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Locating the Binding Sites of Folic Acid with Milk α- and β-Caseins

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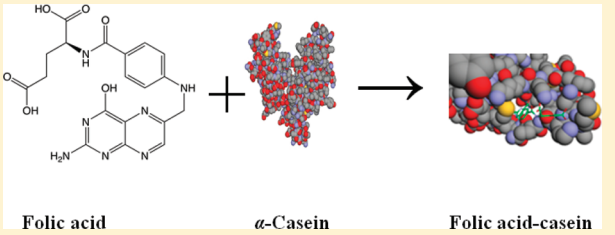
ABSTRACT: We located the binding sites of folic acid with milk α- and β-caseins at physiological conditions, using constant protein concentration and various folic acid contents. FTIR, UV−visible, and fluorescence spectroscopic methods as well as molecular modeling were used to analyze folic acid binding sites, the binding constant, and the effect of folic acid interaction on the stability and conformation of caseins. Structural analysis showed that folic acid binds caseins via both hydrophilic and hydrophobic contacts with overall binding constants of $K_{\text{folic acid}-\alpha\text{-caseins}} = 4.8\ (\pm 0.6) \times 10^4\ \text{M}^{-1}$ and $K_{\text{folic acid}-\beta\text{-caseins}} = 7.0\ (\pm 0.9) \times 10^4\ \text{M}^{-1}$. The number of bound acid molecules per protein was 1.5 (±0.4) for α-casein and 1.4 (±0.3) for β-casein complexes. Molecular modeling showed different binding sites for folic acid on α- and β-caseins. The participation of several amino acids in folic acid−protein complexes was observed, which was stabilized by hydrogen bonding network and the free binding energy of −7.7 kcal/mol (acid-α-casein) and −8.1 kcal/mol (acid-β-casein). Folic acid complexation altered protein secondary structure by the reduction of α-helix from 35% (free α-casein) to 33% (acid-complex) and 32% (free β-casein) to 26% (acid-complex) indicating a partial protein destabilization. Caseins might act as carriers for transportation of folic acid to target molecules.

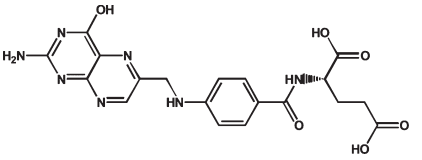


INTRODUCTION

Folic acid, a member of the vitamin B family complexes known as folate, functions primarily as a methyl-group donor involved in many important biological processes, including DNA synthesis.[1] Folate deficiency can contribute to neural tube defects and lowering serum homocysteins causing cardiovascular disease.[2−4] Folic acid is composed of three primary structures, a heterobicyclic pteridine ring, *para*-aminobenzoic acid, and glutamic acid (Scheme 1). Because humans cannot synthesize this compound, it is a dietary requirement used in dietary supplements or fortified foods.[5] However, a limiting factor is the bioavailability of folate from various food sources. The best folate sources in foods are green leafy vegetables, sprouts, fruits, liver, and kidney. Diary products should also be considered as an interesting food category for folate absorption and potential matrix for folate fortification because milk can enhance folate bioavailabilty from our diet (folic acid-milk). It is known that plasma folates are associated with low affinity binding proteins.[6,7] Therefore, it was of interest to study the complexation of folic acid with milk proteins and determine the effect of such interaction on the stability and structures of both folic acid and milk proteins in aqueous solutions.

Caseins are the major phosphoproteins of mammalian milk and exist as micelles made of polypeptides known as α-, β-, and κ-caseins.[8] The three casein components are almost similar in size, molecular weight (24 kD), and net negative charge but differ in their degree of unfoldedness.[9−12] Caseins belong to the rapidly growing family of unstructured proteins that lately attracted much of the interest due to their unique unfolded structure under native conditions, brought about by a combination of high net charge and low intrinsic hydrophobicity.[8,12]

Scheme 1. Chemical structure of folic acid



α-Casein contains two tryptophan (Trp) residues, while β- and κ-caseins have one Trp.[10] Another unique feature of caseins is the large amount of propyl residues, especially in β-casein, which greatly affect the structure of caseins because the proline residues disrupt the formation of α-helixes and β-sheets.[13] In addition, all casein proteins have different hydrophobic and hydrophilic regions along the protein chain. α-Caseins are the major proteins containing 8−10 seryl phosphate groups, while β-casein contains about 5 phosphoserine residues, and it is more hydrophobic than α-casein and κ-casein.[12−14] The structural differences indicate a different affinity for α-, β-, and κ-caseins toward hydrophilic and hydrophobic interactions. Therefore, the structural characterization of the interaction between milk proteins and folic acid is a major step in elucidating the induced effect of folate on milk protein structure and the possibility of folate transportation by caseins in vitro. Recently, casein nanoparticles were used in a drug delivery system.[15]

Fluorescence quenching is considered as a technique for measuring binding affinities. Fluorescence quenching is the decrease of

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the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with a quencher molecule.[16,17] Therefore, it is possible to use quenching of the intrinsic tryptophan fluorescence of Trp-37, Trp-66 in α_{s1}-casein, and Trp-193, Trp-109 in α_{s2}-casein, as well as Trp-143 of β-casein as a tool to study the interaction of folic acid with caseins in an attempt to characterize the nature of the folate−casein complexation.

In this article, we present spectroscopic analysis and docking studies of the interaction of folic acid with milk caseins in aqueous solution at physiological conditions, using constant protein concentration and various folic acid contents. Structural information regarding folic acid binding sites and the effect of folate-protein complexation on the stability and conformation of caseins are reported here.

EXPERIMENTAL SECTION

Materials. Folic acid and α- and β-caseins (with purity of 70 and 98%) were purchased from Sigma-Aldrich Chemical Co (St-Louis, MO) and used as supplied. Other chemicals were of reagent grade and used without further purification.

Preparation of Stock Solutions. Casein was dissolved in aqueous solution (8 mg/mL for α-casein and 11.8 mg/mL for β-casein to obtain 0.5 mM protein content) containing a 10 mM Tris-HCl buffer (pH 7.4). Folic acid 1 mM was prepared in Tris-HCl and diluted to various concentrations in Tris-HCl. The protein concentration was determined spectrophotometrically using the extinction coefficients of 11 000 M^{-1} cm^{-1} (β-casein, MW = 24 00) and 15 000 M^{-1} cm^{-1} (α-casein, MW = 23 600) at 280 nm.[11,18]

FTIR Spectroscopic Measurements. Infrared spectra were recorded on a FTIR spectrometer (Impact 420 model), equipped with a deuterated triglycine sulfate (DTGS) detector and KBr beam splitter, using AgBr windows. A solution of folic acid was added dropwise to the protein solution with constant stirring to ensure the formation of a homogeneous solution and to reach the target folic acid concentrations of 0.125, 0.25, and 0.5 mM with a final protein concentration of 0.25 mM. Spectra were collected from hydrated films after 2 h of incubation of casein with the folic acid solution at room temperature. Interferograms were accumulated over the spectral range 4000−600 cm^{-1} with a nominal resolution of 2 cm^{-1} and 100 scans. The difference spectra [(protein solution + folic acid solution) − protein solution] were generated using the water combination mode around 2300 cm^{-1} as a standard.[19] When producing difference spectra, this band was adjusted to the baseline level in order to normalize the difference spectra.

Analysis of Protein Conformation. Analysis of the secondary structure of caseins and their folic acid complexes was carried out on the basis of the procedure already reported.[20] The protein secondary structure is determined from the shape of the amide I band, located at 1660−1650 cm^{-1}. Fourier self-deconvolution and second derivative resolution enhancement were applied to increase the spectral resolution in the region of 1700−1600 cm^{-1}. The second derivatives were obtained using a point convolution of 11 or 13. The resolution enhancement resulting from self-deconvolution and the second derivative is such that the number and the position of the bands to be fitted are determined. In order to quantify the area of the different components of the amide I contour revealed by self-deconvolution and second derivative, a least-squares iterative curve-fitting was used to fit the Gaussian line shapes to the spectra between 1700 and 1600 cm^{-1}. The details of spectral manipulation regarding curve-fitting have been previously reported.[21] The curve-fitting analysis was performed using the GRAMS/AI version 7.01 software of the Galactic Industries Corporation.

Circular Dichroism. CD Spectra of caseins and their folic acid complexes were recorded with a Jasco J-720 spectropolarimeter. For measurements in the far-UV region (178−260 nm), a quartz cell with a path length of 0.01 cm was used in nitrogen atmosphere. Protein concentration was kept constant (12.5 μM), while varying folic acid concentration (0.125, 0.25, and 0.5 mM). An accumulation of five scans with a scan speed of 50 nm per minute was performed, and data were collected for each nm from 260 to 180 nm. Sample temperature was maintained at 25 °C using a Neslab RTE-111 circulating water bath connected to the water-jacketed quartz cuvettes. Spectra were corrected for the buffer signal, and conversion to the Mol CD ($\Delta\varepsilon$) was performed with the Jasco Standard Analysis software. The protein secondary structure was calculated using CDSSTR, which calculates the different assignments of secondary structures by comparison with CD spectra measured from different proteins for which high quality X-ray diffraction data are available.[22,23] The program CDSSTR is provided in the CDPro software package, which is available at the following web-site: http://lamar.colostate.edu/~sreeram/CDPro.

Fluorescence Spectroscopy. Fluorimetric experiments were carried out on a Perkin-Elmer LS55 Spectrometer. Stock solutions of 1 mM folic acid were prepared at room temperature (24 ± 1 °C). Various solutions of folic acid (2 to 200 μM) were prepared from the above stock solutions by successive dilutions also at 24 ± 1 °C. A solution of casein (1 mM) in 10 mM Tris-HCl (pH 7.4) was also prepared at 24 ± 1 °C. The above solutions were kept in the dark and used soon after. Samples containing 0.4 mL of the above casein solution and various folic acid solutions were mixed to obtain a final acid concentration of 1 to 100 μM with a constant casein content of 10 μM. The fluorescence spectra were recorded at λ_{exc} = 280 nm and λ_{em} from 287 to 500 nm. The intensity at 350 nm (tryptophan) was used to calculate the binding constant (K) according to previous literature reports.[24−29]

On the assumption that there are (n) substantive binding sites for a quencher (Q) on a protein (B), the quenching reaction can be shown as follows:

$$nQ + B \Leftrightarrow Q_nB \quad (1)$$

The binding constant (K_A), can be calculated as

$$K_A = [Q_nB]/[Q]^n[B] \quad (2)$$

Where $[Q]$ and $[B]$ are the quencher and protein concentration, respectively, $[Q_nB]$ is the concentration of the nonfluorescent fluorophore−quencher complex, and $[B_0]$ gives the total protein concentration.

$$[Q_nB] = [B_0] - [B] \quad (3)$$

$$K_A = ([B_0] - [B])/[Q]^n[B] \quad (4)$$

The fluorescence intensity is proportional to the protein concentration as

$$[B]/[B_0] \propto F/F_0 \quad (5)$$

Results from fluorescence measurements can be used to estimate the binding constant of the folic acid−protein complex.

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From eq 4,

$$\log[(F_0 - F)/F] = \log K_A + n \log[Q] \quad (6)$$

The accessible fluorophore fraction (f) can be calculated by the modified Stern−Volmer equation.

$$F_0/(F_0 - F) = 1/(fK[Q]) + 1/f \quad (7)$$

Where F_0 is theinitial fluorescence intensity, and F is th efluorescence intensities in the presence of a quenching agent (or interacting molecule). K is the Stern−Volmer quenching constant, $[Q]$ is the molar concentration of the quencher, and f is the fraction of accessible fluorophore to a polar quencher, which indicates the fractional fluorescence contribution of the total emission for an interaction with a hydrophobic quencher.[17] The plot of $F_0/(F_0 - F)$ vs $1/[Q]$ yields f^{-1} as the intercept on the y axis and $(fK)^{-1}$ as the slope. Thus, the ratio of the ordinate and the slope gives K.

Molecular Modeling and Docking. The docking studies were performed with ArgusLab 4.0.1 software (Mark A. Thompson, Planaria Software LLC, Seattle, WA; http://www.arguslab.com). The casein structures were obtained as reported,[10] and the folic acid three-dimensional structure was generated from PM3 semiempirical calculations using Chem3D Ultra 6.0. The whole protein was selected as a potential binding site since no prior knowledge of such site was available. The docking runs were performed on the ArgusDock docking engine using regular precision with a maximum of 1000 candidate poses. The conformations were ranked using the Ascore scoring function, which estimates the free binding energy. Upon location of the potential binding sites, the docked complex conformations were optimized using the steepest decent algorithm until convergence, with a maximum of 20 iterations. Amino acid residues within a distance of 3.5 Å relative to the folic acid were involved in the complexation.

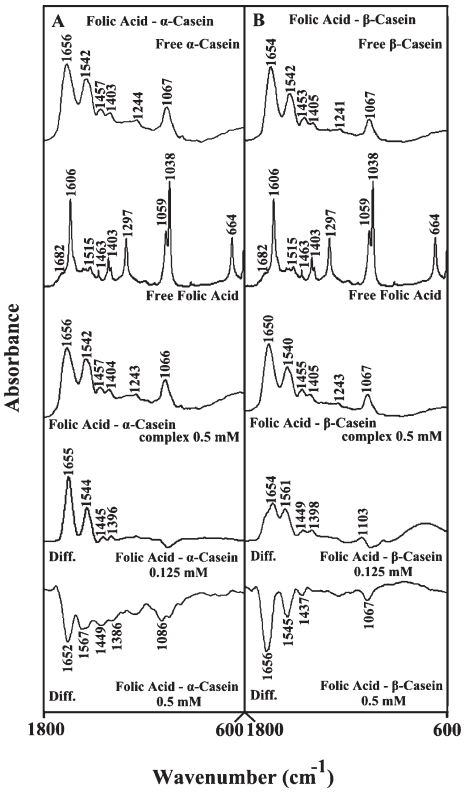

Figure 1. (A) FTIR spectra in the region of 1800−600 cm^{-1} for free α-casein (0.25 mM), free folic acid−α-casein adducts (top three curves), and difference spectra (diff.) of folic acid−α-casein complexes (bottom two curves) obtained at different folic acid concentrations (indicated on the figure). (B) FTIR spectra in the region of 1800−600 cm^{-1} for free β-casein (0.25 mM), free folic acid−β-casein adducts (top three curves) and difference spectra (diff.) of folic acid−β-casein complexes (bottom two curves) obtained at different folic acid concentrations (indicated on the figure).

RESULTS AND DISCUSSION

FTIR Spectra of Folic Acid Complexes with α- and β-Caseins. The folic acid complexation with caseins was characterized by infrared spectroscopy and its derivative methods. Since there was no major spectral shifting for the protein amide I band at 1656−1654 cm^{-1} (mainly C═O stretch) and amide II band at 1542 cm^{-1} (C−N stretching coupled with N−H bending modes),[20,21,30] upon folic acid interaction, the difference spectra [(protein solution + folic acid solution) − protein solution] were obtained. The intensity variations of amide I and amide II bands were monitored upon folic acid complexation, and the results are shown in Figure 1. Similarly, the infrared self-deconvolution with second derivative resolution enhancement and curve-fitting procedures[20] were used to determine the protein secondary structures in the presence of folic acid (Figure 2 and Table 1).

At low folic acid concentration (0.125 mM), an increase of intensity was observed for the protein amide I band at 1656−1654 cm^{-1} and amide II band at 1542 cm^{-1}, in the difference spectra of the folic acid−casein complexes (Figure 1A,B; diff. 0.125 mM). The positive features located in the difference spectra for the amide I and amide II bands at 1655 and 1544 cm^{-1} for folic acid−α-casein and at 1654 and 1561 cm^{-1} for folic acid−β-casein complexes are due to the increase of intensity of the amide I and amide II bands, upon acid complexation (Figure 1A, B; diff., 0.125 mM). The increase in intensity of the amide I and amide II bands is due to folic acid binding to protein C═O, C−N, and N−H groups (hydrophilic contacts). Additional evidence to support the folic acid interactions with C−N and N−H groups comes from the shifting of the protein amide A band at 3300 cm^{-1} (N−H stretching) in the free caseins to higher frequency at 3310 cm^{-1} upon folic acid interaction (spectra not shown). As folic acid concentration increased to 0.5 mM, negative features were observed for amide I and amide II bands at 1652 and 1567 cm^{-1} for folic acid−α-casein and at 1656 and 1545 cm^{-1} for folic acid−β-casein complexes (Figure 1A,B; diff., 0.5 mM). The observed decrease in intensities of the amide I band at 1656−1654 cm^{-1} and amide II band at 1542 cm^{-1}, in the spectra of the folic acid−casein complexes, suggests a reduction of protein α-helical structure, upon acid interaction (Figure 1A,B; diff., 0.5 mM). Similar infrared spectral changes were observed for protein amide I and amide II bands in several drug−protein complexes, where major protein conformational changes occurred.[31]

A quantitative analysis of the protein secondary structure for the free α- and β-caseins and their folic acid adducts in hydrated films has been carried out, and the results are shown in Figure 2 and Table 1. The free α-casein has 35% α-helix (1658 cm^{-1}), 24% β-sheet (1631 and 1617 cm^{-1}), 22% turn structure (1675 cm^{-1}), 5% β-antiparallel (1688 cm^{-1}), and 14% random

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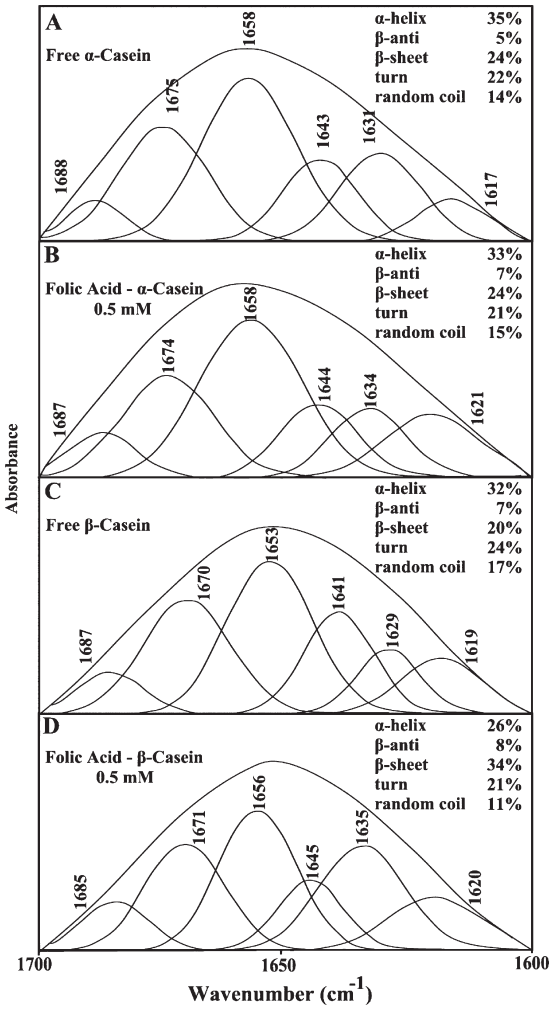

Figure 2. Second derivative resolution enhancement and curve-fitted amide I region (1700−1600 cm^{-1}) of IR spectra for free α- and β-caseins (A and C) as well as in complexes with 0.5 mM folic acid (B and D).

Table 1. Secondary Structure Analysis (FTIR) of Caseins and Their Complexes with Folic Acid in Hydrated Film at pH 7.4

	free casein 0.25 mM (%)		folic acid−casein 0.5 mM (%)	
amide I components (cm^{-1})	α	β	α	β
1692−1680 β-anti (±1%)	5	7	7	8
1680−1660 turn (±2%)	22	24	21	21
1660−1650 α-helix (±2%)	35	32	33	26
1648−1641 random coil (±4%)	14	17	15	11
1640−1610 β-sheet (±2%)	24	20	24	34

coil (1643 cm^{-1}) (Figure 2A and Table 1). The free β-casein contains 32% α-helix (1653 cm^{-1}), 20% β-sheet (1629 and 1619 cm^{-1}), 24% turn structure (1670 cm^{-1}), 7% β-antiparallel (1687 cm^{-1}), and 17% random coil (1641 cm^{-1}) (Figure 2C and Table 1). These results are consistent with spectroscopic studies of caseins previously reported.[32,33] Upon folic acid interaction, a minor decrease of the α-helix occurred for acid−α-casein

Table 2. Secondary Structure of Free Caseins (α and β) (CD Spectra) and Their Folic Acid Complexes at pH 7.4 Calculated by CDSSTR Software

	free casein (%) 0.25 mM		folic acid−casein (%) 0.5 mM	
conformational components	α	β	α	β
turn (±2%)	20	17	22	20
α-helix (±2%)	35	33	32	28
random (±2%)	33	34	32	36
β-sheet (±1%)	12	16	14	16

complexes (Figure 2 and Table 1). Similarly, the α-helix structure was reduced from 32% in the free β-casein to 26% in the acid−β-casein-complexes (Figure 2 and Table 1). The conformational changes observed were more pronounced for β-casein than for α-casein complexes (Table 1). This is indicative of larger perturbations of the β-casein secondary structure by folic acid. This is also consistent with the extra stability of folic acid−β-casein complexes, which will be discussed in fluorescence spectroscopy and modeling further on.

CD Spectroscopy. CD spectroscopy was also used to analyze the protein conformation in the polyphenol−casein complexes, and the results are shown in Table 2. The CD results exhibit marked similarities with those of the infrared data (Table 2). The protein conformational analysis based on CD data suggests that free α-casein has α-helical 35%, β-sheet 12%, turn 20%, and random coil 33%, while free β-casein contains α-helical 33%, β-sheet 16%, turn 17%, and random coil 34% (Table 2). The CD data for free caseins are consistent with the literature reports.[34,35] Upon folic acid interaction, minor reduction of the α-helix was observed from 35 to 33% in free caseins (α and β) and to 32−28% in the acid−casein complexes (Table 2). The CD results are consistent with those of the infrared data (Tables 1 and 2).

Hydrophobic Contacts. The spectral changes of the casein CH_2 antisymmetric and symmetric stretching vibrations, in the region of 3000−2800 cm^{-1} were analyzed in order to locate the presence of hydrophobic contact in the folic−casein complexes. The CH_2 bands of the free α-casein at 2953, 2934, and 2880 cm^{-1} shifted to 2958, 2935, and 2882 cm^{-1} upon folic acid interaction (Figure 3). Similarly, the CH_2 bands of the free β-casein at 2959, 2934, and 2876 cm^{-1} shifted to 2961, 2937, and 2881 cm^{-1} in the spectra of folic acid−casein complexes (Figure 3). The minor shifting of the protein antisymmetric and symmetric CH_2 stretching vibrations suggests the presence of hydrophobic interactions via acid heterocyclic rings and hydrophobic pockets in caseins, which is consistent with fluorescence spectroscopic results discussed below.

Fluorescence Spectra and Stability of Folic Acid Complexes with α- and β-Caseins. α-Casein (mixture of α_{s1}-casein and α_{s2}-casein) has two tryptophan residues, Trp-66, Trp-37 (in α_{s1}-casein), and Trp-109, Trp-193 (in α_{s2}-casein), while β-casein contains one tryptophan Trp-143 with intrinsic fluorescence. These tryptophan residues are located in the protein surfaces.[10] Tryptophan emission dominates casein fluorescence spectra in the UV region. When other molecules interact with casein, tryptophan fluorescence may change depending on the impact of such interaction on the protein conformation.[16,17] The decrease of fluorescence intensity of caseins has been monitored

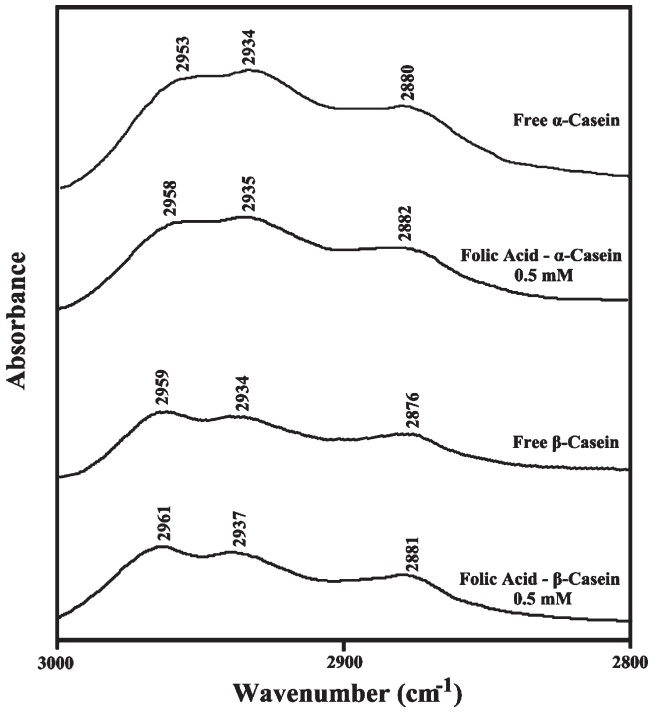

Figure 3. Spectral changes of casein CH_2 symmetric and antisymmetric stretching vibrations upon folic acid complexation.

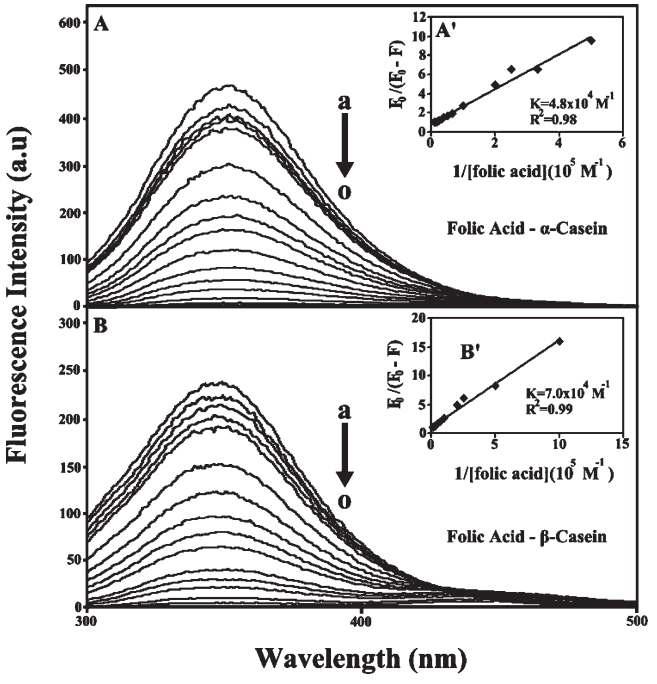

Figure 4. (A) Fluorescence emission spectra of folic acid−α-casein systems in 10 mM Tris-HCl buffer, pH 7.4, at 25 °C: (a) free α-casein (10 μM); (b−o) α-casein with folic acid at 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, 60, 80, and 100 μM. (B) Fluorescence emission spectra of folic acid−β-casein systems in 10 mM Tris-HCl buffer, pH 7.4, at 25 °C: (a) free β-casein (10 μM); (b−o) β-casein, 10 μM, with folic acid at 1, 2, 4, 5, 10, 15, 20, 25, 30, 40, 50, 60, 80, and 100 μM. The plots of $F_0/(F_0 - F)$ as a function of 1/folic acid concentration are shown in the inset. The binding constant K being the ratio of the intercept and the slope for (A′) folic acid-α-casein and (B′) folic acid-β-casein complexes.

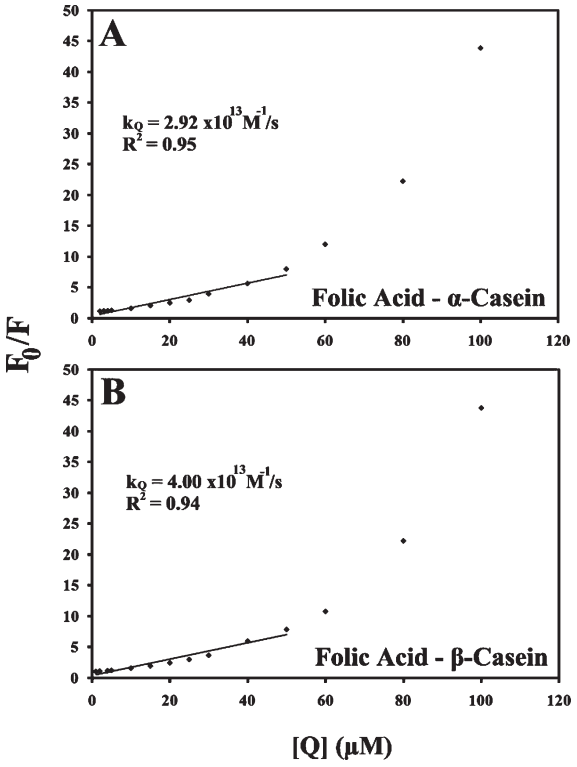

Figure 5. Stern−Volmer plots for fluorescence quenching of the folic acid−casein complexes at different acid concentrations: (A) folic acid−α-casein and (B) folic acid−β-casein.

at 347 nm for folic acid−protein systems (Figure 4A,B shows representative results for each system). The plot of $F_0/(F_0 - F)$ vs 1/[folic acid] (Figure 4A′,B′ shows representative plots). Assuming that the observed changes in fluorescence come from the interaction between folic acid and caseins, the quenching constant can be taken as the binding constant of the complex formation. The K values given here are averages of four-replicate and six-replicate runs for protein−folic acid systems, each run involving several different concentrations of folic acid (Figure 4A,B). The binding constants obtained were $K_{\text{folic acid}-\alpha\text{-casein}} = 4.8 \times 10^4\ \text{M}^{-1}$ and $K_{\text{folic acid}-\beta\text{-casein}} = 7.0 \times 10^4\ \text{M}^{-1}$ (Figure 4A′,B′). The association constants calculated for the folic acid−protein suggest low affinity amine-protein binding, compared to the other strong ligand-protein complexes.[36,37] Stronger binding of folic acid to β-casein is due to the more hydrophobic nature of β-casein, which forms more stable complexes with hydrophobic ligands.

In order to verify the presence of static or dynamic quenching in folic acid−casein complexes, we have plotted F_0/F against Q, and the results are shown in Figure 5. The plot of F_0/F versus Q is straight line at low Q concentrations and nonlinear at high acid contents, for folic acid−casein adducts indicating that the quenching is mainly static in nature with some degree of dynamic quenching at high Q concentrations.[29] The quenching constant (K_Q) was estimated according to the Stern−Volmer equation

$$F_0/F = 1 + k_Q t_0[Q] = 1 + K_D[Q] \quad (8)$$

where F_0 and F are the fluorescence intensities in the absence and presence of a quencher, $[Q]$ is the quencher concentration, and K_D is the Stern−Volmer quenching constant, which can be written as $K_D = k_Q t_0$, where k_Q is the bimolecular quenching rate

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constant and t_0 is the lifetime of the fluorophore in the absence of a quencher; 4.53 ns for α-casein and 3.30 for β-casein.[35] The quenching constants (K_Q) are 2.92×10^{13} M^{-1}/s for folic acid−α-casein and 4.0×10^{13} M^{-1}/s for folic acid−β-casein adducts (Figure 5). Since these values are much greater than the maximum collisional quenching constant (2.0×10^{10} M^{-1}/s), it was believed that static quenching is dominant in these complexes.[38] It should be noted that the overall binding constants (K) estimated for folic acid−casein complexes are coming from low folic acid concentrations, where quenching is mainly static (Figure 4A′,B′).

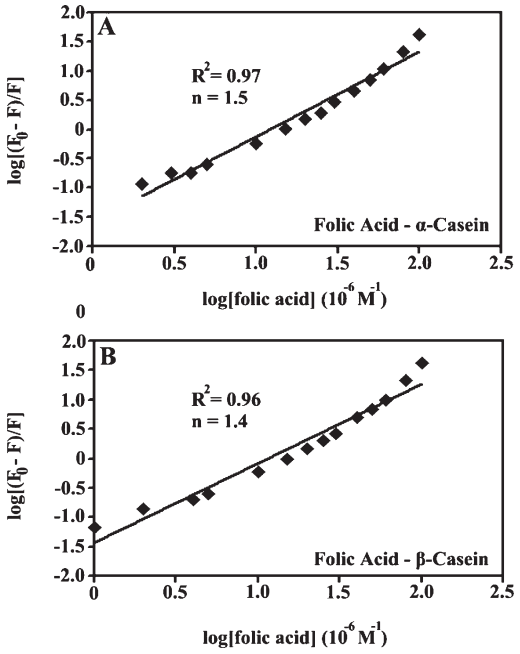

Figure 6. The plot of $\log (F_0 - F)/F$ as a function of log [folic acid] for the calculation of the number of bound acid molecules (n) in folic acid−casein complexes.

The number of folic acid molecules bound per protein (n) is calculated from $\log [(F_0 - F)/F] = \log K_S + n \log$ [folic acid] for the static quenching.[39−43] The linear plot of $\log [(F_0 - F]/F]$ as a function of log [folic acid] was extracted from Figure 6A,B. The n values from the slope of the straight line are 1.5 for folic acid−α-casein and 1.4 for folic acid−β-casein complexes, indicative of 1 to 2 folic acid molecules bound per protein (Figure 6A,B).

Docking Studies. Our spectroscopic results were complemented with docking experiments in which folic acid was docked to α- and β-caseins to determine the preferred binding sites. The docking results are shown in Figure 7 and Table 3. In the folic acid−α-casein complexes, acid is surrounded by Met-4, Tyr-20 (H-bonding), Lys-21, Gln-22, Glu-23, Lys-24 (H-bonding), Asn-25, Met-26, Lys-41 (H-bonding), and Val-43 with average binding distances (folic acid−amino acid) from 1.7 to 2.5 Å and the free binding energy of −7.7 kcal/mol (Figure 7 and Table 3). In the folic acid−β-casein, acid is located in the vicinity of Pro-63, Gly-64 (H-bonding), Pro-65, Ile-66, Pro-67, Leu-70, Thr-128, Asp-129, Val-130 (H-bonding), Leu-139, Leu-140 (H-bonding), Ser-142 (H-bonding), and Pro-186 with average binding distances (folic acid−amino acid) from 1.5 to 2.5 Å and the free binding energy of −8.1 kcal/mol (Figure 7 and Table 3). The binding energy (ΔG) shows the folic acid−β-caseins is more stable than folic acid−α-casein complexes (Table 3), consistent with fluorescence spectroscopic results discussed earlier. However,

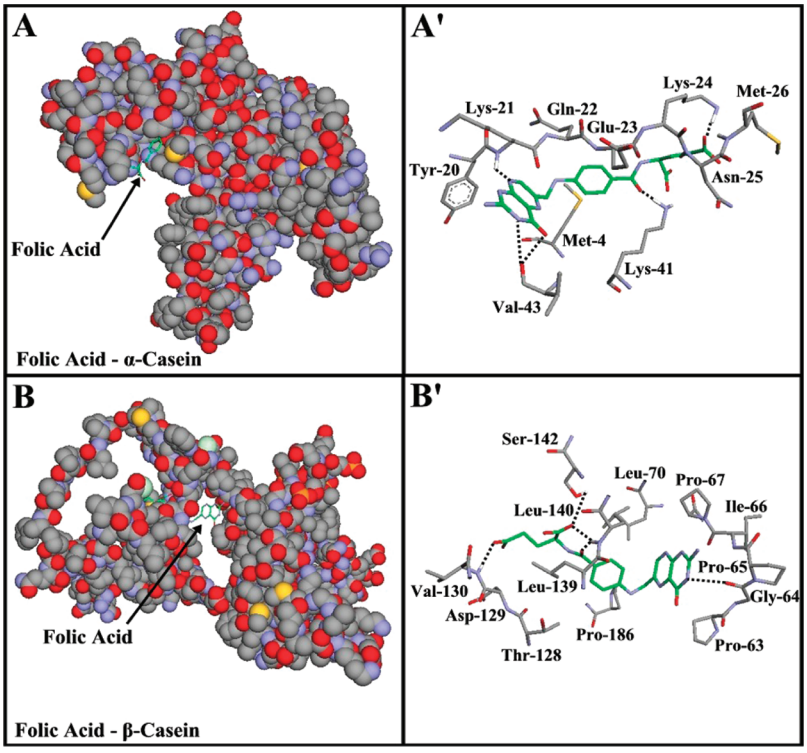

Figure 7. Best conformations for folic acid docked to α- and β-caseins. The acid is shown in green. (A) Whole α-casein in cartoon ribbons with folic acid. (A′) Binding site represented in sticks. (B) Whole β-casein in cartoon ribbons with folic acid. (B′) Binding site represented in sticks. Hydrogen bonds are represented as dashed lines.

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Table 3. Amino Acid Residues Involved in Folic Acid−Protein Interaction with the Free Binding Energy for the Best Selected Docking Positions

complex	amino acids involved in folic acid−protein bindings	$\Delta G_{binding}$ (kcal/mol)
α-casein−folic acid	Met-4, Tyr-20,[a] Lys-21, Gln-22, Glu-23, Lys-24,[a] Asn-25, Met-26, Lys-41,[a] Val-43[a]	−7.7
β-casein−folic acid	Pro-63, Gly-64,[a] Pro-65, Ile-66, Pro-67, Leu-70, Thr-128, Asp-129, Val-130,[a] Leu-139, Leu-140,[a] Ser-142,[a] Pro-186	−8.1

[a] Hydrogen bonding was observed with this amino acid.

the binding sites of folic acid with α-casein are different from those of β-casein complexes (Figure 7 and Table 3).

CONCLUSIONS

Folic acid binds milk α- and β-caseins via hydrophilic and hydrophobic interactions with overall binding constants of $K_{\text{folic acid}-\alpha\text{-casein}} = 4.8 \times 10^4\ M^{-1}$ and $K_{\text{folic acid}-\beta\text{-casein}} = 7.0 \times 10^4\ M^{-1}$. The binding sites of folic acid were different for α- and β-caseins. Stronger binding of folic acid to β-casein is due to the more hydrophobic nature of β-casein, which forms stable complexes with hydrophobic parts of folic acid. Folic acid interaction brings minor alterations of the protein secondary structure. The results suggest that milk caseins can act as carriers for folic acid in delivering it to target molecules.

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ABBREVIATIONS

FA, folic acid; FTIR, Fourier transform infrared spectroscopy; CD, circular dichroism

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