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Mechanism of Interaction of Pb(II) with Milk Proteins: A Case Study of α-Casein

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α-Casein is the major casein protein fraction from bovine milk and is responsible for binding to many ligands. This paper reports the results on the interaction of Pb(II) with α-casein. The interaction studies by spectroscopic titration indicate that Pb(II) has two binding sites with an association constant (k_a) of $(2.3 \pm 0.2) \times 10^5$ M^{-1}. Raman spectra of the α-casein–Pb(II) complex show reduction in the amide I region as well as minor perturbations in the sulfhydryl region of α-casein. Stopped-flow studies show that the reaction mechanism of Pb(II) follows a pseudo-first-order reaction with a rate of 25 ± 6 s^{-1}. The stopped-flow time-resolved spectra show peaks at 330 and 360 nm, correlating to Pb(II)–thiolate bands in the UV absorption spectra. Modification of cysteines present in α-casein does not result in binding of lead, indicating that cysteines could be one of the Pb(II) binding sites.

KEYWORDS: Lead; α-casein; stopped flow; hydrophobicity; cysteines; Raman spectra; milk; circular dichroism

INTRODUCTION

Cow's milk contains about 78% casein, of which 65% is α-casein (α-CN). In bovine milk, 65% of the casein fraction comprises the α_{s1}- and α_{s2}-subunits in a 4:1 ratio with molecular masses of 23615 and 25226 Da, collectively known as α-casein as cited in the literature (*1*). α-CN is a major fraction of milk caseins involved in the transport of calcium and other metal ions to neonates. It is present in all mammalian milk. α-CN is found to be protective against heat coagulation (*2*), having chaperone-like activity (*3*), binds to membrane receptors, and acts as a signal transducer (*4*); it is rheomorphic (plasticity) (*5*), having a poly(L-proline) type II (PPII) helical conformation (*6*), and a transporter of milk proteins to secretary organelles (*7*).

Interaction of Pb(II) ions with biological systems in general and with enzymatically controlled reactions in particular has been extensively studied. It is known that the preferred site for interaction of Pb(II) with proteins is a cysteine residue (*8*). Pb(II) shows clear preference toward sulfhydryl groups, which is characteristic of borderline metals. Pb(II) possesses chemical properties that allow it to interact with a diverse array of ligands. Thus, Pb(II) reacts primarily with nucleophilic ligands such as sulfhydryl, RS^-; amine, RNH^-; phosphate, R (PO_4^-); and carboxyl, $RCOO^-$ (*9, 10*). It is evident from the binding constants for various monodentate ligands that the thoiolate group binds Pb(II) with highest affinity. The distribution of Pb(II) in milk and caseins has been studied by a few techniques (*11, 12*).

Lead is an environmental pollutant with severe toxic effects. Interaction of Pb(II) with milk proteins such as caseins will reveal a few of the underlying mechanisms of lead toxicity. In the present study, an attempt is made to understand the mechanism of interaction of Pb(II) with the purified milk α-casein studied at neutral pH with respect to binding parameters and protein conformational status.

MATERIALS AND METHODS

Materials. Lead nitrate (ACS reagent), 1-anilino-8-naphthalene-sulfonate (ANS salt), and Tris buffer salt were from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dialysis membranes of 23 mm flat width with a molecular mass cutoff in the range of 6–8 kDa were obtained from Thomas Scientific Co. All chemicals were of reagent grade. All buffers and reagents were prepared in triple-distilled water. The concentration of the Pb(II) was determined using a Shimadzu atomic absorption spectrophotometer (AA-6701F, Shimadzu, Kyoto, Japan) calibrated with reference standards (*13*).

Isolation of α-Casein. α-Casein was isolated from fresh cow's milk according to the method of Hipp et al. (*14*). The isolated α-casein consisted of α_{s1}- and α_{s2}-subunits in the ratio of 4:1 having molecular masses of 23.6 and 25.2 kDa with $E^{1\%}_{277nm} \sim 10.1$. The α-CN obtained by the above method was used for further studies. The α-CN obtained was devoid of all intrinsically bound ligands by dialysis against 50 mM EDTA and against deionized water.

Spectrophotometric Titration. The effect of Pb(II) on α-casein was studied by titrating α-casein having a concentration of 4×10^{-5} M against Pb(II) to find out the various parameters of binding. The lead nitrate was prepared in 0.02 M Tris-HCl buffer, pH 6.8, at a concentration of 1×10^{-4} M and is added in increments of 10 μL, with a gastight syringe, to the α-CN taken in a quartz cuvette of 1 cm path length. After the addition and necessary dilution corrections had been made, absorption of the protein was recorded at 277 nm on a double-beam Shimadzu spectrophotometer UV1601. The temperature was kept constant at 25 °C by a circulating water bath connected to the instrument. The experiment was done according to the detailed

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procedure described earlier (*15*). The data were analyzed using Scatchard plots (*16*). All experiments were carried out in triplicate, and the variation in the values is expressed as error bars.

Determination of Hydrophobicity. Protein hydrophobicity of the samples using fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS) was calculated for protein sample by serial dilution with 0.02 M Tris-HCl buffer, pH 6.8, to obtain protein concentrations ranging from 3×10^{-6} to 1×10^{-5} M. ANS of 1×10^{-2} M in 0.02 M Tris-HCl buffer, pH 6.8, was added to the above protein solutions. Fluorescence intensity (FI) was measured 2 h after the addition of ANS in a recording spectrofluorometer (RF5000, Shimadzu) with an excitation wavelength at 375 nm and emission wavelength at 400–600 nm, respectively. The slits were maintained at 5 nm both in the emission and in the excitation wavelengths. The initial slope (S_0) of the net RFI versus protein concentration plot is calculated by linear regression analysis and used as an index of the protein hydrophobicity. The hydrophobicity was measured as described by Kato and Nakai (*17*). All experiments were carried out in triplicate, and the variation in the values is expressed as error bars.

Circular Dichroism (CD) Spectra. Far-UV CD measurements of α-casein were performed in the range of 200–260 nm at 25 ± 1 °C using a Jasco J-810 automatic recording spectropolarimeter fitted with a xenon lamp and calibrated with +*d*-10-camphorsulfonic acid. The instrument slits are programmed to give a 1 nm bandwidth. A protein solution of 1×10^{-5} M was dialyzed against 1×10^{-5} M Pb(II) prepared in 0.02 M Tris-HCl, pH 6.8, and used for CD spectral analysis. The baseline was corrected with buffer containing Pb(II). The mean residue ellipticities were calculated using a mean residue weight of 115 for α-CN based on amino acid composition (*18*, *19*). Data analysis of the secondary structure was carried out by comparison with the standard database of Yang et al. (*20*).

Raman Spectra. Raman spectroscopy of the α-casein and α-casein–Pb(II) complex was performed using a protein concentration of 4.2×10^{-4} M. Protein solutions were taken in sealed borosilicate tubes of 1.4 mL capacity. Spectra were recorded on a Nicolet FT-Raman 950 spectrometer with an indium gallium arsenide detector (Thermo-Nicolet, Madison, WI). The spectra were obtained using 1064 nm laser excitation obtained by a neodydinium yttrium argon laser source. Data were acquired using a laser power of 1.5 W with a total of 512 spectral acquisitions taken for 0.5 h. The spectral resolution is recorded at 4 cm^{-1}. The data spacing is at 2.0 cm^{-1}. Spectral interpretations, buffer subtraction, and the difference spectra were done by using Thermo-Nicolet OMNIC software available with the instrument (*21*). Curve fitting was done using the *Peak Resolve* routine present in the software. The spectra were KBr and baseline corrected. The smoothing was done with Savitsky–Golay 9-point gentle smoothing to prevent peak distortion and peak location. The OMNIC *peak resolve* convergence routine uses the Fletcher–Powell–McCormick algorithm. Spectral curve fitting was done as follows: the second-derivative spectra were generated to identify the peaks. The peaks were integrated with a Gaussian/Lorenzian 50:50 peak width at half-height (PWHH) of 4. The spectral curve fitting was done manually, and numbers of iterations were performed until the convergence point approached 1 (rms of residual approaches noise).

Stopped-Flow Reaction Kinetics. Fast reaction kinetics of the interaction of Pb(II) with α-CN was followed at 250 nm using a stopped-flow spectrophotometer model SX-18 MV, version 4.4 (Applied Photophysics, Leatherhead, U.K.), at 25 ± 1 °C with a path length of 2 mm and a pressure of 125 psi (8 bar), compressed nitrogen. The ligand and protein in equal volumes were mixed, taking from the stock concentrations of 1×10^{-4} of Pb(II) and 1.8×10^{-5} M α-CN prepared in 0.02 M Tris-HCl buffer, pH 6.8, at 25 °C. Also, the rates were monitored at different concentrations of Pb(II) in the range of 2×10^{-8}–2×10^{-6} M. For each data set, 1000 data points were collected. All experiments were repeated at least three times for each of the time regimes. The data scan was recorded three times on an Acorn Risc computer interfaced with the instrument. The averaged data were automatically fit with the single-exponential steady-state equations available with the software (ver. 4.4). The dead time of the instrument is 5 ms. Time-resolved spectra were obtained using the rapid kinetic scan measurements, which were carried out by using the *kinscan* program available with the instrument. The spectral scans were recorded from 200 to 400 nm on a time basis of 1 s with 10 nm intervals and were spliced to get the time-resolved spectra as described in the literature (*22*, *23*). To study the thermodynamic parameters and the temperature dependence of the binding, the kinetic rates at 25–40 °C were recorded on a stopped-flow spectrophotometer.

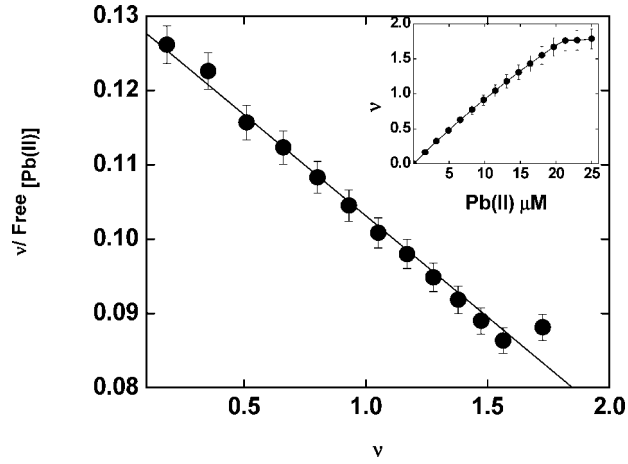

Figure 1. Scatchard plot of α-casein interaction with Pb(II) as monitored by spectroscopic titration at 277 nm. α-Casein (4×10^{-5} M) was titrated against Pb(II) (1×10^{-4} M) in 0.02 M Tris-HCl buffer, pH 6.8, at 25 °C. (Inset) Binding isotherm of α-CN interaction with Pb(II). ν is defined as moles of bound ligand per mole of protein.

Modification of Cysteines. The effects of iodoacetamide on α-casein and the α-casein Pb(II) complex were studied by dialyzing the α-casein at a concentration of 1.8×10^{-5} M for 12 h against iodoacetamide (0.1 M) at pH 6.8, in 0.02 M Tris-HCl buffer at 4 °C with at least three intermittent changes of buffer containing iodoacetamide over 36 h. A Pb(II) concentration of 1×10^{-4} M was used in this study.The rates for the resultant modified protein and unmodified protein were collected using a stopped-flow spectrophotometer.

RESULTS

Spectrophotometric Titration. The binding of Pb(II) with α-casein was studied by spectrophotometric titration. The binding isotherm obtained upon titration of Pb(II) with α-CN is shown in the inset of **Figure 1**. Addition of increasing amounts of Pb(II) gradually quenched the absorbance of α-casein to nearly 17%. The binding isotherm shows saturation of binding of Pb(II) at a concentration of 20 μM. The binding data were analyzed by Scatchard plot to determine number of binding sites and the association constants for the Pb(II)–protein interaction. From the Scatchard plot, the number of binding sites for Pb(II) on α-CN was found to be at least two, as shown in **Figure 1**. The association constant (k_a) calculated from the slope was $(2.3 \pm 0.2) \times 10^5$ M^{-1}. The free energy change $\Delta G°$ calculated from the association constant is -31 ± 5 kJ/mol. To analyze structural changes in α-CN upon interaction with Pb(II), CD and fluorescence experiments were carried out.

CD Spectra. Far-UV CD spectra of α-casein and the α-casein–Pb(II) complex are shown in **Figure 2**. The CD spectra of α-CN and α-CN in the presence of 1×10^{-5} M Pb(II) were recorded to follow the secondary structural changes upon binding. The secondary structural estimation of the far-UV CD spectrum of α-casein recorded in buffer indicates 5% α-helix, 51% β-sheet, and 44% aperiodic structure. In the presence of Pb(II) the secondary structure of α-casein changes to 3% α-helix, 47% β-sheet, and 50% aperiodic structure. Addition of Pb(II) changes the secondary structural component of α-casein. The changes are more significant in the aperiodicity of α-casein.

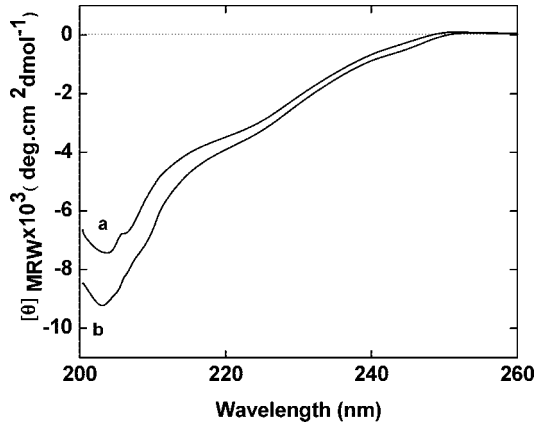

Figure 2. Effect of Pb(II) on the far-UV CD spectra of α-casein: (a) α-CN in buffer (0.02 M Tris-HCl buffer, pH 6.8); (b) α-CN in buffer containing 1×10^{-5} M Pb(II). The protein concentration of 1×10^{-5} M was used for all experiments. Spectra were run from 200 to 260 nm in the far-UV range with a path length of 1 mm. An average of three runs was taken, and the spectra were collected with a scan speed of 10 nm/min. The mean residue weight of 115 was considered for calculating the mean residue ellipticity.

Hydrophobicity. Surface hydrophobicity studies by using fluorescent probe ANS on α-casein and the α-casein–Pb(II) complex show changes in surface hydrophobicity of α-CN upon binding with Pb(II). Fluorescence spectra of α-CN and the α-CN–Pb(II) complex are shown in **Figure 3A**. The emission maximum of α-CN was observed at 455 nm. Interaction of Pb(II) with α-CN resulted in a shift of the emission maximum from 455 to 448 nm. The surface hydrophobicity index of α-CN and α-CN–Pb(II) complex was calculated from the slope of **Figure 3B**. α-Casein shows a hydrophobic index value of 107, whereas the α-casein–Pb(II) complex shows a value of 273 (**Figure 3B**), a nearly 2.5-fold increase in hydrophobic index value compared to the native protein.

Raman Spectra. Raman spectra of α-CN and the α-CN–Pb(II) complex are shown in **Figure 4**. There is a reduction in the amide I (1619 and 1581 cm^{-1}) region after binding of Pb(II). The α-CN–Pb(II) complex shows bands for amide III at 1251 cm^{-1}. The band centered at 1251 cm^{-1} could be due to the increase in aperiodicity in the α-CN upon binding to Pb(II) (**Figure 4A**). Significant changes in the region of thiol groups (2500–2700 cm^{-1}) were also seen in the Raman spectra. The two free cysteines (Cys^{36} and Cys^{40}) of α-CN generate a complex band around 2546 cm^{-1}, which in the case of the α-CN–Pb(II) complex are shifted to 2524 cm^{-1} (**Figure 4B**).

Stopped-Flow Reaction Kinetics. The reaction kinetics of Pb(II) binding with α-CN was followed by stopped-flow spectrophotometer. The kinetics was monitored up to 600 ms, and the rates were analyzed (**Figure 5**). The rates were recorded at various concentrations of Pb(II) from 2×10^{-8} to 2×10^{-6} M. Kinetics follows pseudo-first-order rate constants of 25 ± 6 s^{-1} at 250 nm at different concentrations of Pb(II). Pseudo-first-order rate constants are shown in **Table 1**. The kinetics shows that binding of Pb(II) with thiolates is faster. The stopped-flow time-resolved kinetic spectra were measured for the α-CN–Pb(II) complex in the range of 200–400 nm, which shows significant bands with maxima at 330 and 360 nm in the spectrum (**Figure 6**). The spectrum showed a decrease at 250 nm and formation of new peaks at 330 and 360 nm. These changes start from 25 ms and reach maxima at 1000 ms.

To study the thermodynamic parameters and the temperature dependence of the binding, the kinetic rates from 25 to 40 °C

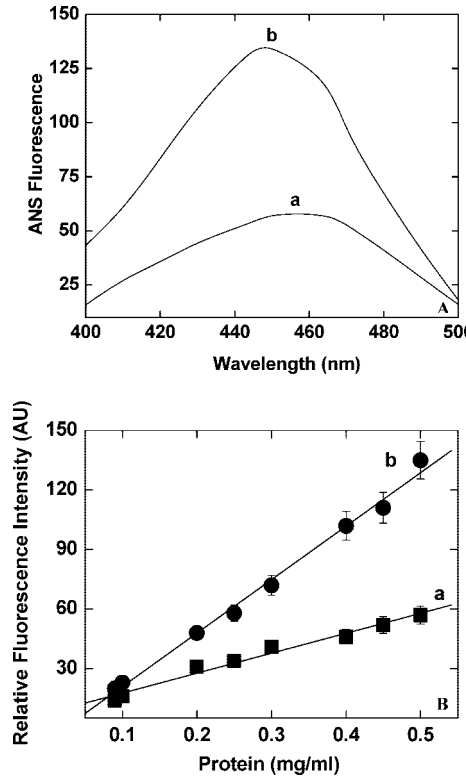

Figure 3. Effect of Pb(II) on surface hydrophobicity of α-casein as studied by ANS dye on spectrofluorometer. (**A**) ANS fluorescence spectra of α-CN in buffer (a) and the α-CN–Pb(II) complex (b). The protein and Pb(II) concentrations used were 1×10^{-5} and 1×10^{-4} M, respectively. (**B**) Plot of surface hydrophobicity index: (a) α-CN in buffer; (b) α-CN–Pb(II) complex. The ANS stock solution of 10 mM was used. A protein concentration of 3×10^{-6}–1×10^{-5} M was incubated with 10 μL of ANS for 2 h and spectra were recorded. The protein was excited at 375 nm, and the emission spectrum was recorded from 400 to 600 nm. Excitation and emission slit widths of 5 nm were used for the experiments. The slopes of lines a and b give the surface hydrophobicity index for α-CN and the α-CN–Pb(II) complex, respectively. α-Casein shows a hydrophobic index value of 107, whereas the α-casein–Pb(II) complex shows a value of 273.

are determined on a stopped-flow spectrophotometer. The rates at different temperatures were collected and plotted as a van't Hoff plot (ln k versus $1/T$) to determine thermodynamic parameters (**Figure 7**). The value of enthalpy ($\Delta H°$) obtained from the slope ($r = -0.99$) of the van't Hoff plot is 15 ± 2 kJ/mol and shows temperature linear dependence of the endothermic molecular association process. The entropy ($\Delta S°$) calculated from the enthalpy and temperature shows 155 ± 2 J/mol/K. The change in Gibbs free energy ($\Delta G°$) from enthalpy and entropy parameters is -31 ± 5 kJ/mol. The association constant obtained from the Gibbs free energy is $(2.3 \pm 0.2) \times 10^5$ M^{-1}.

Modification of Cysteines. Studies on the interaction of the α-CN–Pb(II) complex using iodoacetamide show that iodoacetamide binds to thiolates and blocks them from further interaction. The rapid kinetic rates of the α-CN–Pb(II) complex show a rate of 25 s^{-1}. Addition of Pb(II) to the α-CN–iodoacetamide complex reduces the rate to 0.2 s^{-1} (**Figure 8**). The rate was monitored up to 12 s. This shows that cysteine residues are not available for binding to Pb(II).

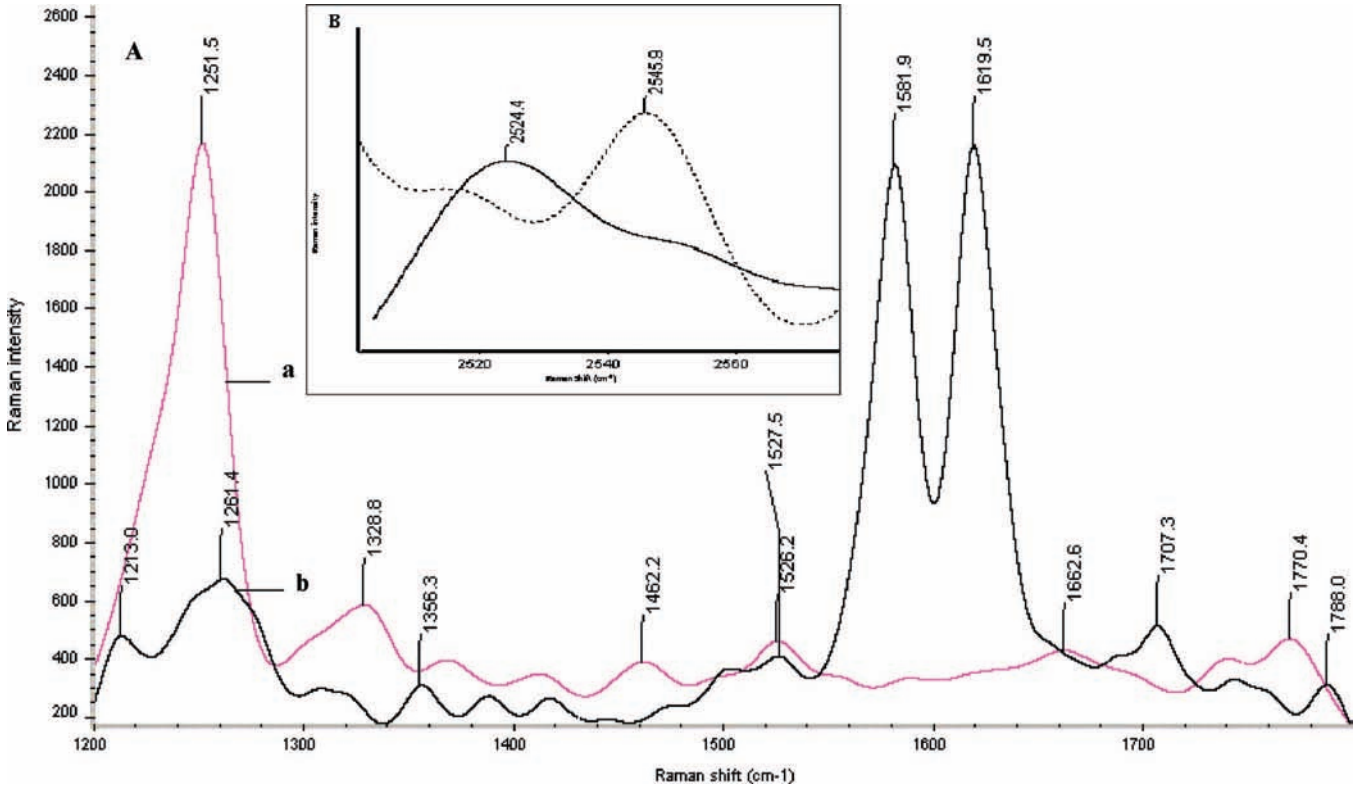

Figure 4. Raman spectra of α-casein and α-casein–Pb(II) complex as studied from 600 to 2600 cm^{-1}: (**A**) Raman spectra of (a) α-CN–Pb(II) complex and (b) α-CN in buffer as studied from 600 to 1200 cm^{-1}; (**B**) Raman spectra of (a) α-CN–Pb(II) complex (b) α-CN in buffer and as studied from 2500 to 2600 cm^{-1}. An α-CN concentration of 4.2×10^{-4} M was used for all experiments. The Raman spectrum was recorded at excitation of 1064 nm laser with a laser power of 1.5 W.

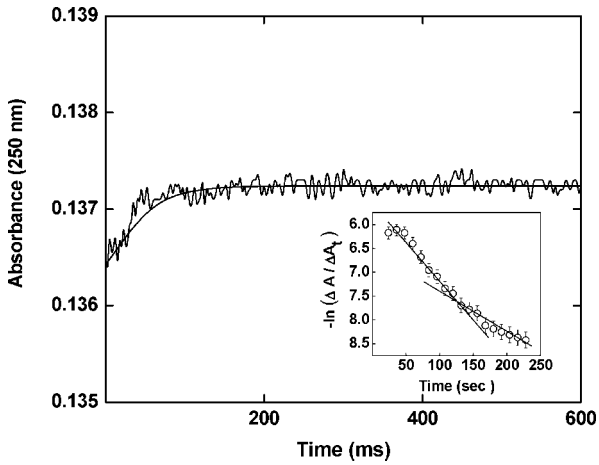

Figure 5. Fast kinetics of interaction of Pb(II) with α-casein as followed on a stopped-flow spectrophotometer at 250 nm and 25 °C. The stock concentrations of Pb(II) and α-CN were at 1×10^{-4} M (0.02 M Tris-HCl, pH 6.8) and 1.8×10^{-5} M, respectively. (Inset) Pseudo-first-order plot for the binding of Pb(II) with α-CN as studied on a stopped-flow spectrophotometer at 25 °C.

DISCUSSION

Among milk proteins, α-casein was identified as having the ability to bind many metal ions. The objective of the present paper is to study the binding of Pb(II) with a major milk protein, α-CN, to understand structural changes upon interaction. Binding of Pb(II) with α-CN was studied by spectrophotometric titration. The number of binding sites for Pb(II) was found to be two on α-CN with an association constant of $(2.3 \pm 0.2) \times 10^5$ M^{-1}. Earlier studies have indicated a similar magnitude in association constant of α-CN and Pb(II) interaction (*12*, *24*–*26*).

Table 1. Pseudo-First-Order Rates for the Binding of Pb(II) with α-Casein As Studied on a Stopped-Flow Spectrophotometer at 25 °C[a]

concentration of Pb(II) (M)	rate (s^{-1}) at 250 nm
2×10^{-8}	23 ± 6
9×10^{-8}	30 ± 7
2×10^{-7}	28 ± 3
9×10^{-7}	23 ± 7
2×10^{-6}	29 ± 4

[a] All readings are the average of three scans ± standard deviation.

The interaction of Pb(II) with α-CN studied by secondary structural estimations using CD and extrinsic fluorescence measurements using ANS probe indicates increase in aperiodicity and enhanced ANS binding to the surface of the α-CN–Pb(II) complex compared with native protein, which may be due to the perturbations in the protein microenvironment. The increase in molar ellipticity values at 205 nm throws some light on such structural changes in the α-CN–Pb(II) complex. Godwin (*27*) reported that Pb(II) induces conformational changes in the protein. Studies by Payne et al. (*28*) have shown that in the case of a few peptides the binding of Pb(II) destabilizes the folding of the peptides.

Raman spectra indicate a decrease in periodic structures and an increase in bands corresponding to aperiodicity. Raman spectral bands for cysteines also show shifts upon Pb(II) binding. The CD, fluorescence, and Raman spectra are indicative of changes and confirm an increase in aperiodicity and possible binding to thiols present in α-CN. The shifts in the bands corresponding to the cysteines in Raman spectra upon Pb(II) binding could be due to the influence of metal ions in the vibrational mode of functional groups to which they are attached (*29*, *30*).

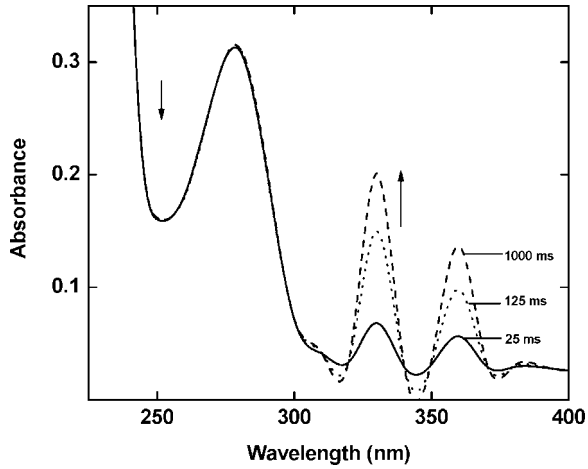

Figure 6. Time-resolved spectra of α-casein with Pb(II) as monitored on a stopped-flow spectrophotometer from 200 to 400 nm in 1 s. The reaction is followed from 25 ms to 1 s at 25 °C. A Pb(II) concentration of 1×10^{-4} M (0.02 M Tris-HCl, pH 6.8) and an α-CN concentration of 1.8×10^{-5} M were used for the experiments. The arrow indicates the direction of change in the α-CN spectrum upon interaction with Pb(II). The data are analyzed as per the software *kinscan* available with the spectrophotometer.

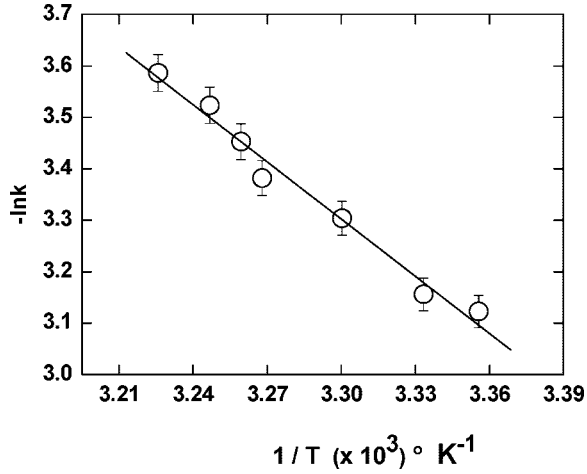

Figure 7. van't Hoff plot to show the effect of temperature on the reaction rates of α-casein interaction with Pb(II) as studied on a stopped-flow spectrophotometer. The temperatures were in the range of 25−40 °C. The value of enthalpy ($\Delta H°$) was obtained from the slope of the van't Hoff plot.

Stopped-flow studies are useful to measure the rate at which the Pb(II) binds with α-CN. Studies indicated formation of the α-CN–Pb(II) complex proceeds rapidly. Stopped-flow time-resolved spectra show formation of two peaks upon Pb(II) binding at 330 and 360 nm. Studies by Busenlehner et al. (*31*) on absorption spectroscopy and spectral behavior of Pb(II) on p1258 *CadC* have shown similar results for absorption bands at 240 and 350 nm, which were due to the binding of Pb(II) to two or three thiolates. Studies on Pb(II) peptide complexes by Payne et al. (*28*) have shown that two characteristic prominent Pb(II)–thiolate bands are positioned at 310 and 260 nm, which are due to the contributions of two sulfur and two nitrogen ligands present in the peptides.

α-Casein is composed of α_{s1}-casein and α_{s2}-casein fractions in the ratio of 4:1. The sequence information shows the presence of two cysteine (Cys^{36} and Cys^{40}) residues in α_{s2}-casein (*32*). These cysteines may be involved in binding to Pb(II). Modification studies of the α-CN and α-CN Pb(II) complex by

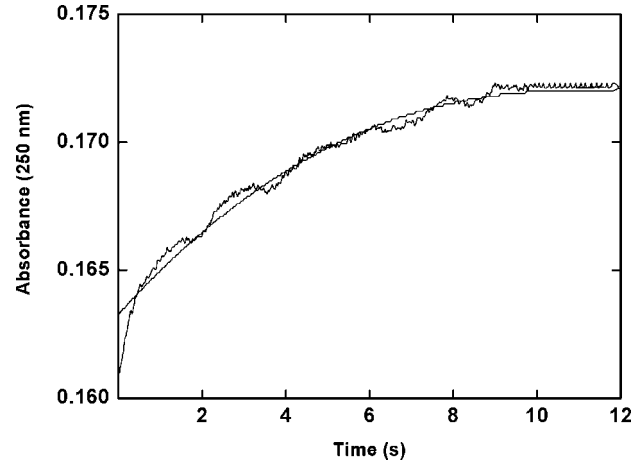

Figure 8. Fast kinetics of interaction of Pb(II) with α-casein–iodoacetamide complex as followed on a stopped-flow spectrophotometer at 250 nm and at 25 °C. The stock concentration of α-CN (1.8×10^{-5} M) was dialyzed against iodoacetamide (0.1 M) at 4 °C with at least three intermittent changes of buffer containing iodoacetamide for 36 h. The kinetics was monitored for the α-CN–iodoacetamide complex with Pb(II) at 1×10^{-4} M concentration.

iodoacetamide and measuring the rates of binding of the native protein and iodoacetamide complex show reduction in the rate for α-CN–iodoacetamide complex, which may be due to the blocking of cysteine residues of α-CN by iodoacetamide, which in turn are not available for interacting with Pb(II).

The measurements of rates at different temperatures have shown the temperature dependence for the complex formation. The changes in the entropic and enthalpic parameters during the interaction of Pb(II) with α-CN indicated contributions of binding from hydrophobic and charged regions of α-casein (*33*).

In conclusion, the major protein fractions of bovine milk α-casein interaction with Pb(II) through cysteine and sulfhydryl residues are of strong binding sites as indicated by spectroscopic titration and Raman spectra, and such binding follows a pseudo-first-order kinetics, which has a component of very rapid reaction. The energetics of interaction show the reaction is not reversible because of the nature of the interaction between Pb(II) and amino acid residues.

ABBREVIATIONS USED

CD, circular dichroism; α-CN, α-casein; ANS, 8-anilino-1-naphthalenesulfonic acid; k_a, association constant; Pb(II), lead.

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