Stabilizing vitamin D₃ using the molten globule state of α -lactalbumin

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ABSTRACT

α -Lactalbumin (α -LA) is the second most abundant bovine whey protein. It has been intensively studied because of its readiness to populate the molten globular (MG) state, a partially folded state with native levels of secondary structure but loss of tertiary structure. The MG state of α -LA exposes a significant number of hydrophobic patches that could be used to bind and stabilize small hydrophobic molecules such as vitamin D₃ (vitD). Accordingly, we tested the ability of α -LA to stabilize vitD in a pH interval from 7.4 to 2; over this pH interval, α -LA transitions from the folded state to the MG state. The MG state stabilized vitD better than the folded state and was superior to the major bovine whey protein β -lactoglobulin (β -LG), which is known to stabilize vitD. At pH 7.4, β -LG and α -LA stabilized vitD to the same extent. Tryptophan fluorescence quenching measurements indicated that α -LA has one binding site at pH 7.4 but acquires an additional binding site when the pH is lowered to pH 2 to 4. Stability measurements of the vitD in the α -LA–vitD complex at different temperatures suggest that UHT processing would lead to little loss of vitD. This study demonstrates the potential of α -LA as a component in vitD fortification, particularly for low pH applications. **Key words:** α -lactalbumin, molten globule, vitamin D, stability, fortification

INTRODUCTION

One of the most important functions of fat-soluble vitamin D₃ (**vitD**) is to regulate calcium and phosphorus concentrations (Prentice et al., 2008). Vitamin D is produced in the skin upon sun exposure and is a natural constituent in some foods (Mattila et al., 1995; Lu et al., 2007). Nevertheless, a large part of the population lacks sufficient amounts of vitD (Huotari and

Herzig, 2008), and fortification of food with vitD is an important alternative means to obtain adequate vitD levels. The low stability and solubility of vitD makes fortification of clear beverages a challenging task. Complexation of vitD with, for example, proteins can address the low solubility and stability of vitD but the low pH of many beverages means that the final complex also needs to be acid-stable.

Several different ways of stabilizing vitD using milk proteins have been established (Wang et al., 1997; Lee and Hong, 2009; Haham et al., 2012; Diarrassouba et al., 2013, 2015a,b). One of the simplest is to complex vitD with β -LG (Wang et al., 1997; Diarrassouba et al., 2013). Increased stability is seen at both intermediate and low pH and is thought to arise by simple binding of vitD to a binding site of β LG.

Another potential vitD-complexing protein is α -LA, a globular, acidic (isoelectric point: 4.5) milk protein (14.2 kDa) that is involved in the synthesis of lactose. Conformationally, it is very versatile—mildly destabilizing conditions such as low pH, heat treatment, removal of calcium, or exposure to trifluorethanol and oleic acid induce a molten globule (**MG**) state (Permyakov and Berliner, 2000; de Laureto et al., 2002; Kaspersen et al., 2014), a flexible state with some secondary structure but no persistent tertiary structure. Experiments with the hydrophobic fluorophore 8-anilidonaphthalene-1-sulfonic acid (ANS) have shown that hydrophobic patches in the protein become more solvent exposed in the MG state, particularly in the state formed at pH 2 (Kim et al., 2016). Vibrational circular dichroism (**CD**) experiments indicate that the transition from native state to MG state occurs between pH 4.75 and pH 2.75 (Ryu et al., 2012).

We previously exploited the ability of α -LA to bind oleic acid to form so-called lipotides (complexes of lipids and partially unfolded protein) and concluded that these complexes stabilize different hydrophobic compounds at pH 7.4 (Pedersen et al., 2015, 2016). However, below pH 7, the lipotides become unstable and precipitate (Pedersen et al., 2016). α -Lactalbumin is known to bind vitD at pH 7 (Delavari et al., 2015), where α -LA has one strong binding site for vitD; excess

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amounts of vitD lead to aggregation of the protein. Here, we investigated whether α -LA could bind and stabilize vitD in the low-pH MG state through its exposed hydrophobic patches. We showed that vitamin D is indeed partially protected from degradation in the presence of α -LA at low pH. Strikingly, at low pH, α -LA stabilized vitD to a greater extent than β -LG, whereas β -LG was superior to α -LA at intermediate pH. Fluorescence and CD experiments suggested that the increased stability at low pH was linked to partial unfolding of α -LA.

MATERIALS AND METHODS

Materials and Buffers

Calcium-depleted α -LA from bovine milk ($\geq 85\%$ pure, L6010), β -LG ($\geq 90\%$ pure, L3908), and cholecalciferol (vitD, $\geq 98\%$, C9756) were from Sigma-Aldrich (St. Louis, MO). All other reagents were of high purity or HPLC grade.

To keep conditions close to those that vitD is likely to encounter in fortified drinks, we used low concentrations (5 mM) of the following buffers in combination with 15 mM NaCl: glycine (pH 2–3), acetate (pH 4–5), and Tris (pH 7.4). pH was adjusted with HCl or NaOH.

Turbidity

Sample turbidity was evaluated by measuring the light scattering at 600 nm. Vitamin D was dissolved in 96% ethanol at 115 mM and diluted in buffer to the desired concentration. Triplicates of 1 mg/mL β -LG and α -LA solutions containing 0 to 210 μ M vitD were measured at different pH at 23°C. Means and standard deviations are reported based on triplicate measurements.

Stability

The 1 mg/mL α -LA or β -LG solution was mixed with 70 μ M vitD ($\sim 1:1$ molar ratio), incubated at 37°C, and covered from light. Samples were collected at different time points. The content of intact vitD was evaluated by HPLC (Pedersen et al., 2016). To determine stability at high temperature, samples were placed on an Eppendorf block heater (SBH130DC, Stuart, Camberley, UK) at pH 3.5 and the desired temperature, with samples taken out regularly. All samples were in triplicate and means and standard deviations are reported based on triplicate measurements. The time to reach 50% of the initial vitD concentration ($t_{1/2}$) was determined by fitting vitD concentrations

against time to an exponential decay function with amplitude $A \left(A \times \exp^{-\ln(2) \times t/t_{1/2}} \right)$.

HPLC

The vitD content was determined using HPLC as described (Pedersen et al., 2016). An UltiMate 3000 HPLC (Dionex, Sunnyvale, CA) with a Kinetex 2.6u C18 100 Å column, 75 $\times$ 2.1 mm (Phenomenex, Torrance, CA) was loaded with 20 μ L of sample. A gradient from 20% of 0.1% trifluoroacetic acid in MilliQ water to 100% of 0.1% trifluoroacetic acid in acetonitrile was used to elute vitD at a flowrate of 0.3 mL/min. Elution of vitD was followed by absorbance at 265 nm, and the concentration determined based on a calibration curve, which was linear ($R^2 = 0.99$) at concentrations from 1 to 120 μ M vitD.

Circular Dichroism

Spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco Spectroscopic Co. Ltd., Tokyo, Japan) at wavelengths from 180 to 250 nm for far UV and from 250 to 350 nm for near UV, with a bandwidth of 2 nm and a scanning speed of 50 nm/min. Measurements were conducted at a concentration of 1 mg/mL protein in a 0.1- or 2-mm quartz crystal cuvette at 23°C for far and near UV, respectively. Three scans of the same sample were averaged and the buffer background subtracted.

Fluorescence

Tryptophan fluorescence was measured on a LS-55 luminescence spectrometer (Perkin-Elmer Instruments, Chalfont St. Giles, UK), using excitation at 280 nm and emission at 300–450 nm and excitation/emission slits of 10/10 nm. The protein had an initial concentration of 0.015 mg/mL (1.1 μ M) and was titrated with vitD. A total of 60 μ L of vitD was added to the protein, diluting the protein by up to 4%. This was corrected in the final data.

RESULTS AND DISCUSSION

Reduced Aggregation of Vitamin D in Presence of α -LA

The low solubility of vitD in buffer solution makes the solution turbid, as measured by scattered light at 600 nm. We previously reported that lipotides completely solubilize vitD, resulting in a clear solution with no light scattering (Pedersen et al., 2015) and we used

the same procedure to evaluate the ability of α -LA to solubilize vitD. We tested the ability of α -LA and β -LG to solubilize vitD in a pH range from 2 to 7.4, with particular focus on the low pH range where α -LA attains its MG state. When vitD was added to a buffer of pH 7.4 in increasing concentrations, a linear increase in scattered light was seen, giving a slope of $0.19 \text{ (mM vitD)}^{-1}$ (Figure 1A and Table 1). At pH 2 and 4, vitD seemed to aggregate more, resulting more light scattering (0.28 mM^{-1} and 0.23 mM^{-1} , respectively). Addition of α -LA and β -LG led to only modest overall decreases in turbidity, and under no condition was vitD completely solubilized. This might suggest that a decrease in light scattering is largely caused by the ability of these proteins to interfere with collisions between vitD molecules by blocking the encounters rather than by directly forming complexes with vitD. The trend was seen, however, for both α -LA and β -LG, although β -LG is known to bind vitD at a ratio of 1:2 (Wang et al., 1997). However, α -LA showed the greatest ability to reduce vitD scattering, with the highest light scattering at pH 7.4 (0.14 mM^{-1}) and lowest at pH 2 (0.10 mM^{-1}). The opposite trend was seen for β -LG, with the lowest light scattering at pH 7.4 (0.15 mM^{-1}) and the highest at pH 2 (0.24 mM^{-1}). Turbidity measurements of β -LG and α -LA at pH 5 are not shown because both protein

solutions gave rise to light scattering. The isoelectric points (pI) of α -LA and β -LG are 4.5 and 5.1, respectively, and aggregation of the proteins is therefore likely to occur at pH 5.

At pH 2, α -LA is known to precipitate to some extent (Kim et al., 2016) and it is possible that this contributes to light scattering. However, the amount of salt used might have a large influence on the degree of aggregation, and we did not detect any significant light scattering by α -LA alone at pH 2 (compare the intercepts at $0 \text{ } \mu\text{M}$ vitD in Figure 1A). α -Lactalbumin generally shows a stronger ability than β -LG to decrease light scattering of vitD solutions, and this is especially true at low pH. Although it is not clear whether the proteins directly contribute to light scattering of vitD, it is evident the addition of vitD leads to light scattering. Centrifugation of the samples at $13,000 \times g$ did not lead to pelleting of the protein in any of the samples.

MG State of α -LA Can Increase Stability of Vitamin D

Neither α -LA nor β -LG was able to solubilize vitD completely to yield a clear solution at the concentrations used. Nevertheless, the decrease in turbidity prompted us to investigate the stability of vitD in these complexes. We incubated vitD at 37°C in α -LA,

Figure 1. Stability and turbidity of vitamin D₃ (vitD) : (A) light scattering measurements (at 600 nm) of solutions containing different concentrations of vitD in the presence of α -LA (aLA), β -LG (bLG), or buffer at different pH values; (B) change in residual vitD over time in presence of α -LA, β -LG, or buffer at different pH values. Measurements were made at 23°C , and SD of triplicates are shown. Color version available online.

Table 1. Turbidity and stability of vitamin D₃ in different mixtures at 23°C

Mixture	Change in scattered light ¹ [1/(10 × mM)]	t _{1/2} (h) ²
α-LA		
pH 2	1.0	19.2 ± 1.1
pH 3	1.2	20.0 ± 1.0
pH 4	1.2	13.6 ± 0.3
pH 5	ND ³	12.3 ± 0.2
pH 7.4	1.4	9.8 ± 0.2
β-LG		
pH 2	2.4	5.9 ± 0.3
pH 3	2.0	7.0 ± 0.3
pH 4	1.9	12.6 ± 0.9
pH 5	ND	14.7 ± 0.4
pH 7.4	1.5	10.0 ± 0.9
Buffer		
pH 4	2.3	0.97 ± 0.1
pH 2	2.8	1.04 ± 0.1
pH 7.4	1.9	0.90 ± 0.1

¹The slope of the linear fits from Figure 1A.

²The t_{1/2} values (time required for 50% degradation of vitamin D₃) were determined from Figure 1B based on exponential decay fits. All fits were with R² > 0.97. Standard errors of all values in the second column are 0.1.

³Not determined due to intrinsic protein aggregation at this pH.

β-LG, or buffer at pH 2 to 7.4 in the absence of light (to minimize photochemical reactions) and measured the amount of intact vitD in solution over time with HPLC (Figure 1B and Table 1). Vitamin D was rapidly degraded when no protein was present in the solution, and little difference in stability could be seen from pH 2 to pH 7.4. By fitting to an exponential decay function, the time to reach 50% of the initial vitD concentration (t_{1/2}) was determined to be 0.9 to 1.0 ± 0.1 h for the buffer solutions. β-Lactoglobulin increased the stability of vitD at all pH values but was most effective at pH 5 (t_{1/2} = 14.7 ± 0.4 h) at which the protein also aggregated. This is consistent with the observation that binding of vitD to β-LG was strongest at pH 5 (Diarrassouba et al., 2013). At pH 4 to 7.4, α-LA stabilized vitD to a similar degree as β-LG, but a large change in stability was seen at pH 2 to 3. At low pH, β-LG stabilized vitD poorly (t_{1/2} = 5.9 ± 0.3 and 7.0 ± 0.3 h at pH 2 and 3, respectively), whereas α-LA worked best in stabilizing vitD at low pH (t_{1/2} = 19.2 ± 1.1 and 20.0 ± 1.0 h for pH 2 and 3, respectively; Table 1).

Binding of Vitamin D to α-LA

The increased stability of vitD when using α-LA at low pH suggests a role for the acid-induced MG state of α-LA. We therefore turned to far- and near-UV CD and Trp fluorescence measurements to elucidate the structural changes that α-LA undergoes when vitD is added.

α-Lactalbumin contains around 47% α-helical and 12% β-sheet (Kaspersen et al., 2014) structures and shows a typical α-helix CD spectrum with local minima at 208 and 220 nm (Figure 2A-B). At pH 7.4, the 2 minima are almost equal in intensity but decreasing the pH to 4 amplified the 208-nm minimum to a greater extent than the 220-nm minimum. This increase in the 208/220 nm ratio is characteristic for the α-LA MG state (Dolgikh et al., 1981) and for dissociation of helices in general (Lau et al., 1984). Little change in the CD signal was seen when vitD was added to α-LA at pH 7.4; at lower pH, the CD signal did not change the profile but underwent an overall decrease in intensity. Although the decrease in intensity could be caused by light scattering from vitD, it is unclear why this was only seen for α-LA at pH 2 to 5, but not at higher pH where vitD also led to light scattering (Figure 1A). β-Lactoglobulin, in contrast, retained the same far-UV CD irrespective of pH or addition of vitD (Figure 2C-D). β-Lactoglobulin changes from a dimeric to a monomeric form when pH is lowered (Uhrinová et al., 2000) but shows only minor changes in the far-UV CD signal in this pH range (Tau-lrier and Chalikian, 2001).

The MG state of α-LA lost most of its tertiary structure, as seen by near-UV spectra, which underwent a marked decline in intensity as the pH decreased from 7.4 to 2 (Figure 3A). However, adding vitD had no significant effect, indicating that the MG state was retained in the presence of vitD. β-Lactoglobulin underwent only a small change in tertiary structure at low pH (Figure 3B) and, again, vitD had little effect on its overall tertiary structure.

Tertiary structure may also be investigated by Trp fluorescence; vitD absorbs light at 265 nm and showed some light scattering at the investigated concentrations (Figure 1A). Furthermore, vitD can quench Trp fluorescence when in proximity. All of these effects reduce the Trp fluorescence signal as increasing amounts of vitD are added. The wavelength with maximum fluorescence intensity can still reveal whether the environment of the Trp residues changes. At pH 7.4, α-LA had a defined tertiary structure despite the lack of Ca²⁺ (Figure 3), and the maximum intensity (λ_{max}) was at 331 nm, indicating a folded α-LA structure (Figure 4A). As a large excess of vitD was added (7 μM or 7 times molar excess), the maximum intensity red-shifted slightly (333 nm), in good agreement with previous results showing that α-LA remains fairly stable when vitD is added (Delavari et al., 2015). At pH 4, the protein has lost part of its native tertiary structure (λ_{max} = 335 nm) and the signal is further changed when vitD is added (λ_{max} = 340 nm). At pH 3 and 2, the protein has already lost its native tertiary structure and is exposed to the

Figure 2. Secondary structural changes of α -LA (aLA) and β -LG (bLG): far-UV circular dichroism (CD) signal of (A, B) α -LA and (C, D) β -LG at pH 2 to 7.4 in the absence or presence of vitamin D₃ (vitD). (A, C) Far-UV CD signals, and (B, D) far-UV CD signal intensity at 208 nm and intensity at 208/220 nm as a function of pH. Color version available online.

solvent ($\lambda_{\max} = 346$ and 347 nm, respectively) and no further change is seen when vitD is added ($\lambda_{\max} = 346$ and 347 nm, respectively). Because vitD induced very little change in tertiary structure according to near-UV CD (Figure 3A), we attributed the change in $\lambda_{\max}$ at pH

7 and 4, when vitD is added, to vitD binding close to Trp residues. To obtain further information on binding of vitD to α -LA at different pH values, we measured Trp fluorescence at different vitD: α -LA molar ratios. α -Lactalbumin has 4 Trp residues and quenching can

Figure 3. Tertiary structural changes of α -LA (aLA) and β -LG (bLG): near-UV circular dichroism (CD) signal of (A) α -LA and (B) β -LG at pH 2 to 7.4 in the absence or presence of vitamin D₃ (vitD). Color version available online.

be used to resolve how large a fraction of the Trp residues is accessible (Lehrer, 1971). To determine this, we used a modified form of the Stern-Volmer equation:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a K_a [\text{vitD}]} + \frac{1}{f_a}, \quad [1]$$

where F_0 is the fluorescence in the absence of vitD, F is the fluorescence at a given vitD concentration, K_a is the Stern-Volmer quenching constant of the accessible fraction, $[\text{vitD}]$ is the vitD concentration, and f_a is the fraction of fluorophore population accessible to quencher. In the case of α -LA at pH 2 to 4, a linear correlation was seen for the range of vitD concentrations used (Figure 4B). This gave a value of f_a of 0.87 to 1.2, suggesting that most of the Trp residues are accessible to the quencher, in good agreement with loss of tertiary structure and formation of the MG state at low pH. At pH 7.4, a linear relation was also seen at low concentrations of vitD (Figure 4B). Here, f_a was 0.54, suggesting that half of the Trp residues are protected when α -LA is folded. At high concentrations of vitD at pH 7.4, it was clear, however, that there was no linear relationship between $F_0/(F_0 - F)$ and $1/[\text{vitD}]$ (Figure 4C). Because the data were not linear at the lowest values of $1/[\text{vitD}]$, we fitted data to a cubic polynomial to empirically extrapolate to 0 $1/[\text{vitD}]$ and thus to estimate the total fraction of accessible Trp (f_a). We are aware

that extrapolation is highly sensitive to the order of the polynomial but found that a cubic polynomial was the lowest order that gave a satisfactory fit for all 4 data sets (Figure 4C). This showed that all Trp residues became accessible at high concentrations independent of the pH ($f_a = 1.11, 1.21, 1.00$, and 1.03 for pH 2, 3, 4, and 7.4, respectively).

The Trp quenching data were also used to obtain a measure of the dissociation equilibrium constant (K_d) by fitting data to the equation (González-Mondragon et al., 2004):

$$1 - \frac{F}{F_0} = \frac{a}{2E_t} \left[\frac{(E_t + [\text{vitD}] + K_d)}{-\sqrt{(E_t + [\text{vitD}] + K_d)^2 - 4[\text{vitD}]E_t}} \right], \quad [2]$$

where F_0 is the fluorescence in the absence of vitD, F the fluorescence at a given vitD concentration, E_t is the concentration of α -LA, and a is the asymptotic value to which $1 - F/F_0$ tends at high $[\text{vitD}]$. The equation was fitted to data for vitD titration in α -LA (Figure 5). A good fit ($R^2 > 0.99$) was obtained for measurements at pH 2 to 4 (Figure 5), whereas a clear deviation from the fit was seen at pH 7.4 (Figure 5; inset). This was most likely caused by the different Trp quenching regions seen at pH 7.4 for high and low vitD concentrations (Figure 4B). For this reason, data measured at pH 7.4 were also fitted to a modified equation [2] with an ad-

ditional term that accounts for a second binding step at vitD concentrations sufficiently high to assume that vitD is present in excess. This means that we can ap-

ply a simple Michaelis-Menten-type binding isotherm. The 2 binding steps are constrained through a total amplitude set to 1 through the factor $(1 - a)$:

Figure 4. Intrinsic quenched fluorescence intensity: (A) Trp fluorescence (arbitrary units, a.u.) of α -LA (aLA) at pH 7.4 and 2 with (8.2 and 8.4 μM , respectively) and without vitamin D₃ (vitD); (B) modified Stern-Volmer plot [$F_0/(F_0 - F)$] for determination of fluorescence accessible to the quencher, where F_0 is the fluorescence in the absence of vitD, F is the fluorescence at a given vitD concentration. Data were measured at pH 2, 3, 4, and 7.4 and fitted to equation [1]; (C) detail of panel B ($1/\text{vitD}$ of 0–0.4 μM) for high concentration of vitD. Data were fitted to a cubic polynomial to determine the fraction of initial fluorescence accessible to the quencher. Color version available online.

$$1 - \frac{F}{F_0} = \frac{a}{2E_t} \left[\frac{(E_t + [\text{vitD}] + K_d)}{-\sqrt{(E_t + [\text{vitD}] + K_d)^2 - 4[\text{vitD}]E_t}} \right] [3] \\ + (1-a) \frac{[\text{vitD}]}{K'_d + [\text{vitD}]},$$

where K'_d denotes the dissociation equilibrium constant associated with high vitD concentrations at pH 7.4. Using equation [3] for pH 7.4 and equation [2] for pH 2 to 4, we determined K_d to be 0.048 ± 0.059 , 1.39 ± 0.07 , 1.06 ± 0.05 , and 1.11 ± 0.05 μM for pH 7.4, 4, 3, and 2, respectively, whereas K'_d at pH 7.4 was estimated to be 10.2 ± 1.2 μM . However, the apparent higher affinity at pH 7.4 suggested by the lower K_d value is misleading, because the degree of quenching does not increase more steeply at pH 7.4 than at the lower pH values (Figure 5), and a fit using the simpler equation [2] leads to an apparent K_d value of 0.98 ± 0.14 μM . Consistent with this, an earlier determination of K_d for α -LA in 20 mM Tris, pH 7.0, yielded a value 0.7 μM (Delavari et al., 2018). The 2-state quenching at pH 7.4 complicates data interpretation, and the large error associated with K_d brings into question the validity of the value. We also tried purifying the complex between α -LA and vitD using gel filtration, but α -LA and vitD eluted in different fractions at all pH tested (pH 2, 3, 4, and 7.4; data not shown). Furthermore, small-angle x-ray scattering measurements of α -LA at pH 2, 3, 4, and 7.4 did not show any difference with or without vitD (data not shown). This further supports the hypothesis that the stabilization of vitD by α -LA, particularly at low pH, does not arise from stable binding between α -LA and vitD but rather by flexible and dynamic interactions between the 2 components.

It is worth noting that the Trp fluorescence measurements were made at 0.015 mg/mL protein, which is much lower than the 1 mg/mL used for CD, light scattering, and stability measurements; the degree of vitD aggregation, in particular, might be affected by this difference in concentration.

UHT Processing in Presence of α -LA

During processing of food and beverages, it is common to use UHT processing to increase shelf life. Products can be heated to 135°C for a few seconds, during which time vitD might be degraded because of its poor stability. To estimate the stability of vitD at 135°C, we measured its stability at 20 to 80°C, pH 3.5 (i.e., with α -LA in the MG state; Figure 6A). These measurements showed a reasonably linear correlation in an Arrhenius-type plot (Figure 6B). Extrapolation to

135°C gives an estimated $t_{1/2}$ of 281 s. Such an extrapolation assumes that the MG state of α -LA is stable at higher temperatures and can retain the local binding environment of vitD. Although we cannot demonstrate this experimentally, we note that the MG state of α -LA is stable to at least 90°C (Dolgikh et al., 1981). A $t_{1/2}$ of around 5 min indicates that only a small amount of vitD would be lost during a brief UHT treatment (~1% after 5 s). Earlier studies of UHT treatment of lipotides made with α -LA showed that $t_{1/2} = 21$ s (Pedersen et al., 2015; i.e., 15% loss after 5 s). It is likely that the practical $t_{1/2}$ of the lipotides is in fact higher, because high temperatures might destabilize the lipotide and release α -LA that can improve vitD stability at high temperatures. The lipotide studies, however, were conducted at pH 7.4 and the results are therefore not directly comparable.

CONCLUSIONS

α -Lactalbumin is able to stabilize vitD to the same extent as β -LG at pH 7.4 and to a greater extent at pH 2 to 3. We attribute this ability of α -LA to stabilize vitD to the existence of the MG state at low pH,

Figure 5. Equilibrium binding constant from intrinsic fluorescence: $1 - (F/F_0)$ as a function of vitamin D₃ (vitD) concentration at pH 7.4 to 2, where F_0 is the fluorescence in the absence of vitD, F is the fluorescence at a given vitD concentration. Data were fitted to equation [3] for pH 7.4 and equation [2] for pH 2–4. The stippled vertical line indicates the concentration of α -LA (1.1 μM). Inset: Fitting of data at pH 7.4 using equation [3] (solid line) and equation [2] (stippled line). Color version available online.

Figure 6. Vitamin D₃ (vitD) stability at increased temperatures: (A) decline in residual vitD with time at different temperatures; solutions contained vitD and α -LA at pH 3.5; (B) Eyring plot showing the temperature dependence of vitD degradation as seen by the time for 50% vitD degradation ($t_{1/2}$) calculated from (A) in the presence (●) or absence (○) of α -LA, giving a fit of $y = -5.5(\pm 1.0) + 2.5(\pm 0.3)x$. In panel A, SD are shown from triplicates. Color version available online.

where hydrophobic patches of the protein become more solvent exposed and accessible for vitD binding. At pH 7.4, α -LA is quenched by vitD in 2 steps, suggesting 2 different binding sites. At pH 2 to 4, α -LA reaches the MG state and all Trp residues become exposed for quenching. Stability measurements at different temperatures suggest that the α -LA–vitD complex might withstand UHT treatment and is a good candidate for vitD fortification in low pH beverages. Commercial fortified drinking products typically contain a few nanograms of vitD per milliliter, which is 3 to 5 orders of magnitude less than the concentration range used in the present study (3–200 μ g/mL vitD). Under these conditions, turbidity (an aesthetic rather than a functional issue from the consumer viewpoint) would be negligible. However, vitD should still benefit from the higher stability and solubility conferred by α -LA, leading to longer shelf life and perhaps greater physiological accessibility. Future studies under application-relevant conditions may resolve whether this is the case.

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