surround this site. At pH 4.6, where the tightest binding occurred, β-sheet content decreased with a concomitant increase in β-turn and random structures, indicating that the introduction of the bulky moiety in the tight cavity disturbed some secondary structural elements such as β-I or β-A strands which surround this site (*23*).

Near-UV CD spectra of β-LG A, with and without vitamin D_3, also suggested that the structure was preserved in most samples except those at pH 8.0. At this pH, Glu_{89} of the EF loop is completely exposed to solvent thus permitting full ligand access (*23*). Near-UV CD spectra suggested that vitamin D_3 was positioned in close proximity to a phenylalanine residue (Phe 82, 105, 136, or 151). Binding is believed to have occurred near Phe_{105} located in the interior of the calyx based on published results by Zsila et al. (*18*) who were able to describe the close proximity of the bound ligand to this residue.

The present results indicate that β-LG A is able to bind vitamin D_3 at both proposed binding sites. In addition, it would appear that independent and simultaneous ligand binding occurs when the EF loop is in an open conformation, since the internal site is accessible; otherwise, ligands loosely bind in the monomeric hydrophobic surface patch or tightly at the dimeric interface.

On the basis of the present results, further insight regarding the strength of individual binding sites can be deduced. At pH 6.6 and 8.0, where simultaneous binding occurred, the affinity ($K'_d \approx 0.15$ μM) was stronger than that at pH 2.5 ($K'_d \approx 0.25$ μM), where binding occurred at the external hydrophobic surface patch, yet weaker than at pH 4.6 ($K'_d \approx 0.04$ μM), where binding occurred between monomers. Accordingly, this indicates that the weakest affinity site is found at the hydrophobic surface patch. Conversely, this same site found at the dimer interface is the highest affinity site as previously proposed (*14*, *15*).

***β*-Casein.** The binding constants support that vitamin D_3 binding was dependent on pH and ionic strength. At pH 6.6, binding increased as a function of ionic strength, similar to the findings of Lietaer et al. (*29*). Presumably, the increased masking of the charges on the protein by the ions lead to reduced solubility of the protein and enhanced hydrophobic interactions, creating more surface area available for binding (*29*). However, increased binding was associated with a weaker affinity (K'_d = 0.26 μM), compared to the lower ionic strength where binding was stronger (K'_d = 0.13 μM). These results suggest that weaker binding was associated with the protein interface where more protein−protein interactions occurred. Monomers likely predominated in solution since the concentration used ($\sim$ 0.2 mg/mL) did not exceed the CMC (0.5 to 2 mg/mL) (*25*). It is presumed that the rheomorphic nature of β-CN allowed the hydrophobic portion to tightly interact with the vitamin's

hydrophobic moiety in the most thermodynamically stable conformation. However, it is believed that interactions were stronger at the lower ionic strength where less protein interactions occurred. On the other hand, the results at pH 8.0 were contrary to the results found at pH 6.6 since binding decreased as a function of ionic strength. No explanation for the discrepancy can be given.

On the basis of the far-UV CD results, it was evident that β-CN did not contain a high percentage of secondary structure content since all samples contained ~50% random structures, as has been noted by others (*41*). Despite β-CN's low amount of regular secondary structure, it was worthwhile to investigate if ligand binding could induce more ordered structure. The addition of vitamin D_3 did induce some significant ($P \leq 0.05$) secondary structural changes at pH 6.6 (i.e., increased α-helix and decreased β-sheet); however, no structural changes were induced at pH 8.0 ($P > 0.05$). Caessens et al. (*41*) found similar results with adsorbed β-CN, whereby lowering the pH increased structure induction upon adsorption. They assumed the reduction of net charge created favorable conditions for a more ordered structure. Presumably, the same theory could be applied to ligand binding.

It is widely accepted that β-CN does not contain any stable tertiary structure (*42*); however, near-UV CD experiments were employed to assess if vitamin D_3 induced any changes. Near-UV CD spectra indicated that vitamin D_3 was positioned in close proximity to phenylalanine, as evidenced by the increased energy of one or several of the nine phenylalanine residues spread throughout the hydrophobic domain (*25*). No changes were evident at pH 8.0 ($I = 0.15$ M) where the least binding occurred, potentially indicating that phenylalanine perturbation increased as a function of *n*. It should be noted that samples prepared for near-UV CD experiments had a concentration of ~1 mg/mL, which was within the CMC range, indicating that micelles may have been present. If micelles predominated in solution, it is speculated that β-CN could potentially trap the nonpolar vitamin D_3 within the micellar structure. This concept has previously been reported in a study examining the effect of sodium dodecyl sulfate (SDS) on β-CN self-association. At low SDS concentrations, where the highest proportions of polymers were found, the author suggested that the hydrophobic tail of the SDS molecule was involved in the β-CN self-association (*28*).

The present results suggest that from ~1 to 2 molecules of vitamin D_3 were bound to each β-CN monomer. Hydrophobic interactions were largely responsible, as evidenced by the quenching of tryptophan and by the perturbation of phenylalanine, which are both located in the hydrophobic domain (*25*). Furthermore, the strength and degree of binding varied as a function of pH and ionic strength, by influencing the degree of hydrophobicity. Finally, binding also induced more ordered secondary structure as the pH was lowered from pH 8.0 to 6.6.

The results found in the present study indicated that vitamin D_3 was bound to both β-LG A and β-CN as a function of pH and ionic strength. Binding occurred under the conditions naturally found in milk (pH 6.6, $I = 0.08$ M). In addition, it is believed that vitamin D_3 can bind to β-LG A following the production of fermented milk products (i.e., cheese and yogurt) or inside the acidic stomach, since vitamin D_3 was strongly bound under all experimental conditions.

Despite the structural and functional dissimilarities between the two proteins, vitamin D_3 binding constants were comparable. Depending on the solution conditions (i.e., pH and ionic strength), β-LG A binding affinities ranged between 0.02 and 0.29 μM, whereas β-CN affinities ranged between 0.06 and 0.26 μM. These values suggest that each protein can strongly bind vitamin D_3, where strong binding is defined as $K'_d < 10$ μM (*33*). The number of binding sites was also comparable, where 0.51 to 2.04 and 1.16 to 2.05 mol of vitamin D_3 were bound per mol of β-LG A and β-CN, respectively.

If vitamin D_3 were void of its protective fat matrix (e.g., fat-free dairy products), it is believed that vitamin D_3 found in the whey or casein fraction would be stabilized by β-LG A and β-CN, since these proteins would protect vitamin D_3 from an otherwise polar environment. If vitamin D_3 stability is maintained, it can be further assumed that vitamin D_3 would be available for absorption within the human body. However, these strong binding affinities may impact on its bioavailability, which can only be assessed with clinical trials.

ABBREVIATIONS

K'_d, apparent dissociation constant; *n*, apparent mole ratio; CD, circular dichroism; CMC, critical micellar concentration; NATA, *N*-acetyl-L-tryptophanamide.

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