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Hu, YiLin
Sood, Satish M
Slattery, Charles W

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Thermal Cycling Aids Folding of a Recombinant Human β-Casein with Four Extra N-Terminal Amino Acid Residues

YiLin Hu,[1] Satish M. Sood, and Charles W. Slattery[2]
Department of Biochemistry, School of Medicine, Loma Linda University, Loma Linda, California 92350

Due to the limited secondary structure, it is believed that the caseins of milk, particularly the β-caseins (β-CN), may be in a mostly random-coil conformation or in various structures that result from random association of hydrophobic residues. However, the self-association of the human proteins with increasing temperature (T) and in the presence of Ca^{2+} is reproducible, implying that they normally fold into fixed tertiary structures. A nonphosphorylated recombinant human β-CN with four extra amino acids at the N-terminus (GSHM−) was prepared and studied by laser light scattering, analytical ultracentrifugation, fluorescence spectroscopy, turbidity, and circular dichroism. In 3.3 M urea or at 4°C, the protein was monomeric, as expected. Increasing T both without and with the addition of Ca^{2+} ions caused self-association as it does for the nonphosphorylated native β-CN but with a somewhat different interaction pattern. However, returning the protein to its monomeric state by reequilibration at 4°C followed again by increasing T caused a shift in the pattern. Such thermal cycling eventually caused the protein to equilibrate to a particular conformation where no more change could be observed. The resulting interaction pattern was similar to that of the native protein but differed particularly in that there was more extensive self-association for the recombinant mutant. The equilibration to a stable conformation was more rapid in the presence of Ca^{2+} ions. This suggests that the native protein normally folds into a particular conformation which may be aided by Ca^{2+} in the mammary gland. Further study of a recombinant form with the native amino acid sequence is needed. © 2000 Academic Press

Key Words: human β-casein; protein folding; thermal cycling; laser light scattering; analytical ultracentrifugation; fluorescence spectroscopy; turbidity; circular dichroism.

The major casein component of human milk is β-casein (β-CN)[3] which comprises ~80% of the total casein (1). It is a 24-kDa amphipolar molecule and exists in six differently phosphorylated forms (zero up to five phosphates per molecule) with 60–70% of the total being divided between the forms with 2 (β-CN-2P) and 4 (β-CN-4P) phosphates (2, 3). Along with κ-CN (~15% of total casein) for stabilization, these β-CN molecules form micelles to carry the otherwise insoluble calcium and phosphate which are indispensable nutrients for the growth and development of newborns (4). In addition, human β-CN molecules, or fragments thereof, are found to have biological effects such as enhancement of calcium absorption (5, 6), inhibition of angiotensin I-converting enzyme (7), opioid agonism (8), immunostimulating and immunomodulating effects (9), and antibacterial action (10, 11). Because of these biological effects, some effort has gone into producing recombinant human β-CN in both its nonphosphorylated (12) and phosphorylated (13) forms. The nonphosphorylated form, produced in *Escherichia coli,* was shown to have a primary structure identical to native β-CN except for an N-terminal methionine residue and it was claimed, although the data were not shown, that they were the same with regard to the formation of micelles and solubility in water and urea-containing buffers (12). Phosphorylation was performed *in vivo,* also in *E. coli,* by coexpression of human casein kinase II. However, the phosphorylation pattern of the recombinant form was different than the native in that the forms

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[3] Abbreviations used: β-CN, β-casein; CD, circular dichroism; NTA, nitrilotriacetic acid; IPTG, isopropyl-β-D-thiogalactosidase; FI, fluorescence intensity; FP, fluorescence polarization; ANS, 8-anilino-1-naphthalenesulfonic acid.

with 1 and 2 phosphates were predominant (13). Since the phosphorylation sites for human β-CN are within the first 10 residues of the molecule, one is left to wonder what effect extra N-terminal residues might have on the molecule where it might affect both the phosphorylation process and on the ability of the β-CN to bind minerals and form micelles and thereby deliver mineral nutrients to the infant.

On the basis of Raman spectroscopy (14) and CD (15), it has been proposed that bovine β-CN has secondary structure elements comprising 8–10% α-helix and 20–25% β-sheet. On the other hand, calculations of structure from the amino acid sequences of six different species, including human, are interpreted to indicate no α-helix and almost all of the molecule consisting of β-strands (16). It is further suggested that other constraints within the sequence prevent condensation of these strands into β-sheets but that a rheomorphic open and mobile conformation (or random-coil) of various structures that result from random association of hydrophobic residues are formed rather than a fixed tertiary structure (16). However, the self-association of the human β-CN proteins with increasing temperature, T, and in the presence of Ca^{2+} ions is reproducible (17–21), implying that they may normally fold into fixed tertiary structures. Furthermore, an analysis of the amino acid sequences of β-CN from several different species also suggests that this may be so because there are a number of places where particular residues are essentially invariant across the range of species (16, 22). This raises the possibility that these residues are important in the folding process to form a functional molecule.

Recently, to study the folding of proteins, mutants containing substitutions for residues considered important or having extra residues at some point have been prepared and used in different laboratories (23, 24). In the present investigation, a nonphosphorylated recombinant human β-CN was expressed and purified from *E. coli* and used to study this protein. This recombinant has four extra amino acids (GSHM−) at the N-terminus and is thus termed recombinant native plus 4 (rn + 4). Turbidity studies, sedimentation velocity, as well as the study of the change of the association behavior with thermal cycling (repeated heating and cooling) were performed on this recombinant protein and the results were compared with those of the native nonphosphorylated human β-CN. It was found that the properties of the molecule were not identical to those of β-CN-0P but dependent upon the previous treatment conditions, implying that the extra N-terminal amino acids had an effect on protein folding.

MATERIALS AND METHODS

Materials. The construct pGEM-3Z/β-CN, which carries the mature human β-CN cDNA, was kindly provided by Dr. Ravi Menon (University of Colorado, Boulder, CO). Other materials were purchased as follows: all restriction enzymes and T4 DNA ligase from New England BioLabs (Beverly, MA) and Promega (Madison, WI), the *pfu* DNA polymerase from Stratagene (La Jolla, CA), the Rapid DNA Ligation Kit from Boehringer Mannheim (Indianapolis, IN), the 1-kb DNA ladder from Gibco BLR (Grand Island, NY), a Zero Blunt PCR cloning kit and One Shot Top 10 cells from Invitrogen (Carlsbad, CA), the expression vector pET-28a(+), nonexpression host cells NovaBlue and expression host cells NovaBlue (DE3), thrombin (biotinylated or regular), and streptavidin resin from Novagen (Madison, WI), the QIAprep Spin Miniprep kit and Ni–NTA (nickel nitrilotriacetic acid) resin from Qiagen (Valencia, CA), the GeneClean kit from Bio101 (Vista, CA), LB Broth (Miller) from Difco Laboratories (Detroit, MI), DIAFLO ultrafiltration membranes, YM-30, from Amicon (Beverly, MA), Centricon-30 concentration units from Millipore (Bedford, MA), Kontes glass columns from Scientific Glassware/Instruments (Vineland, NJ), the Mono Q 10/10 anion-exchange HPLC column from Pharmacia Biotech (Piscataway, NJ), SeaKem GTG agarose from FMC BioProducts (Rockland, ME), acrylamide, bis-acrylamide, ammonium persulfate, TEMED (*N,N,N′,N′*-tetramethylenediamine), Coomassie brilliant blue and Bradford protein determination reagent from Bio-Rad Laboratories (Richmond, CA), the VectaStain ABC (avidin:biotinylated enzyme complex) kit, the VectaStain ABC/AP (avidin DH conjugated to alkaline phosphatase) kit and the VectaStain DAB–Ni (diaminobenzidine plus nickel chloride) substrate kit were purchased from Vector Laboratories (Burlingame, CA).

All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO), BWR (Brisbane, CA), or Fisher Scientific (Tustin, CA).

Plasmid construction. The mature human β-CN cDNA sequence in the construct pGEM-3Z/β-CN was amplified using PCR and first cloned into a PCR-Zeroblunt vector (Invitrogen). Two primers were designed to create a *Nde*I site at the 5′ end and a *Hin*dIII site at the 3′ end of the amplified portion of the β-CN sequence. The *Nde*I and *Hin*dIII sites were then used to subclone the desired sequences into the expression vector pET-28a(+) (Novagen) and the sequences of these constructs were checked by automated DNA sequencing. The final construct of rn + 4 has a T7/lac promoter which is inducible with isopropyl β-D-thiogalactopyranoside (IPTG), a 6xHis tag for purification and a thrombin cleavage site at the N-terminus of the coding sequence for human β-CN in order to remove the His tag. The construct is organized so that after thrombin cleavage, four amino acids in the sequence of Gly-Ser-His-Met (GSHM) will remain in front of the N-terminus of the mature human β-CN sequence.

Protein expression. A single colony of strain NovaBlue competent cells (Novagen) carrying the expression construct was picked up and cultured overnight, inoculated into a fresh LB media with a 1:5 ratio and incubated at 37°C with shaking (225 rpm) until the absorbance, A_{600}, reached approximately 0.6. Expression was induced with 1 mM IPTG and the culture was incubated at 37°C with shaking for another 3 h before harvest. Three hours of IPTG induction was found to be optimal and the existence of the human β-CN entity was confirmed by Western blotting using antibodies kindly supplied by Dr. E. Clifford Herrmann at Loma Linda University. The total expression was estimated at around 150 mg/L.

Purification. After 3 h of IPTG induction, a cell pellet was obtained by centrifugation and resuspended in lysis buffer containing 6 M guanidinium hydrochloride (Gu–HCl). Clear lysate was obtained by centrifugation and Triton X-100 detergent and β-mercaptoethanol were added to final concentrations of 0.5% and 10 mM respectively before mixing with Ni–NTA (Qiagen, Valencia, CA) resin with gentle stirring for 1 h. The lysate–resin mixture was then loaded onto a column and the resin with the protein bound was sequentially washed with pH 8.0 buffers (0.1 M NaH_2PO_4, 0.01 M Tris–HCl, 0.5 M NaCl) containing 8, 6, and 4 M urea and then eluted with 4 M urea-buffers (same as above) with pH of 6.3, 5.9, and 4.5. The presence of protein in each collected fraction was determined and frac-

tions containing protein were pooled and run through a Ni–NTA column for a second time. Fractions containing pure protein were pooled and dialyzed at 4°C extensively against thrombin cleavage buffer (20 mM Tris–HCl, 150 mM NaCl, pH 8.4) while slowly reducing the urea concentration stepwise to near zero.

Removal of the 6xHis tag. The recombinant protein was cleaved with thrombin (Novagen) at a ratio of 1 U thrombin/mg protein at 20°C for 12 h. Anion-exchange HPLC (Mono Q 10/10, Pharmacia) was used to remove the enzyme and the 6xHis tag. The cleaved protein was then dialyzed against low salt buffer, LSB (0.02 M NaCl, 0.01 M Imidazole, pH 7.0), and subjected to physicochemical studies.

N-terminal amino acid sequencing. Determination of the N-terminal amino acid sequence was based on the Edman method. The cleaved protein was electrophoresed on a 10% tricine–polyacrylamide gel and the protein band was transferred to a polyvinylenedifluoride (PVDF) membrane by electroblotting in CAPS buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid) at pH 11 and 90 mA for 90 min. The protein band was stained with 0.25% Coomassie Brilliant Blue R250 solution for 30 s, followed by destaining of the background in 100% methanol for 2 min. The recombinant human β-CN bands were cut from the membrane and applied to a PE Applied Biosystems 470A gas-phase protein/peptide sequencer equipped with an online phenylthiohydantoin (PTH) amino acid analyzer (Foster City, CA) and the N-terminal sequence determined following the manufacturer's protocol.

Phosphate content determination. The phosphate content of the recombinant protein was determined by the method of Van Veldhoven and Mannaerts (25). Protein phosphate in the nanomolar range can be determined by this method. Recombinant human β-CN was heated at 230°C in the presence of sulfuric acid to extract the phosphates, which were then reacted with ammonium heptamolybdate and malachite green. The final reaction gives a green color, which was quantified spectrophotometrically at 610 nm and compared to a standard curve to determine the amount of phosphate in the recombinant protein.

Laser light-scattering measurements. Particle size distributions and diffusion coefficients of the recombinant mutant rn + 4 were determined by laser light scattering using a Nicomp Model 370 submicron particle sizing system and Nicomp analysis software (Pacific Scientific, Silver Spring, MD) as described in earlier papers (19–21).

Turbidity studies. For rn + 4, turbidity of protein solutions (3 mg/mL in LSB) containing 0.0, 2.5 and 5.0 mM Ca^{2+} at increasing temperatures (4 up to 37°C) was determined by measuring A_{400}. The solutions were then reequilibrated at 4°C overnight and again warmed the following day. This was repeated several times and curves of A_{400} vs T were plotted in order to study the temperature-dependent association as well as the effect of Ca^{2+} on the association behavior of this recombinant protein.

Sedimentation velocity analysis. Sedimentation velocity studies were carried out using a Beckman Model E analytical ultracentrifuge (Fullerton, CA) using methods described earlier (27). Observed sedimentation coefficients (s_{obs}) were measured using schlieren optics, and were calculated from the movement of the maximum ordinate of the schlieren curve. All s_{obs} values were corrected to standard conditions at 20°C and water solution ($s_{20,w}$). Monomer characterization was performed at two temperatures (23 and 37°C) on samples prepared in a denaturing buffer (3.3 M urea, 0.01 M imidazole, pH 7.08), whereas self-association behavior of the recombinant protein was studied on thermally cycled protein at increasing temperatures (from 4 up to 37°C) and/or different pH values (6.5 up to 10.0) on samples prepared at 2 mg/mL in LSB.

Fluorescence measurements. Fluorescence intensity (FI) and fluorescence polarization (FP) were measured in a Perkin–Elmer LS-3 fluorescence spectrometer (Norwalk, CT). The β-CN mutant was prepared in buffer (0.02 M NaCl, 0.002 M sodium cacodylate, pH 7.0)

with or without 5 mM $CaCl_2$, at a protein concentration of 0.075% (w/v) as determined by absorbance at 280 nm. Samples were thermally cycled before the fluorescence measurements. The FI of the single intrinsic tryptophan was determined at an excitation wavelength of 290 nm and an emission wavelength of 350 nm. Hydrophobic interaction sites were probed at different T between 5 and 45°C with 8-anilino-1-naphthalenesulfonic acid (ANS) in concentrations ranging from 6 to 38 μM, added after temperature equilibrium was reached. Excitation for these was at 385 nm and emission was measured at 470 nm. The FP of the intrinsic tryptophan and the extrinsic ANS were determined using the same excitation and emission wavelengths respectively as described above and with the excitation and emission polarizers at 0 or 90° as appropriate (19).

Circular dichroism studies. Far-ultraviolet CD (260–180) measurements were carried out using a JASCO J-600 spectropolarimeter (Easton, MD). Samples were prepared at 1 mg/mL in LSB with 3.3 M urea with the addition of 0.0, 2.5, and 5.0 mM Ca^{2+}, and subjected to thermal cycling before the spectroscopic measurements. Measurements were performed on samples equilibrated at different T: 4, 25, and 37°C.

Values for θ, or observed ellipticity, at varying wavelengths were obtained. The molar ellipticity ($[\theta]$) in units of degree-square centimeters per decimole (deg.cm^2.dmol^{-1}) was calculated from θ using Eq. [1]:

$$[\theta] = \frac{MW \times \theta}{10 \times d \times c},\qquad [1]$$

where MW is the molecular weight of the protein molecule, θ is the observed ellipticity, d is the pathlength (cm), and c is the concentration (g/mL). Values for $[\theta]$ (deg.cm^2.dmol^{-1}) were plotted versus wavelength (nm) and curves thus obtained were compared.

RESULTS

Expression and purification. The expressed protein was found primarily in the insoluble cytoplasmic portion, i.e., the inclusion body fraction of the cells. Metal-chelating affinity chromatography (MCAC) under denaturing conditions was used for protein purification, which utilized the binding between the polyhistidine tag and the Ni–NTA resin. The protein began to elute from the column at pH 5.9 and peaked at pH 4.5, indicating that this protein existed in both monomeric (usually eluted at pH 5.9) and aggregated forms (usually at pH 4.5) under the experimental conditions. A second column chromatography seemed to further improve the purity to over 90% with a yield of about 40 mg/L. Thrombin digestion of this recombinant protein was performed using 1 U enzyme per milligram of protein at 20°C for 12 h and the cleavage was complete and specific.

N-terminal amino acid sequence. The N-terminal sequence of rn + 4 was determined as follows: Gly-Ser-His-Met-Arg-Glu-Thr-Ile-Glu-Ser-Leu-Ser-Ser-Ser-Glu-Glu-Ser. The sequence GSHM- in front of Arg, which is the first amino acid in the native protein, was left behind by thrombin cleavage. The remaining sequence is identical to that of the N-terminal portion of native β-CN as would be expected.
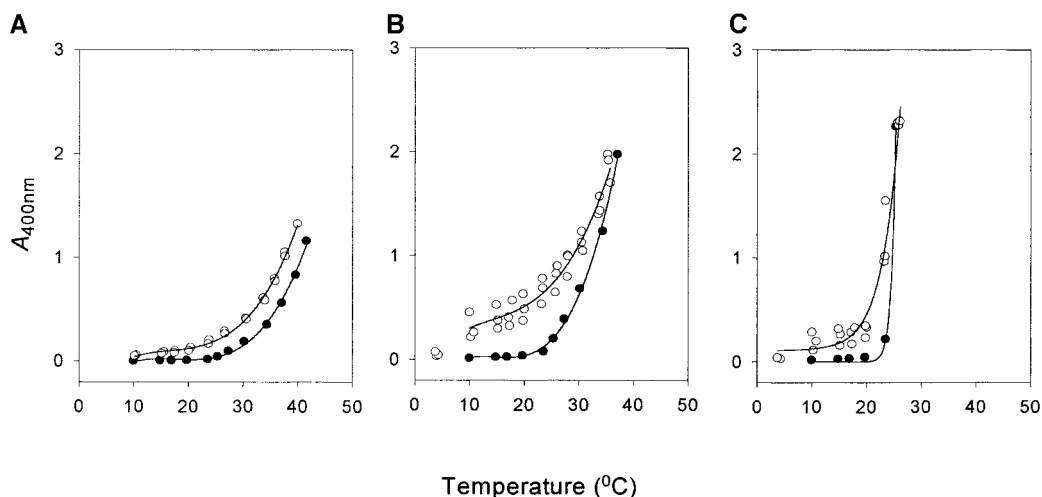
FIG. 1. Turbidity studies of rn + 4 in the presence of (A) 0, (B) 2.5, and (C) 5.0 mM CaCl₂. Absorbance at 400 nm was plotted versus temperature. Each sample was prepared in LSB at a protein concentration of 3 mg/mL and subjected to repeated thermal cycling. In each graph, the filled circles represent the first run, whereas the open circles represent the points obtained after various temperature cycles. The lines are regression curves for the first and of pooled data for the repeated runs. An overnight cooling of the sample at 4°C was carried out between cycles.

Phosphate content. Virtually no phosphate was found in the rn + 4 recombinant human β-CN, which was as expected since the *E. coli* system performs no posttranslational modification.

Turbidity studies. In LSB, the solubility behavior of rn + 4 may be compared with that of the native human β-CN-0P (18). As temperature was increased, solutions of both proteins stayed clear up to a certain point before turbidity started to increase. In both cases, the turbidity disappeared and clear solutions were obtained on cooling, indicating that the temperature-dependent polymerization was generally reversible. As with the native β-CN-0P molecule (18), the solubility of rn + 4 appears to depend on the protein concentration as well as on *T*. In the presence of up to 5 mM Ca²⁺ both proteins behaved in a similar manner. Absorbance measurements at 400 nm as a function of *T* for rn + 4 in solutions with different Ca²⁺ concentrations are shown in Fig. 1. Repeating the measurements by reequilibration at low *T* resulted in a shift in the readings not found for the native β-CN-0P (18). For each solution with a different Ca²⁺ concentration, the data for the repeated runs which were close together were pooled and a regression curve was drawn through those data points to represent the final curve. This is compared with the curve of the first run in each case. Aggregation of the rn + 4 seemed to start at around 10°C, where the solution began to get a milky tinge. Also, rn + 4 seemed to attain a conformational equilibrium by thermal cycling, through which it eventually stabilized in a fixed pattern. It is worth noting that this equilibrium was attained faster as the Ca²⁺ concentration was increased. A comparison of the regres-

sion curves for the final runs representing rn + 4 solutions with different Ca²⁺ concentrations (Fig. 2) shows that, similar to that for the native protein (18), the transition *T* at which significant association of rn + 4 occurred shifted to a lower value when Ca²⁺ concentration was increased.

Hydrodynamic studies. Observed and corrected sedimentation coefficients of the rn + 4 in 3.3 M urea at 23 and 37°C are shown in Table I along with the diameter and $D_{20,w}$ values from laser light scattering
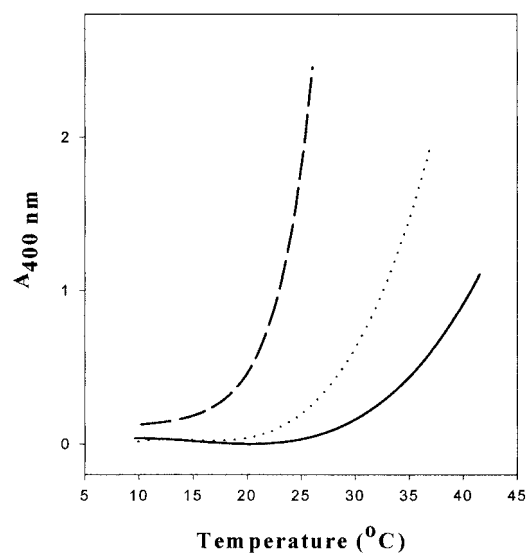

FIG. 2. Regression curves from data of Fig. 1 after conformation equilibration by temperature cycling for 0.0 (solid line), 2.5 (dotted line), and 5.0 (dashed line) mM CaCl₂.

TABLE I

Characterization of rn + 4 Human β-CN[a] Monomer Expressed in *E. coli*

Temperature (°C)	s_{obs} (S)	$s_{20,w}$ (S)	D_{obs} (10^{-7} cm^2/s)	$D_{20,w}$ (10^{-7} cm^2/s)	Diameter (nm)
23	1.82 ± 0.13	2.48	12.3 ± 0.3	12.5	7.6 ± 0.2
37	2.83 ± 0.03	2.63	15.8 ± 0.1	12.0	8.4 ± 0.1
37	2.67 ± 0.07[b]	2.50	Not determined	—	—

[a] Recombinant β-CN was prepared in 3.3 M urea, 0.01 M imidazole, pH 7.08, at 9 mg/mL.
[b] The concentration of this sample was 3.5 mg/mL.

experiments. The difference in the $s_{20,w}$ values as T is increased and as concentration of protein is decreased suggests that there is some protein aggregation even in urea solution. However, the values appear to be approximately those of the monomeric molecule and are quite comparable to those reported for different monomeric forms of the native human β-CN (3, 17, 18, 26). With the assumption that the molecule is spherical, a combination of the $s_{20,w}$ and $D_{20,w}$ gave a monomeric M_r of nearly 22,000 for the recombinant mutant, which is quite close to the values reported for the different monomeric forms of the native human β-CN (3, 17, 20, 21, 26) using similar data and also from sedimentation equilibrium. No significant change in diameter or in the $s_{20,w}$ value was observed when the temperature was increased to 37°C, which suggests that the molecule is still in the monomeric form under those conditions. The $s_{20,w}$ value at 37°C at a protein concentration of 9 mg/mL also shows no significant change.

In LSB without urea, a value of 2.3 S was obtained for the $s_{20,w}$ at 4°C which increased to 3.1 S when T was raised to 23°C (Table II). As with the native molecule, high-molecular-weight material was present and deposited on the bottom of the centerpiece of the ultracentrifuge cell. This deposit thickened as T was increased to 37°C. At 30°C, two peaks were obtained with

1.7 and 46 S values, respectively, for the slow and the fast peaks. Under similar conditions, the native molecule exhibited a single peak of 26 S. At 37°C the native molecule showed a $s_{20,w}$ of 42 S compared to the 65 S for the recombinant protein. From these results, it is apparent that the solubility and the temperature-dependent association behavior of rn + 4 is significantly different from the native human β-CN-0P, although the monomeric properties do not appear to be much different. The reason for this difference is probably in folding because of the extra residues.

To study the role of electrostatic interactions, the sedimentation coefficient of the recombinant molecule was studied in LSB at a protein concentration of 2 mg/mL but at four different pH values in the range of 6.5 to 10.0. At pH 6.5 and 7.0, measurements were also made at different T (Table III). At pH 8.5 and 37°C, two peaks were obtained, the fast peak had a value of nearly 25 S for $s_{20,w}$ whereas that for the slow peak was that of monomer. The sedimentation coefficient value for the fast peak is significantly higher than that (13.5 S) reported for the native β-CN-0P (27). Both the rn + 4 and the native β-CN-0P molecule were found to be monomeric at pH 10 and 37°C, which implies that electrostatic interactions predominate over hydrophobic interactions under these conditions. At pH 6.5, both proteins behaved in a similar manner, i.e., at low tem-

TABLE II

Sedimentation Coefficient of rn + 4 Human β-CN[a] at Different Temperatures

Temperature (°C)	s_{obs} (S)	$s_{20,w}$ (S)
4	1.5 ± 0.01	2.3
7	1.9 ± 0.01	2.4
10	2.0 ± 0.03	2.4
15	2.5 ± 0.02	2.8
23	3.3 ± 0.03	3.1
25	3.5 ± 0.03	3.1
30	57.1 ± 0.02 (fast peak)	45.5
30	2.0 ± 0.06 (slow peak)	1.7
33	62.6 ± 0.16	49.6
37	88.9 ± 0.17	65.5

[a] Recombinant human β-CN was prepared in LSB (0.01 M imidazole, 0.02 M NaCl, pH 7.1) at a concentration of 2 mg/mL.

TABLE III

Effect of pH on Sedimentation Coefficient at Different Temperatures of rn + 4 Human β-CN[a] Expressed in *E. coli*

pH	Temperature (°C)	s_{obs} (S)	$s_{20,2}$ (S)
6.5	4	1.6 ± 0.03	2.5
	23	3.3 ± 0.06	3.1
	37	Heavy deposit (no peak)	—
7.1	4	1.5 ± 0.01	2.3
	23	3.3 ± 0.03	3.1
	37	88.9 ± 0.1	65.5
8.5	37	35.9 ± 0.5 (fast peak)	24.7
	37	3.5 ± 0.03 (slow peak)	2.5
10.0	37	3.8 ± 0.09	2.6

[a] Recombinant human β-CN was prepared in LSB (0.01 M imidazole, 0.02 M NaCl, pH 7.0) at a concentration of 2 mg/mL.
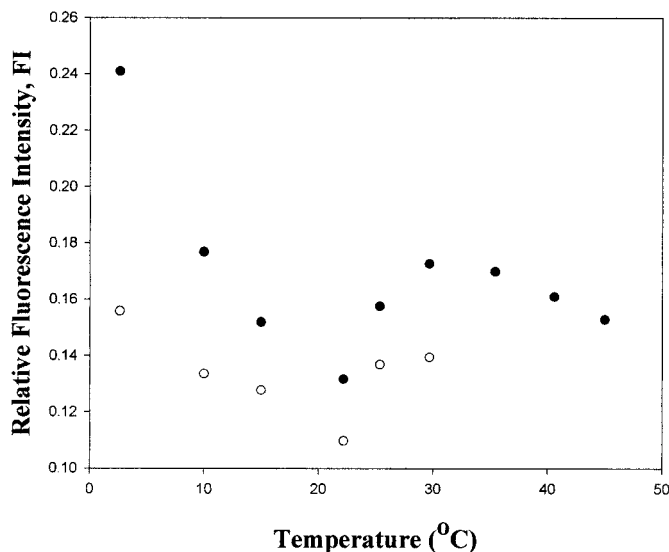
FIG. 3. Relative fluorescence intensity of the intrinsic tryptophan of rn + 4 as a function of temperature with 0.0 (●) or 5.0 (○) mM CaCl$_2$. Protein concentration was 0.075% (w/v) in a buffer of 2 mM Na cacodylate, 20 mM NaCl, pH 7.0, and subjected to four temperature cycles before fluorescence measurements.

perature both are monomeric, some association occurs at room temperature, but at 37°C there was a thick precipitate at the bottom of the cell and no peak was obtained.

Fluorescence studies. In general, the results of fluorescence measurements on rn + 4 that had been thermally cycled were qualitatively similar to those for native human β-CN-0P (18). The relative fluorescence intensity (FI, in arbitrary units) for the intrinsic tryptophan in rn + 4 in the presence or absence of 5 mM CaCl$_2$, from about 5 to 45°C, is shown in Fig. 3. In the absence of Ca^{2+}, the FI of the intrinsic tryptophan decreased steadily between 5 and 22°C, increased to a new level between 22 and 30°C with an inflection point of approximately 25°C, compared to 29°C for the native β-CN-0P (18), then decreased again above 30°C. The decline of FI in the low temperature region (5–22°C) is characteristic of a fluorophore in a polar environment (28). The subsequent increase in FI between 22 and 30°C could be attributed to the tryptophan shifting into a more nonpolar environment. Such a shift may be due to a conformational change or to aggregation at this T and protein concentration but with a difference between rn + 4 and the native protein.

At low T, the FI with Ca^{2+} was lower than without, suggesting that the tryptophan may be sensitive to the electrostatic environment. Above 25°C, the aggregation of the molecules to a size where there was extensive light scattering in the presence of Ca^{2+}, made accurate FI measurements very difficult. This is again different than for the native protein where such aggre-

gation was not extensive enough to prevent fluorescence measurements (18). However, the transition temperature at which the conformational change took place also seemed to be slightly lower in the presence of calcium than when it was absent.

The samples were subjected to thermal cycling before comparative measurements were performed. However, there was still a conformational change on going from low to high T. The FI values as a function of temperature at various ANS concentrations were compared when ANS was added to the solution either before or after equilibration at the final T of measurement. The magnitude of FI change at the transition T was smaller with ANS added before T equilibration than after, indicating that ANS interacted with and participated in the equilibration of protein conformation and aggregation when it was added before equilibrium was reached. The FI values for the rn + 4/ANS complexes at various levels of ANS increased significantly above 20°C, reaching maxima around 30°C (Fig. 4A). In the presence of 5 mM Ca^{2+} (Fig. 4B), the aggregation of the molecules again led to heavy precipitation between 25 and 30°C, making accurate FI measurement above about 30°C impossible. The increase in FI at the transition T was sharper in the presence of Ca^{2+} than in its absence. These properties again differed in a similar manner to those of the native protein.

The FI measurements at each T, shown in Fig. 4 for 6 up to 38 μM ANS, were subjected to analysis for the determination of the ANS dissociation constant K_d. Double-reciprocal plots of 1/FI vs 1/[ANS] were constructed, values for K_d were calculated from these and plotted vs T as shown in Fig. 5, with the assumption that the number of ANS binding sites stayed constant. Both in the presence and absence of Ca^{2+}, K_d increased slightly between 2 and 20°C but increased drastically at the transition temperature (20°C), indicating a conformational change at this point. The magnitude of the increase in K_d could be larger in the presence of Ca^{2+} than without, but heavy precipitation occurred when 5 mM Ca^{2+} was added which hindered accurate measurement.

Tryptophan FP of rn + 4 measured as a function of T is shown in Fig. 6. Again, protein conformation seemed to approach an equilibrium after repeated thermal cycling. The FP value increased steadily between 5 and 20°C, then dramatically between 20 and 30°C, and finally started to plateau (or decline) above 30°C. The FP curve of the equilibrated protein was shifted upward compared with that of the sample without thermal cycling. Also, the transition point at which the conformational change took place shifted toward a slightly lower T. Hence, thermal cycling seemed to have caused the stabilization of the protein conformation into one which limited the tryptophan mobility at all T. The equilibrium conformation may have more
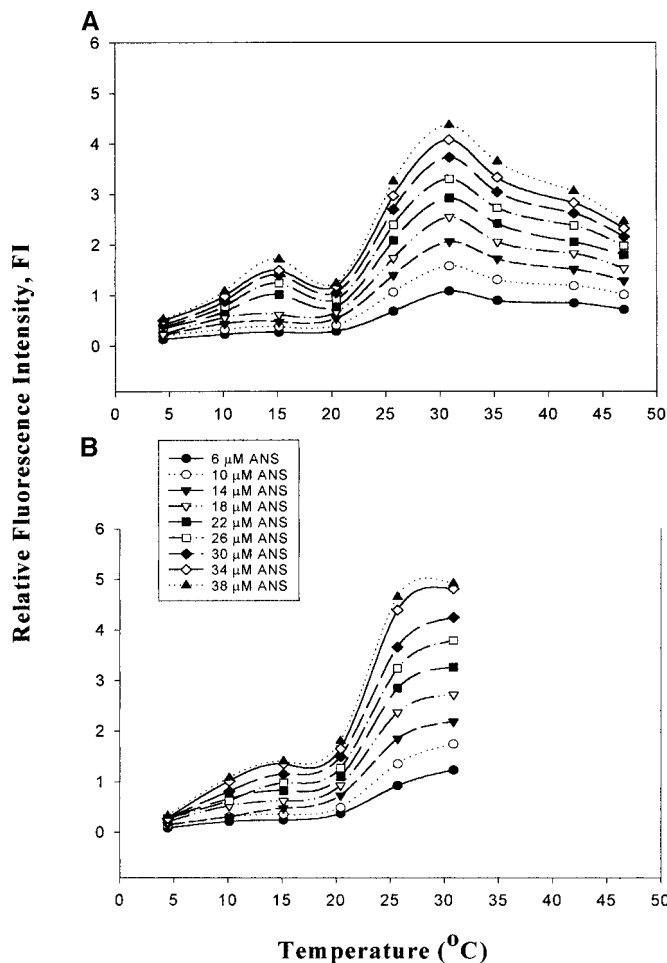
FIG. 4. Relative fluorescence intensity of different concentrations of extrinsic ANS complexed with rn + 4 as a function of temperature with 0.0 (A) and 5.0 (B) mM CaCl$_2$. Protein concentration was 0.075% (w/v) in a buffer of 2 mM Na cacodylate, 20 mM NaCl, pH 7.0, and subjected to four temperature cycles before fluorescence measurements. For each fluorescence measurement, ANS was added to the final concentration indicated after temperature equilibrium.

FIG. 5. Dissociation constant, K_d, of ANS from rn + 4 as a function of temperature with 0.0 (●) and 5.0 (○) mM CaCl$_2$.

accordance with the results shown in Fig. 5 in which the ANS dissociation constant did not change much as the transition T was approached but increased drastically at that T. When ANS concentration was increased, the same trend was observed, although the FP curve shifted downward. Also, the magnitude of the decrease in FP at the transition T was smaller, and the transition seemed to happen at a slightly lower T. The reason for this phenomenon was unclear, although it could be ascribed to the binding of more ANS to the available hydrophobic areas on the surface of the protein which were relatively flexible. In the presence of 5 mM CaCl$_2$, a similar type of FP change was observed, though the magnitude of the drop in the FP value at

hydrophobic sites exposed so that there was a greater tendency toward aggregation starting at a lower T and in turn confined the mobility of the tryptophan residue to a greater extent.

The FP of ANS added at various concentrations as a function of T with or without 5 mM CaCl$_2$ is shown in Fig. 7. In the absence of Ca^{2+}, the FP increased steadily between 2 and 20°C with the addition of 10 μM ANS, probably due to some minor aggregation of the molecule which did not affect the amount of ANS binding but limited ANS mobility. There was a drop of FP between 20 and 25°C, indicating that a conformational change occurred during which aggregation took place and some hydrophobic areas originally on the surface were buried in the interior of the molecule, leading to the decrease in the population of bound ANS. This is in
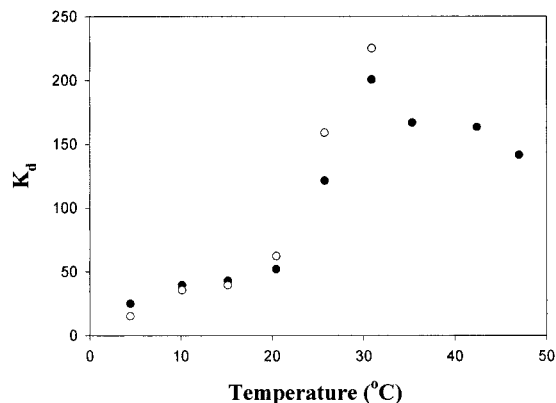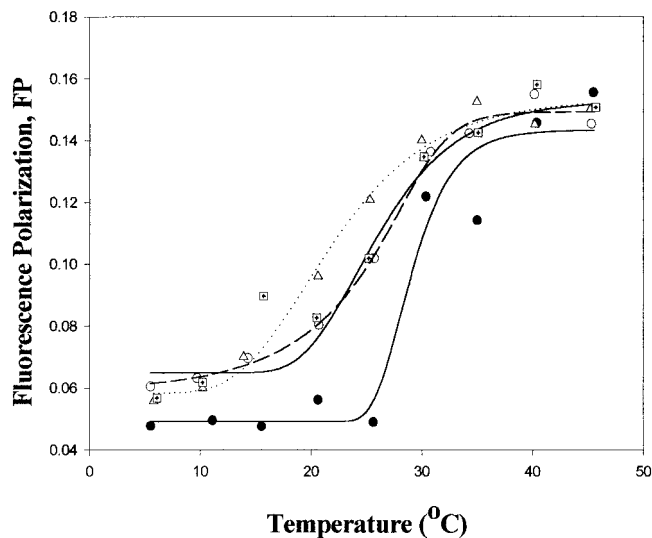

FIG. 6. Fluorescence polarization, FP, of the intrinsic tryptophan as a function of temperature for different temperature cycles with an overnight cooling at 4°C between cycles. Protein concentration was 0.075% (w/v) in a buffer of 2 mM Na cacodylate, 20 mM NaCl, pH 7.0. Cycle 1 (●), cycle 2 (○), cycle 3 (△), cycle 4 (□).
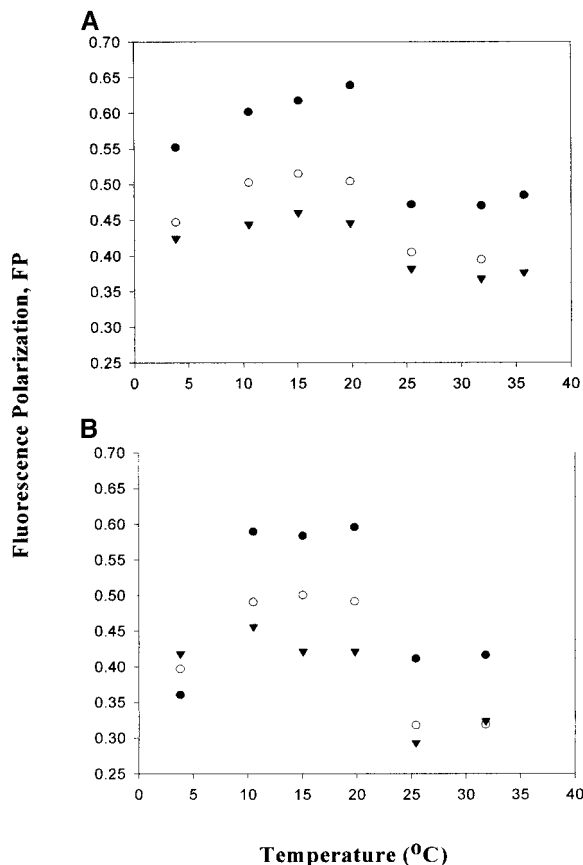
FIG. 7. Fluorescence polarization, FP, of different concentrations of extrinsic ANS complexed with rn + 4 as a function of temperature with 0.0 (A) and 5.0 (B) mM CaCl$_2$. Protein concentration was 0.075% (w/v) in a buffer of 2 mM Na cacodylate, 20 mM NaCl, pH 7.0, and subjected to four temperature cycles before fluorescence measurements. For each fluorescence measurement, ANS was added to the final concentration indicated after temperature equilibrium. The concentration of ANS was 10 (●), 22 (○), or 34 (▼) μM.

the transition point was larger with Ca^{2+} than without. This suggested that the Ca^{2+} binding might enhance protein aggregation through hydrophobic interactions, leading to less exposure of hydrophobic sites and hence to less binding of ANS and lower FP.

Circular dichroism. The CD spectra of rn + 4 in LSB with 3.3 M urea at different *T* are compared in Fig. 8. Temperature did not seem to affect the spectrum much when protein was denatured by urea, although there was a slight decrease in the magnitude of the inverted peak when *T* was increased from 4 up to 37°C, possibly due to the fact that urea is a weak denaturant and protein was probably not fully denatured. From comparison with standard curves (28), the structure appears to be a mixture of mostly β-sheet with some random coils.

A comparison of CD spectra in LSB with different Ca^{2+} concentrations is shown in Fig. 9. At all three Ca^{2+} concentrations, there was a significant decrease

in the magnitude of the inverted peak when *T* was increased, suggesting the loss of regular structure of the protein. Also, the position of the peak seemed to shift toward higher wavelengths, and the extent of the shift seemed to be larger for samples with higher Ca^{2+} concentrations at higher *T*, indicating that a possible conformational change occurred when *T* was increased, probably aided by the presence of Ca^{2+} although changes in the spectra with increasing Ca^{2+} concentration are much smaller than changes with *T*.

DISCUSSION

The effect of temperature on folding. As was stated above, the characteristics of rn + 4 to attain a conformational equilibrium by repeated heating and cooling, or thermal cycling, is quite different from the native β-CN-0P, the self-association of which is reproducible. The properties eventually seemed to stabilize in a characteristic pattern. This indicates the possible role temperature plays in the induction of "correct" folding of these recombinant proteins.

In a comparison of the data from turbidity studies of native human β-CN-0P with rn + 4, it should be noted that even when the recombinant protein behaved similarly to the native protein, the self-association of the mutant seemed to be stronger than its native counterpart, with the A_{400} values obviously higher. The fact that "small-scale" associations of rn + 4 started at lower *T* (10–15°C), with the physical appearance of the protein solution as a slightly milky tinge, plus the much higher values of A_{400} compared with those of the native protein above the transition *T* indicated that with the aid of temperature the recombinant protein might have folded into a certain conformation which is similar to but different from the native protein. The turbidity at the transition point of recombinant protein is much greater than its native counterpart, especially
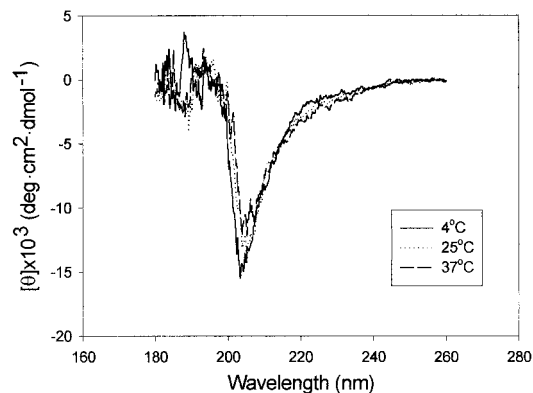

FIG. 8. The CD spectrum of rn + 4 in the presence of 3.3 M urea. The sample was prepared at 1 mg/mL in LSB, with the addition of urea to the final concentration of 3.3 M.
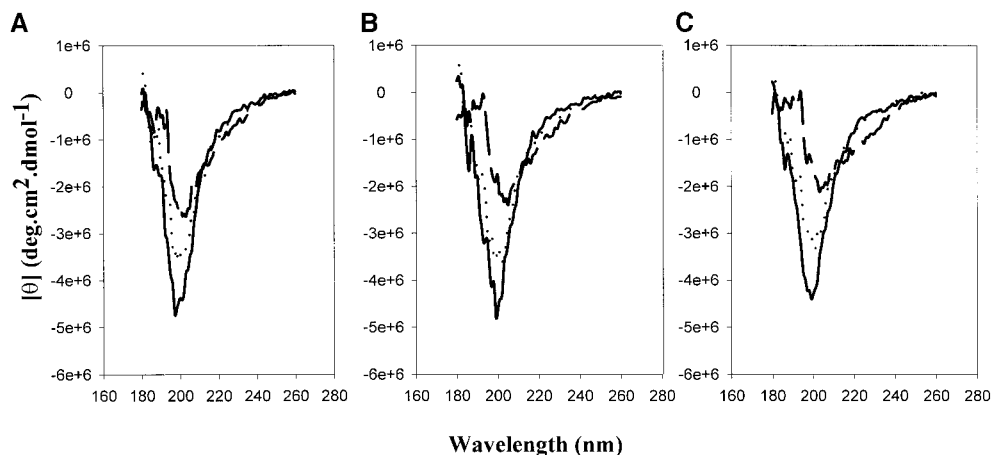
FIG. 9. Comparison between CD spectra of rn + 4 in the presence of different concentrations of Ca^{2+}. Each sample was prepared in LSB, with the addition of 0.0 (A), 2.5 (B), and 5.0 (C) mM $CaCl_2$, respectively. Thermal cycling of the sample was performed before CD measurements.

in the presence of Ca^{2+}. This suggests that the recombinant protein may have higher affinity for Ca^{2+}.

The effect of calcium on folding. When rn + 4 is compared with the native β-CN-0P, one can see that it behaved very similarly to the native protein in the absence of Ca^{2+}, with significant aggregation occurring above 27°C. This pattern was again observed when Ca^{2+} was added to the solution, although the increase in turbidity was not as sharp as that of the native protein for 2.5 mM Ca^{2+} but came very close when the Ca^{2+} concentration was increased to 5.0 mM. One assumption that could be made, based on the above observation, is that Ca^{2+} not only binds to this molecule and enhances aggregation, but also might induce the correct folding with binding. The Ca^{2+} in human mammary gland may have played an important role in inducing the folding of native protein into the "correct" conformation, together with other possible chaperones in the human system, while recombinant protein was deprived of Ca^{2+} and expressed in an environment completely different from the human system. Further, Ca^{2+} was not involved in any phase of the sample preparation process.

It is apparent that this recombinant human β-CN with four extra N-terminal amino acids exhibits similar but quantitatively different self-association patterns and other characteristics with changes in temperature when compared with the native β-CN-0P. The results of the experiments described here imply that molecular folding of the mutant may arrive at different conformations that may be changed to a more fixed structure by thermal cycling. The question still presents itself as to how much of the differences noted are due to the extra amino acid residues and how much may be due to the recognized problems with folding found often in recombinant systems. We are in the process of preparing material and making similar measurements on a wild-type recombinant human β-CN-0P in order to help answer that question.

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