β-Lactoglobulin/Folic Acid Complexes: Formation, Characterization, and Biological Implication

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β-Lactoglobulin/Folic Acid Complexes: Formation, Characterization, and Biological Implication

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β-Lactoglobulin (β-LG), the major whey protein in bovine milk, binds to a wide range of compounds. Folic acid (FA) is a synthetic form of the B group vitamin known as folates, which are essential cofactors for a variety of physiological processes. The interaction of β-LG with FA was studied using fluorescence spectroscopy to determine the FA binding constant and mode and the influence of the protein on FA photodegradation. At ≤20 μM FA, which may be the critical self-association concentration, the binding constant and number are 2.0 (±0.6) × 10^6 M^{-1} and 1.30 (±0.03) when excited at 280 nm and 4.3 (±2.2) × 10^5 M^{-1} and 1.17 (±0.04) at 295 nm, as determined by protein intrinsic fluorescence. FA binds to the surface of β-LG, possibly in the groove between the α-helix and the β-barrel. Fluorescence analysis of the pterin portion of FA shows that complexation with β-LG improves FA photostability. It is suggested that β-LG complexes could be used as an effective carrier of FA in functional foods.

Introduction

Preventing or delaying the onset of chronic diseases has become an attractive strategy for improving the cost-effectiveness of public health spending.[1] Increasing intake of bioactive compounds in the form of so-called functional foods to provide benefits beyond basic nutrition has been proposed as an alternative to classical pharmacology for improving health and well being as well as lessening the burden of disease.[2,3] However, most bioactive compounds are sensitive to environmental factors associated with food processing and storage, such as light, oxygen and heating.[4] A promising approach to addressing the problem is to use nanocomplexes of ligand-binding proteins as carriers for encapsulation and protection of bioactive compounds because the proteins generally exhibit a high affinity to their ligands.[5]

β-Lactoglobulin (β-LG) is one of the most abundant proteins in bovine milk whey. β-LG contains endogenously bound fatty acids when isolated from milk using nondenaturing technique.[6] The binding ability of fatty acids to β-LG is dependent on the hydrocarbon chain and is strongest for the 16-carbon palmitate.[7] The ability of β-LG to binding fatty acids has been developed for use as an emulsifying agent in food technology or as a fatty acid carrier in cell culture.[8]

β-LG has been reported to bind a variety of hydrophobic and amphiphilic compounds. The binding constants for different compounds with β-LG vary widely from 1.5 × 10^2 M^{-1} for 2-heptanone and 6.8 × 10^5 M^{-1} for palmitate to as high as 5 × 10^7 M^{-1} for retinol.[9] Under physiological conditions, β-LG exists as a dimer with each monomer containing 162 amino acid residues and having a molecular mass of 18 kDa.[10] β-LG folds into a central calyx formed by eight antiparallel β-strands and an α-helix located at the outer surface of the β-barrel.[11,12] Three portions of β-LG have been suggested as sites for ligand binding: the internal cavity of the β-barrel, the surface hydrophobic pocket in a groove between α-helix and the β-barrel, and the outer surface near Trp19-Arg124.[13]

The impact of binding to β-LG on the physical and chemical properties and biological activity of bioactive compounds has been reported. β-LG can form water-soluble complexes with retinol and β-carotene and could thus protect these lipophilic compounds from degradation by heat, oxidation, and irradiation.[14] Complexation with the protein provides a significant increase in the hydro-solubility of the amphiphilic compound *trans*-resveratrol and a slight increase in its photostability.[15]

Folate constitutes a group of water-soluble B-vitamins present in many chemically related derivatives. Folic acid (FA), a synthetic form of folate, is composed of pterin, *p*-aminobenzoyl, and L-glutamic acid (Figure 1). It is the oxidized and most stable form of the folates.[16] In vivo, FA is reduced on the pteridine ring at positons 5, 6, 7, and 8 by dihydrofolate reductase to form biologically active tetrahydrofolate (THF).[17] THF and its derivatives are cofactors for enzymes in one-carbon transfer reactions and are required in the biosynthesis of purines, thymidylate, and several amino acids.[18] Folate is therefore essential for the proper functioning of a variety of physiological processes in humans. This vitamin plays an important role in the prevention of neural tube defects in infants and possibly in the intervention on vascular diseases and several types of cancers.[19]

Humans cannot synthesize folate de novo and depend entirely on their diet to provide this vitamin.[20] Folate nutritional status is dependent on intake with food and supplements and on the bioavailability of the various ingested forms.[21] Products fortified with folate and food with added folate are substantially more effective at improving this vitamin nutrition status than folate naturally present in foods.[22] FA fortification and periconceptional supplementation has thus been reported to reduce the incidence of neutral tube defects.[23]

However, FA is known to be sensitive to ultraviolet (UV) radiation, which degrades it rapidly by breakage of the C9−N10 bond to yield inactive pteridine and *p*-aminobenzoyl glutamate.[24]

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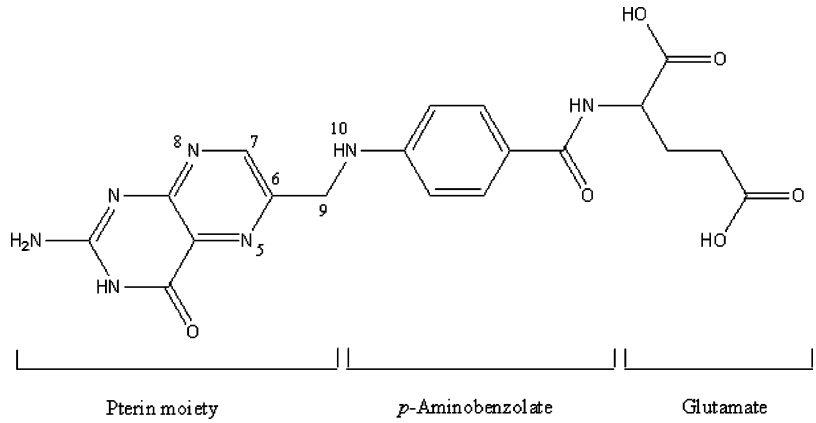

Figure 1. Structure of folic acid.

In the present study, the interaction between β-LG and FA was investigated using fluorescence spectroscopy. The binding constant, number of, and site of FA on β-LG were determined, and the influence of interaction with β-LG on FA photodegradation was discussed. The data gathered from these experiments could be useful in the application of β-LG as a carrier for water-soluble active compounds.

Material and Methods

Materials. β-LG (B variant, Purity ≥90%), folic acid (98%), and retinol (≥99.0%) were purchased from Sigma-Aldrich Chemical Co. and used without further purification.

Samples Preparation. β-LG stock solution was made by dissolving the protein in 10 mM phosphate buffer at pH 7.4 to obtain a concentration of 100 μM, measured as absorbance at 278 nm using a molar extinction coefficient of 17 600 M^{-1} cm^{-1}.[25] Folic acid stock solution was prepared freshly for each experiment by dissolving in 10 mM phosphate buffer at pH 7.4 at a concentration of 500 μM. β-LG-FA mixtures were prepared by adding β-LG and FA stock solution to phosphate buffer in varying proportions.

Retinol stock solution was prepared freshly by dissolving in ethanol at a concentration of 500 μM. Samples containing retinol were prepared by adding β-LG and retinol stock solution to 10 mM phosphate buffer at pH 7.4, followed by FA stock solution after 1 h. All samples were prepared at room temperature in plastic tubes and covered with aluminum foil.

Steady-State Fluorescence Measurement. Fluorescence was measured using a Cary Eclipse fluorescence spectrophotometer (Varian Inc.). The spectral resolution of both excitation and emission was 5 nm. FA fluorescence emission spectra were recorded from 290 to 545 nm with an excitation wavelength of 280 nm and from 360 to 600 nm with an excitation wavelength of 348 nm. Protein intrinsic fluorescence emission spectra were recorded from 300 to 550 nm with an excitation wavelength of 295 nm and from 290 to 500 nm with an excitation wavelength of 280 nm.

UV Irradiation. Samples were exposed to UV light (peak λ = 365 nm) using a UVL-21 ultraviolet lamp equipped with the mode lamp stand (VWR International Inc.) with the fluence rate set at 1 mW·cm^{-2}. Samples were placed on the lamp stand and about 9 cm away from light source and analyzed every 5 min for up to 60 min.

Results and Discussion

Structure of Folic Acid in Aqueous Solution by Fluorescence. FA pterin moieties possess both H-bond donor and acceptor groups and have the potential for self-recognition and self-assembly.[26] Fluorescence can be used to study fluorophore self-assembly because self-aggregation induces fluorescence self-quenching.[27,28] Figure 2 shows the fluorescence of FA measured at the emission maximum (λ_{max}, ~455 nm, see inset) with an excitation wavelength of 348 nm over a 1000-fold range of concentrations. The emission maximum is attributed to pterin moieties.[29] Fluorescence intensity increased with FA concentration up to 100 μM, beyond which the intensity begins to decrease, suggesting intermolecular quenching by self-association of pterin moieties. The critical concentration is lower than determined previously (600 μM) using circular dichroism for the formation of chiral columnar aggregates composed of a stacked array of FA tetramers.[30] We therefore speculate that association starting at 100 μM may involve the formation of dimers, trimers, or tetramers but not stacking into chiral columnar aggregates.

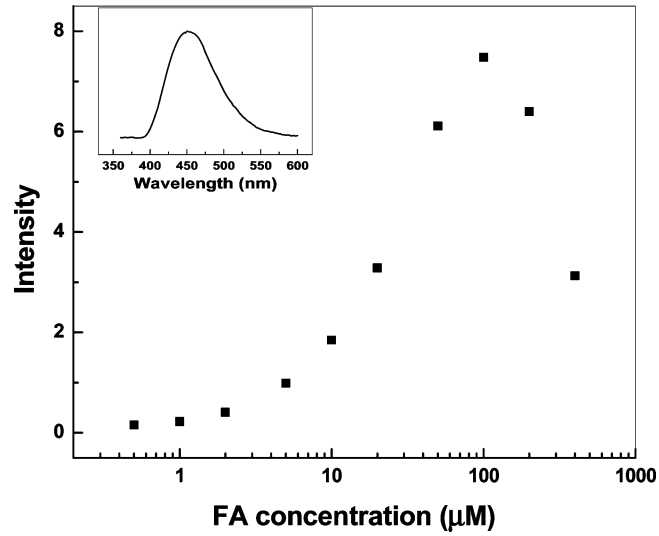

Figure 2. Fluorescence (at λ_{max}) of folic acid at 0.5, 1, 2, 5, 10, 20, 50, 100, 200, and 400 μM in phosphate buffer (10 mM, pH 7.4) with excitation λ = 348 nm. The inset shows emission λ_{max}.

When excited at 280 nm, both the pterin and *p*-aminobenzoyl glutamate moieties of the FA molecule emit fluorescence producing peaks at 455 and 360 nm (inset in Figure 3) with the latter associated with *p*-aminobenzoyl glutamate.[16] The intensities increased with FA concentration up to 20 μM and then began to decrease, suggesting self-quenching due to intermolecular association. Internal quenching of pterin moiety fluorescence by *p*-aminobenzoyl glutamate[24,29] may explain why the intensity at 455 nm also began to decrease at 20 μM. FA self-aggregation thus appears to occur in two steps: association of

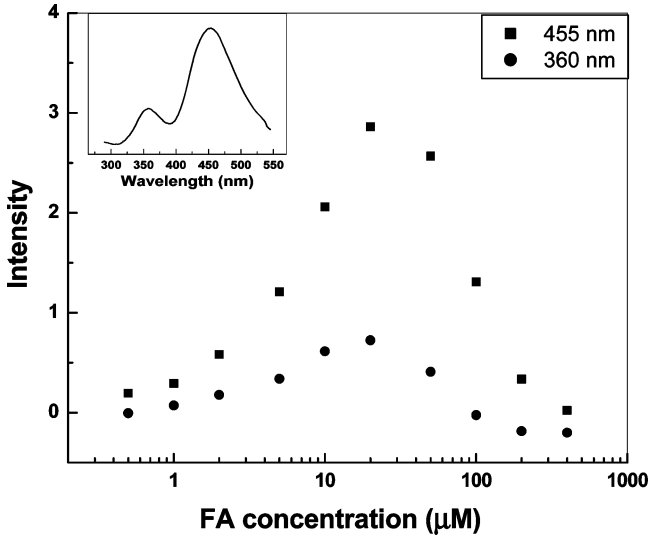

Figure 3. Fluorescence (at both λ_{max}) of folic acid at 0.5, 1, 2, 5, 10, 20, 50, 100, 200, and 400 μM in phosphate buffer (10 mM, pH 7.4) with excitation $\lambda = 280$ nm. The inset shows emission λ_{max} around 360 and 455 nm.

p-aminobenzoyl glutamate above 20 μM followed by association of pterin moieties above 100 μM. It has been reported that the formation of chiral columnar aggregates of FA did not substantially change glutamic chain mobility even above 600 μM.[30] The first step of FA association may therefore be due to the benzyl group rather than glutamic acid.

Study of FA/β-LG Interaction by Protein Intrinsic Fluorescence. β-LG has two tryptophan (Trp) residues and four tyrosine (Tyr) residues per monomer.[31] Only Trp residues produce a fluorescent emission with an excitation wavelength of 295 nm, while Trp and Tyr residues both emit fluorescence when excited at 280 nm, Trp having the higher quantum yield. Figure 4 shows the intrinsic fluorescence emission spectra of β-LG at excitation wavelengths of 280 nm (A) and 295 nm (B) in the presence of different concentrations of folic acid at pH 7.4. The FA concentration was increased only up to 20 μM because of its fluorescence self-quenching and self-aggregation at the higher concentrations. β-LG has an emission λ_{max} of about 336 nm at both excitation wavelengths, but the intensity obtained at 280 nm was about 1.6 times that at 295 nm. The λ_{max} did not change at either excitation wavelength as the FA concentration increased to 20 μM, suggesting that the interaction with FA does not change the microenvironment of the Trp residues in the β-LG native state at a FA/β-LG molar ratio ≤2.

Figure 4 also shows that the β-LG fluorescence intensity decreased with FA concentrations at both excitation wavelengths of 280 and 295 nm. It decreased to 42% and 46%, respectively,

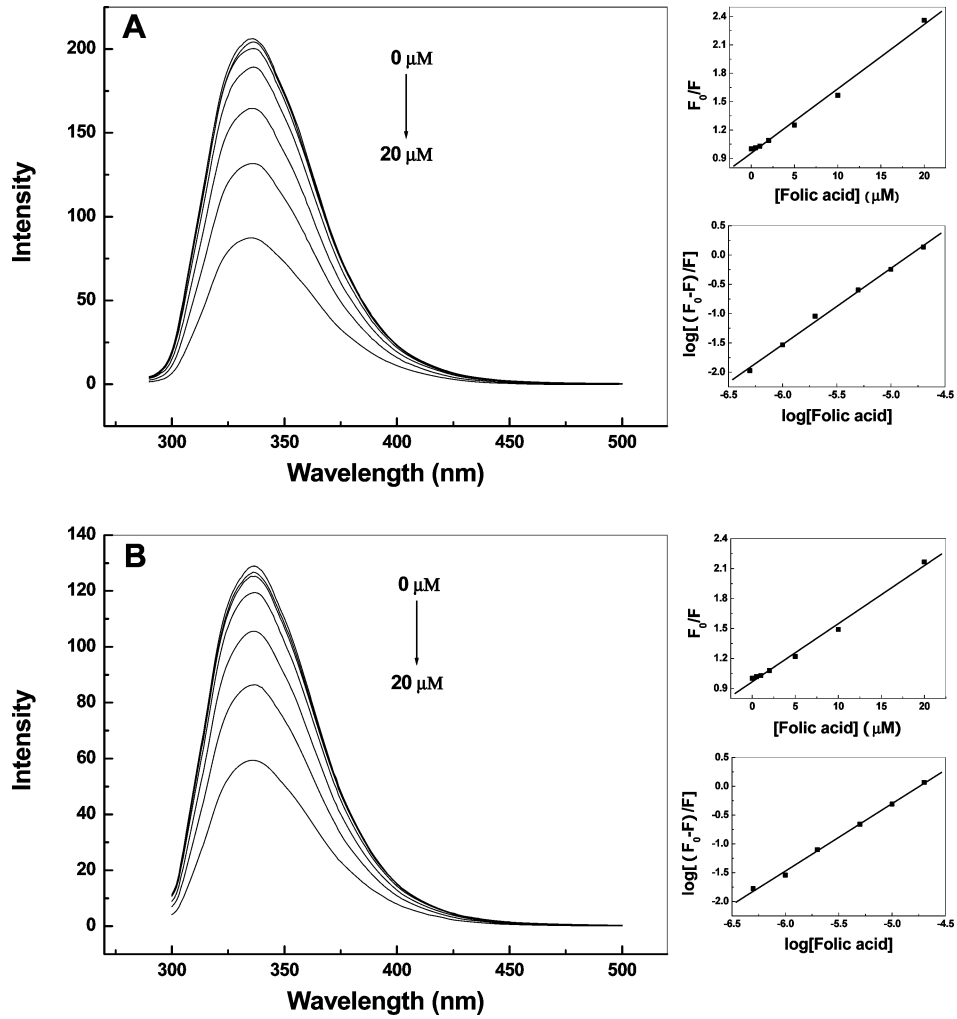

Figure 4. Fluorescence emission spectra of 10 μM β-LG in the presence of 0, 0.5, 1, 2, 5, 10, and 20 μM folic acid in phosphate buffer (10 mM, pH 7.4) at excitation wavelengths of 280 nm (A) and 295 nm (B): (upper inset) F_0/F versus [FA] (Stern−Volmer equation); (lower inset) $\log[(F_0 - F)/F]$ vs log [FA] (eq 2).

when the FA concentration reached 20 μM. These results indicate the occurrence of FA-induced protein fluorescence quenching. β-LG has an overall radius of ~2 nm, with nearly 60% of its mass within 0.5 nm of the surface and almost 90% within 1 nm.[32] Ligand-induced β-LG fluorescence quenching might occur through diffusion of free ligand within the distance for fluorescence resonant energy transfer between two fluorophore groups (i.e., dynamic quenching). The concentration dependence of the fluorescence intensity was thus analyzed using the Stern−Volmer equation[33]

$$F_0/F = 1 + k_q \tau_0 [\mathrm{FA}] = 1 + K [\mathrm{FA}] \quad (1)$$

F_0 and F are the fluorescence emission intensities in the absence and presence of quencher FA, respectively; k_q is the fluorescence quenching rate constant; τ_0 is the fluorescence lifetime of fluorophone in the absence of quencher; [FA] is the concentration of FA; and K is a constant equal to the reciprocal of the quencher concentration when the fluorescence intensity decreases by half. The linear plot of F_0/F as a function of [FA] is given in the upper inset of Figure 4A and B. Calculated from the slop of the straight line, K values are 6.8 (±0.2) × 10^4 and 5.8 (±0.1) × 10^4 M^{-1} at excitation wavelengths 280 and 295 nm, respectively. τ_0 has been reported to be 1.28 ns for the Trp residues of β-LG at neutral pH.[34] Therefore, k_q can be calculated to be 5.3 × 10^{13} and 4.5 × 10^{13} M^{-1} s^{-1} at 295 and 280 nm, respectively. The k_q are much higher than the maximal dynamic quenching constant (2.0 × 10^{10} M^{-1} s^{-1}),[35,36] indicating that the change in fluorescence intensity of β-LG is attributable mainly to the formation of its complexes with FA (static quenching).

For static quenching, the concentration dependence of β-LG fluorescence intensity can be analyzed according to the following equation[36,37]

$$\log[(F_0 - F)/F] = \log K_s + n \log [\mathrm{FA}] \quad (2)$$

K_s is the binding constant and n is binding number of FA to β-LG. The linear plot of $\log[(F_0 - F)/F]$ as a function of log [FA] is shown in the lower inset of Figure 4. From the slope of the straight line, n values are 1.30 (±0.03) and 1.17 (±0.04) at 280 and 295 nm, respectively. Calculated from the intercept, K_s values are 2.0 (±0.6) × 10^6 and 4.3 (±2.2) × 10^5 M^{-1} when excited at 280 and 295 nm, respectively. At neutral pH, β-LG exists as a mixture of monomers and dimers and the proportion of dimer is 29% at 10 μM protein.[38] This was not taken into account when protein intrinsic fluorescence were analyzed according to eqs 1 and 2, possibly leading to an overestimation of the binding constant.[32]

β-LG reportedly has a high affinity for the water-insoluble compound retinol, with a binding constant of about 5.0 × 10^7 M^{-1} and one binding site at the hydrophobic cavity.[9,39] The influence of retinol on FA-induced changes in β-LG fluorescence emission spectra was thus studied in an attempt to identify the binding site of FA on β-LG. Figure 5 shows β-LG fluorescence emission spectra in the absence and presence of FA, retinol or both at excitation wavelengths of 280 (A) nm and 295 (B) nm. Normalized relative to fluorescence intensity of pure β-LG at λ_{max} (left ordinate axis), FA decreased β-LG fluorescence intensity to about 68% at 280 nm (curve b in Figure 5A). The protein fluorescence intensity decreased to about 51% in the presence of 10 μM retinol, with a new λ_{max} appearing around 480 nm (curve c in Figure 5A), attributed to retinol.[40] Addition

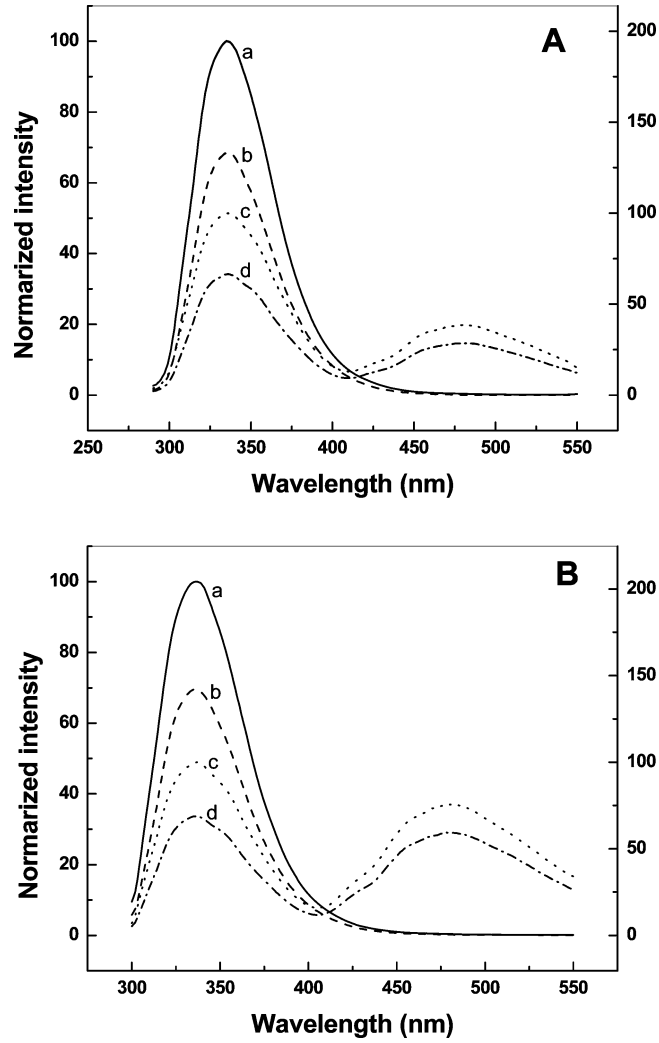

Figure 5. Fluorescence emission spectra of β-LG alone (a), in the presence of folic acid (b), retinol (c), and both retinol and folic acid (d) in phosphate buffer (10 mM, pH 7.4) at excitation wavelengths of 280 nm (A) and 295 nm (B). The left ordinate axis is normalized relative to the fluorescence intensity of pure β-LG at λ_{max}, and the right ordinate axis is normalized relative to the fluorescence intensity of pure β-LG in the presence of retinol at λ_{max}. The concentrations of β-LG, retinol, and folic acid are all 10 μM.

of FA decreased the retinol fluorescence intensity, indicating the occurrence of FA-induced retinol fluorescence quenching. Normalized relative to fluorescence intensity of the β-LG-retinol mixture at λ_{max} (right ordinate axis), FA decreased the β-LG fluorescence intensity in the presence of retinol to about 66% (curve d in Figure 5A), very close to the value obtained in the absence of retinol. The results are similar for excitation at 295 nm (Figure 5B). FA decreased the β-LG fluorescence intensity to about 70% (left ordinate axis, curves a and b) and decreased the protein fluorescence in the presence of retinol to about 69% (right ordinate axis, curves c and d). These results indicate that retinol has no apparent influence on FA-induced β-LG fluorescence quenching at either excitation wavelength, suggesting that the binding site of FA is different from that of retinol.

Two different binding sites at the surface of β-LG have been reported: the outer surface near Trp19-Arg124 and the surface hydrophobic pocket in a groove between the α-helix and the β-barrel.[13] The β-LG monomer contains two Trp residues at positions 19 and 61 (Trp19 and Trp61). Trp19 is in an apolar environment and contributes about 80% of the total fluorescence, while the fluorescence of the partly exposed Trp61 is probably

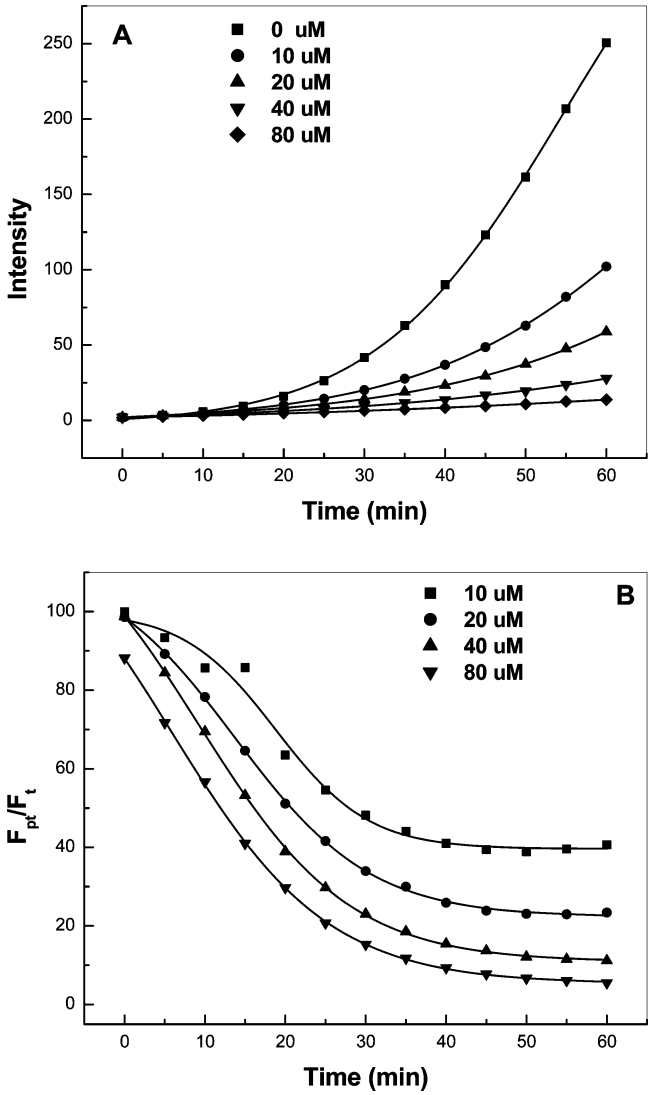

Figure 6. (A) Fluorescence of 10 μM folic acid around 455 nm in the presence of 0, 10, 20, 40, and 80 μM β-LG in phosphate buffer (10 mM, pH 7.4) as a function of UV radiation time. (B) Ratio of fluorescence intensity in the presence (F_{pt}) and absence (F_t) of β-LG. The excitation wavelength is at 348 nm.

quenched by the proximity of the Cys66−Cys160 disulfide bond.[39] When the excitation wavelength is 295 nm, only Trp produces a fluorescent emission. The β-LG monomer also contains four Tyr residues at positions 20, 42, 99, and 102,[41] with Tyr102 located in the groove between the α-helix and the β-barrel.[42] All Trp and Tyr residues emit fluorescence when excited at 280 nm. As discussed above, FA bound to the surface of β-LG with a greater binding constant at 280 than at 295 nm. However, in the case of resveratrol bound at the surface of β-LG, the binding constant is greater at 295 than at 280 nm.[15] Generally, the closer the quencher to the fluorophore, the greater the quenching effect. We therefore speculate that the binding site of resveratrol may be on the outer surface near Trp19-Arg124, while FA binds to the surface hydrophobic pocket in a groove between the α-helix and the β-barrel.

Influence of Binding to β-LG on Photodegradation of Folic Acid. An extremely low quantum yield of FA fluorescence (<0.005) has been reported in comparison with pterin (~0.30), which has been attributed to internal quenching.[24,29] Figure 6A shows a marked increase in pterin-associated fluorescence intensity near 455 nm when FA was exposed to UV radiation, no doubt due to cleavage of the C9−N10 bond.[24] After 60 min of exposure, the intensity was about 123 times as strong as without UV radiation. The increase in FA fluorescence intensity induced by photodegradation was attenuated by addition of β-LG, more so as the protein concentration increased from 10 to 80 μM. These results indicate that interaction with β-LG improves the photostability of FA significantly. Figure 6B shows the ratio of the fluorescence intensity in the presence (F_{pt}) and absence (F_t) of β-LG at various exposure times. The F_{pt}/F_t ratio gradually decreases over time, reaching a plateau of about 40% in the presence of 10 μM β-LG after 40 min and about 22%, 12%, and 6%, respectively, in the presence of 20, 40, and 80 μM β-LG after 50 min. Due to the negligible fluorescence intensity of FA not exposed to UV radiation, the plateau ratio should be proportional to the degree of FA photodegradation. Complexation with β-LG can therefore be expected to decrease FA photodegradation to 40%, 22%, 12%, and 6%, respectively, at protein concentrations equal to twice, 4, and 8 times the FA concentration.

Conclusions

Self-association of FA at pH 7.4 occurs possibly by association of the benzyl group above 20 μM, followed by association of pterin moieties above 100 μM. At concentrations less than 20 μM, FA binds to the surface of β-LG. The binding constant and number are 2.0 (±0.6) × 10^6 M^{-1} and 1.30 (±0.03) when analyzed at an excitation wavelength of 280 nm and are 4.3 (±2.2) × 10^5 M^{-1} and 1.17 (±0.04) at 295 nm. Complexation with β-LG decreases FA photodegradation to 40% and 6% when β-LG concentrations are equal to and 8 times the FA concentration. β-LG may thus be considered a good carrier of FA and FA-β-LG complexation a useful model of interactions between proteins and bioactive compounds.

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