A Recombinant C121S Mutant of Bovine β-Lactoglobulin Is More Susceptible to Peptic Digestion and to Denaturation by Reducing Agents and Heating†

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ABSTRACT: The lipocalin β-lactoglobulin (BLG) is the major whey protein of bovine milk and is homodimeric at physiological conditions. Each monomer contains two disulfide bonds and one cysteine at position 121 (C121). This free thiol plays an important role in the heat-induced aggregation of BLG and, possibly, in its conformational stability. We describe here the expression in the yeast *Pichia pastoris* of a mutant bovine BLG, in which C121 was changed into Ser (C121S). Circular dichroism and high-performance liquid chromatography experiments, together with the X-ray crystal structure, show that the C121S mutant retains a nativelike fold at both neutral and acid pH. The mutation completely blocks the irreversible aggregation induced by heat treatment at 90 °C. Compared to the recombinant wild-type protein, the mutant is less stable to temperature and disulfide reducing agents and is much more sensitive to peptic digestion. Moreover, its affinity for 1-anilino-8-naphthalenesulfonate is increased at neutral and acid pH. We suggest that the stability of the protein arising from the hydrophobic effect is reduced by the C121S mutation so that unfolded or partially unfolded states are more favored.

Bovine β-lactoglobulin (BLG)[1] is the major whey protein of cow milk. It is a small (18400 Da) globular protein composed of 162 amino acids including two disulfide bonds (C66−C160 and C106−C119) and one cysteine at position 121 (*1*, *2*). BLG is dimeric at neutral pH; each monomer is composed of eight antiparallel β-strands (named A−H) forming a hydrophobic core and one α-helix standing alongside the barrel. A ninth β-strand, named I, forms part of the homodimer interface (*1*, *3*).

When heated at neutral pH to above about 60 °C, BLG is irreversibly denatured, due to its polymerization through inter- and intramolecular disulfide bond formation and exchange promoted by C121 (*4*, *5*). The molecular detail of the heat-induced denaturation and polymerization process of BLG has not been fully elucidated yet, but it clearly involves several successive reactions and intermediates (*6*−*8*) leading to irreversible modifications of BLG structure at different levels (*9*−*11*). C121 plays a crucial role in this heat-induced polymerization of BLG: chemical blocking of the free thiol group prevents the protein from aggregating and renders its denaturation reversible (*4*, *5*, *10*, *12*, *13*).

Chemical blocking of the free thiol has also been shown to exert a destabilizing effect. The secondary and tertiary structures of the reacted BLG have been found to be modified (*13*). In some cases, the equilibrium between the monomer and dimer forms of the modified BLG has been observed to be shifted toward the monomer (*13*, *14*). Finally, some chemically blocked BLG has been found to be less resistant to denaturants (*12*, *15*) and heating (*13*), or their affinity for fatty acids was reduced (*16*). In all of these studies, C121 has been suggested to play an important role in stabilizing the BLG structure.

However, because C121 has a rather low accessibility, partial denaturation is required in order to expose this residue to thiophilic reagents. Such denaturation can be, in some cases, partially irreversible. For example, when studying BLG with its thiol groups blocked by iodoacetamide, McKenzie et al. (*17*) have suggested the presence of equimolar amounts of free C119 and C121 in the native protein. This result arose from the harsh conditions used during the alkaline treatment, which allowed disulfide bond interchange to occur between C106, C119, and C121 (*18*, *19*). Moreover, chemical modifications are never 100% effective, and the blocking

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[1] Abbreviations: BLG, β-lactoglobulin; ANS, 1-anilino-8-naphthalenesulfonate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; RP, reversed phase; GP, gel permeation; rBLG, recombinant β-lactoglobulin; WT, wild type; CD, circular dichroism; β-ME, β-mercaptoethanol; DTT, dithiothreitol; HEPES, sodium salt of *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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groups can react with other residues than C121. Thus, the destabilizing effect of thiol blocking may be due to the modifying groups introduced and/or to the stress caused by the chemical treatments rather than to the simple elimination of the free thiol group.

To avoid the side effects of blocking the thiol group of BLG in partially irreversible denaturing conditions, it is of great interest to carry out studies on a genetic mutant of bovine BLG, in which C121 is substituted by an amino acid with approximately the same side chain characteristics. The aim of this work was to produce in the yeast *Pichia pastoris* a wild-type (WT) and a mutant BLG, in which the free Cys at position 121 was changed into Ser. The structural characterization of this C121S mutant has been carried out, as well as studies on the effect of the mutation on the heat-induced unfolding and aggregation of the protein. Studies on the impact of C121S mutation on BLG stability have been carried out under heating, reducing conditions, and peptic digestion. Finally, the accessibility of the hydrophobic regions of the mutant has been probed by ANS binding measurements. During the preparation of this paper a study of the urea-induced denaturation of the Ala, Val, and Ser mutants of C121 was reported (*20*). Our results for the stability of the C121S mutant, studied by the distinct methods reported here, are complementary to, and in good agreement with, those in this recent report.

MATERIALS AND METHODS

Preparation of Bovine BLG. A BLG purified from cow's milk according to Mailliart and Ribadeau Dumas (*21*) was used as a standard in all experiments. As revealed from RP-HPLC and SDS−PAGE, BLG is a mixture of genetic variants A and B (ratio 1:1), more than 95% pure.

Mutant Design and Cloning. The WT recombinant BLG expression system previously constructed by Wilson et al. (*22*) was used as a template. In this system, the cDNA coding for WT (variant A) was inserted into the *Xho*I/*Xba*I site in the multiple cloning site of the expression vector pGAPZαA (Invitrogen, Groeningen, The Netherlands). The gene of interest is placed under the control of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, allowing constitutive expression of the recombinant protein. pGAPZαA also contains the α-factor signal peptide from *Saccharomyces cerevisiae*, which allows secretion of the protein into the culture medium. A gene conferring resistance to Zeocin was used as a selection marker. The C121S mutation was performed according to the QuickChange protocol from Stratagene (La Jolla, CA) with the following primers: 5′ primer, 5′-GC-CTG-GTC-TGC-CAG-AGT-CTG-GTC-AGG-ACC-CC-3′; 3′ primer, 5′-GG-GGT-CCT-GAC-CAG-ACT-CTG-GCA-GAC-CAG-GC-3′ (mismatched bases are underlined). Amplified DNA was transformed into Epicurian *Escherichia coli* XL1-blue cells (Stratagene) and plated for selection on Luria broth (LB)−agar plates supplemented with Zeocin (25 μg/mL). Plasmids from positive clones were purified with the miniprep kit from Qiagen (Venlo, The Netherlands) and sequenced to confirm the correct introduction of the mutation (MWG Biotech, Ebersberg, Germany). The positive plasmid was linearized with *Bsp*HI and electroporated into *P. pastoris* according to the EasySelect *Pichia* Expression Manual from Invitrogen. Selection for multicopy transformants was carried out by plating clones on increasing levels (100−800 μg/mL) of Zeocin. The best producing clones were selected by culture for 48−72 h in YPD medium (1% yeast extract, 2% peptone, 2% glucose), and protein expression was assayed by SDS−PAGE.

Protein Expression in P. pastoris. A fresh colony of *P. pastoris* was grown overnight (30 °C, 200 rpm) in 40 mL of buffered glycerol complex medium (BMGY) supplemented by glucose (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.04% biotin, 2% glucose, 100 mM potassium phosphate, pH 7.0) and supplemented with Zeocin (100 μg/mL). This culture was used to inoculate 2 L culture flasks containing 250 mL of BMGY each. Constitutive expression of the protein was performed at 30 °C under shaking (200 rpm) during 3 days with occasional pH readjustment with 1 M phosphate buffer, pH 7.0, and glucose addition. The protein expression during the culture was followed by SDS−PAGE. After 72 h, the supernatant was collected by centrifugation (10 min, 2000*g*) for further purification of the protein.

Protein Purification. All preparative chromatography steps were carried out at 20 °C on an Äkta purifier system and followed with the Unicorn control software (Amersham Biosciences, Piscataway, NJ). The supernatant was first filtered through paper to remove any remaining free cells and dialyzed against distilled water on a modular tangential flow system (Vivaflow 50; Vivascience, Hanover, Germany). The retentate was loaded onto an anion-exchange column, Q-Sepharose XL Streamline (200 × 26 mm; Pharmacia, Peapack, NJ). The protein was eluted at a flow rate of 5 mL/min with 20 mM Tris-HCl, pH 7.6, buffer by increasing steps of NaCl concentration (0.1/0.3/0.5/1 M). The protein content of each fraction was checked by SDS−PAGE. The BLG was mainly eluted at 0.3 M NaCl. This fraction was concentrated by ultrafiltration (Vivaspin 20, 10 kDa MWCO; Vivascience) and passed through a Superdex 75 gel filtration column, type XK26 (700 × 26 mm, Pharmacia) equilibrated with 50 mM sodium phosphate and 0.1 M NaCl, pH 7, at a flow rate of 4 mL/min. The purity of each fraction was checked by RP-HPLC and SDS−PAGE. The fractions containing the pure protein were pooled, dialyzed against distilled water, and lyophilized for storage at 4 °C.

Protein Characterization. Protein sequencing was performed on a protein sequencer, 477A, from Applied Biosystems (Foster City, CA).

Electrospray ionization mass spectrometry (ESI-MS) experiments were performed on a Thermo Finnigan LCQ Advantage (San Jose, CA) by infusion at a flow rate of 2.5 μL/min. Samples were at 5 pmol/μL in H_2O−acetonitrile (1/1 v/v) plus 0.5% formic acid.

Free thiol titration was performed with DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] according to Ellman (*23*).

HPLC. Chromatography was carried out at 20 °C on a 2695 separation module with a 996 photodiode array detector and followed with the Millenium software, all from Waters (Milford, MA). Protein samples were prepared at 1 mg/mL.

GP-HPLC was run on a TSK gel 3000 SWXL column (300 × 7.8 mm i.d.; TosoHaas, Montgomeryville, PA) equilibrated with 50 mM sodium phosphate and 0.1 M NaCl, pH 7.0, or 10 mM glycine hydrochloride, pH 2.0. Elution was performed at a flow rate of 0.8 mL/min, and peak detection was monitored at 220 nm. The column was

calibrated with six protein standards covering a molecular mass range from 13.7 to 150 kDa.

RP-HPLC was run on a Nucleosil column (C_{18}, 300 Å, 10 μm, 250 × 3 mm i.d.) from Macherey-Nagel (Hoerdt, France). The column was equilibrated with solvent A [5% aqueous acetonitrile, 0.1% trifluoroacetic acid (TFA)], and elution was performed at a flow rate of 1 mL/min with a linear gradient from 0 to 100% solvent B (70% aqueous acetonitrile, 0.09% TFA) over 25 min. Peak detection was monitored at 220 nm.

PAGE. SDS−PAGE was performed according to Schägger and von Jagow (*24*) on a 15% acrylamide gel (0.33 M Tris-HCl, pH 8.8, buffer). The samples were mixed with 2× loading buffer (0.18 M Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 3% bromophenol blue, ±6% β-ME) and boiled for 2 min. The anode buffer was 0.2 M Tris-HCl, pH 8.9, and the cathode buffer was 0.1 M Tris-HCl, 0.1 M Tricine, and 0.1% SDS. The electrophoresis was performed at 10 mA (stacking gel) and 20 mA (running gel). The gels were stained with Coomassie Blue R250. After destaining, plates were scanned, and intensities of bands were quantified using Quantity One software (Bio-Rad, Hercules, CA).

Alkaline (native) electrophoresis was performed on a 10% acrylamide gel (0.33 M Tris-HCl, pH 8.8, buffer). Samples were mixed with 2× loading buffer (50 mM Tris-HCl, pH 6.8, 40% glycerol, 3% bromophenol blue, ±6% β-ME). Samples with β-ME were incubated for 1 h at 30 °C for complete reduction of the disulfide bonds. The electrophoresis buffer was 50 mM Tris-HCl and 0.384 M glycine. Migration, staining, and destaining were carried out under the same conditions as for SDS−PAGE.

Circular Dichroism. CD spectra were measured using a CD6 dichrograph and recorded with the CDmax software (Jobin Yvon, Longjumeau, France).

Protein samples were prepared at 1 mg/mL in 10 mM sodium phosphate, pH 6.7, or in 10 mM glycine hydrochloride, pH 2. Near-UV (250−320 nm) and far-UV (185−260 nm) spectra were measured in a 5 mm path length cylindrical cell and in an 0.1 mm path length square cell, respectively. Eight spectra were accumulated with 1 nm steps and 1 s integration time. The spectra were corrected by subtracting the buffer spectrum before calculating the molar ellipticity. For all experiments, the BLG concentration was determined spectrophotometrically assuming $\epsilon_{278} = 17600\ M^{-1}\ cm^{-1}$.

Heat Treatment of the BLG. Proteins were prepared at 1 mg/mL in 50 mM sodium phosphate, pH 7.0, submitted to heating at 90 °C for 10 min using a cover-heating thermocycler, and then cooled at 20 °C for 1 h. Samples were loaded on GP-HPLC and prepared as described above for electrophoresis.

Effect of Temperature on the Near-UV CD Spectrum of the BLG. The protein was prepared at 1 mg/mL in 10 mM sodium phosphate, pH 6.7. The cell was heated from 20 to 90 °C in increasing steps of 10 °C. The sample was equilibrated for 3 min at each temperature, and five spectra were accumulated. After the spectra were recorded at 90 °C, the sample was cooled to 20 °C and equilibrated for 30 min before remeasuring the spectrum at 20 °C.

Peptic Digestion. Protein samples were prepared at 2 mg/mL in 20 mM citrate−phosphate, pH 2.8. The enzyme was porcine pepsin from Sigma (St. Louis, MO). Digestion was performed at 37 °C with 2% enzyme/substrate ratio (w/w). Aliquots were taken after 0, 2, 6, and 24 h, and the reaction was stopped by addition of 0.2 M Tris-HCl, pH 8.0 (125 μL/100 μL reaction). The extent of digestion was then determined by RP-HPLC.

ANS Fluorescence Spectroscopy. Fluorescence experiments were carried out in triplicate at 20 °C using an Aminco SLM 4800C spectrofluorometer (Thermo Spectronic, Rochester, NY) in the ratio mode. Protein samples were prepared at 7 μM in H_2O−HCl, pH 7.0 or 2.0. ANS was prepared at 40 μM in dimethylformamide (DMF) buffer; the ANS concentration was measured spectrophotometrically assuming $\epsilon_{350} = 5000\ M^{-1}\ cm^{-1}$. Excitation was set at 390 nm, and emission was measured at 485 nm (slits, 4 nm). ANS fluorescence upon binding to BLG was measured at a ligand/protein ratio of 100 in order to saturate all of the binding sites (*25*, *26*).

Protein Crystallization and X-ray Structure Determination. Protein crystals of the orthorhombic lattice Y form (*1*) were grown from approximately 2 M ammonium sulfate solution, pH 7.6, by the hanging drop technique. Drops contained 5 μL of well solution and an equal volume of 20 mg/mL protein in 10 mM HEPES, pH 7.5. Crystals were cryoprotected by transfer to microscope immersion oil (Cargille B) for a few seconds and then flash frozen by rapid immersion into liquid N_2. Data were collected on station 14.1 of the SRS at the CLRC Daresbury Laboratory in two passes. A high-resolution data set (1.95 Å resolution) was collected as 60 images of 1.5° rotation and 60 s exposure on the ADSC CCD detector followed by 51 images of 3 s exposure covering the same rotation range. The wavelength of the radiation was 1.488 Å, and the crystal to detector distance was 70 mm in both cases. Processing of the images was done with MOSFLM and SCALA (*27*). The unit cell dimensions were $a = 55.69$ Å, $b = 80.47$ Å, and $c = 66.59$ Å, and the space group was $C222_1$ with 1 monomer in the asymmetric unit. A total of 212945 observations resulted in 11241 reflections being observed between 46 and 1.95 Å resolution with an $R_{merge} = 0.076$ (0.243 in the highest resolution shell). The data set was 99.5% complete with an overall multiplicity of 5.7 and an $I/\sigma(I)$ of 5.7 (2.6). The overall temperature factor was 26.29 Å^2.

The PDB data set 1qg5 (*28*) was used as a molecular replacement model in the program CNS (*29*), which produced a clear single solution, rigid body refinement of which provided a starting point for refinement. Ten cycles of least-squares refinement produced an *R*-factor of 0.31 ($R_{free} = 0.34$ for 5% of the reflections randomly removed from the data set). A SIGMAA weighted map (*30*) calculated at this stage showed clearly that Ser121 was essentially identically positioned to that of the Cys121 in the native protein and that the core of the protein was, as expected, unaltered. Refinement continued with alternating cycles of REFMAC (*31*) and manual model building with O (*32*) until no further improvement could be achieved. A total of 1325 atoms (residues 1−158), including 70 water molecules, were refined against 10574 (783) reflections in the resolution range between 45.6 and 1.95 (2.00−1.95) Å. The final $R = 0.211$ (0.231) with the $R_{free} = 0.267$ (0.290). The rms bond and angle deviations were 0.025 Å and 2.06°, respectively, with an average *B*-factor of 35.6 Å^2. The coordinates have been deposited in the PDB with accession code 1UZ2 together with the structure factors, code R1UZ2SF. Figures were

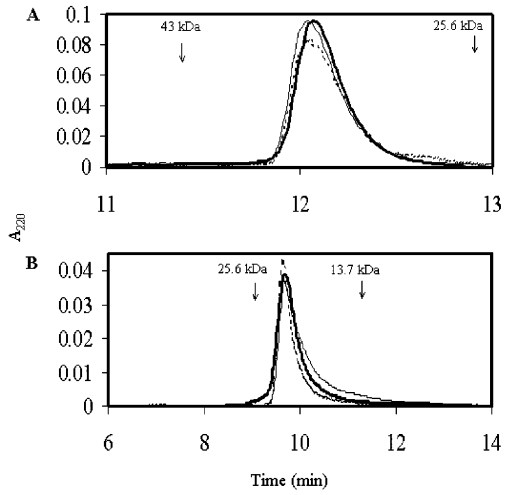

FIGURE 1: Gel permeation HPLC (TSK G3000 SKWL, 300 × 7.8 mm i.d.) of rBLGs eluted with 50 mM sodium phosphate and 0.1 M NaCl, pH 7.0 (A) or 10 mM glycine hydrochloride, pH 2.0 (B). The flow rate was 0.8 mL/min. Dashed line: standard BLG. Solid line: WT-rBLG. Thick line: C121S mutant. Standards were ribonuclease A (13.7 kDa), chymotrypsinogen (25.6 kDa), and ovalbumin (43 kDa).

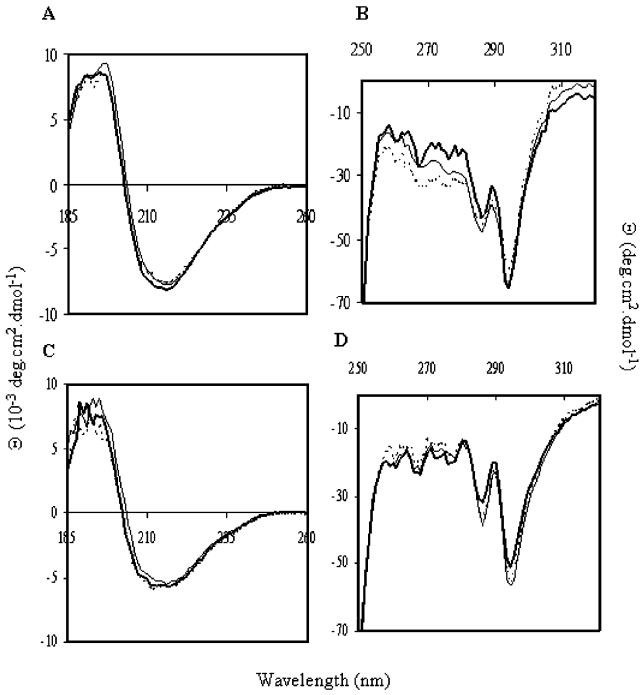

FIGURE 2: Circular dichroism of rBLGs at pH 6.7 (up) and pH 2.0 (down) in the far-UV (A, C) and near-UV (B, D). Dashed line: standard BLG. Solid line: WT-rBLG. Thick line: C121S mutant. Buffer solutions were 10 mM sodium phosphate, pH 6.7, and 10 mM glycine hydrochloride, pH 2.0.

produced by BOBSCRIPT (*33*, *34*) and rendered with RASTER3D (*35*).

RESULTS

Production of Recombinant Proteins. WT and C121S recombinant BLGs (rBLGs) were produced and purified as described in the Materials and Methods section. Our protocol allowed the proteins to be expressed at a concentration of 100 mg/L of culture medium, which is in agreement with previously described yields for recombinant BLG production in shake flasks (*22*, *36*). The yield of purified protein was 70 mg/L of culture medium.

Structural Characterization. The identity of the proteins was confirmed by N-terminal sequencing and mass spectrometry (data not shown). Titration of free SH groups with DTNB showed one cysteine per monomer in WT and none in C121S.

(A) Structure at Neutral pH. The quaternary structure of the rBLGs was measured by GP-HPLC at pH 7.0 (Figure 1A). Both WT and C121S eluted as a single peak at almost the same time as standard BLG, corresponding to a molecular mass of 36 kDa. This result indicated that both WT and C121S were dimeric under the conditions used (1 mg/mL, 0.1 M NaCl, pH 7.0).

The secondary structure of the rBLGs was analyzed by far-UV CD. The spectra of WT and C121S were identical to that of the standard (Figure 2A), showing that the rBLGs had the same secondary structure as native BLG. The near-UV CD spectrum of WT was also very similar to that of the standard (Figure 2B), indicating a similar tertiary structure. The C121S mutant spectrum showed only small differences compared to the WT spectrum in the 260−280 nm region, showing that the mutation did not affect the BLG tertiary structure to any large extent.

The fluorescence emission spectra of the WT and the C121S mutant were very similar to that of the native protein (data not shown), suggesting that the hydrophobic environment of W19, the main BLG fluorophore (*37*), is conserved in both rBLGs. Thus, removing the free thiol group from BLG has no obvious effect on the protein structure at neutral pH and does not impede correct formation of the two native disulfide bonds.

(B) Structure at Acid pH. The BLG structure is resistant to acid conditions. At pH <3, low protein concentration, and low ionic strength, the dimer ↔ monomer equilibrium is strongly shifted toward the monomer form (*38*), and BLG adopts a somewhat altered but still globular conformation (*39*, *40*). The ability of rBLGs to maintain their tertiary structure at pH 2.0 was checked by GP-HPLC (Figure 1B) and CD (Figure 2C,D). Both rBLGs had the same retention time as the standard BLG in GP-HPLC, indicating that they were in the monomeric form at acid pH, with the same apparent size as native BLG. The spectra of both rBLGs in the far-UV (Figure 2C) and near-UV (Figure 2D) were superimposable on those of the standard. These results indicate that, under the conditions used here, both rBLGs were monomeric and had similar structures to native BLG at acid pH.

Heat Treatment of rBLG. The effect of the C121S mutation on the irreversible denaturation and aggregation of BLG induced by the heat treatment at 90 °C was studied. Changes to the proteins were followed by native PAGE and SDS−PAGE (Figure 3) and GP-HPLC (results not shown).

The heat-treated WT native PAGE profile was identical to that of the similarly treated standard (Figure 3A, lanes 2 and 4). Manderson et al. (*8*) showed that this profile is composed of native monomers (zone a, here composed of variants A and B in the standard), larger aggregates (dimers, trimers, and larger, zone c), and intermediate forms (zone b). These intermediates are monomers with various non-native disulfide bonds, among which is C106−C121, arising from thiol−disulfide exchange, leaving C119 as the free thiol

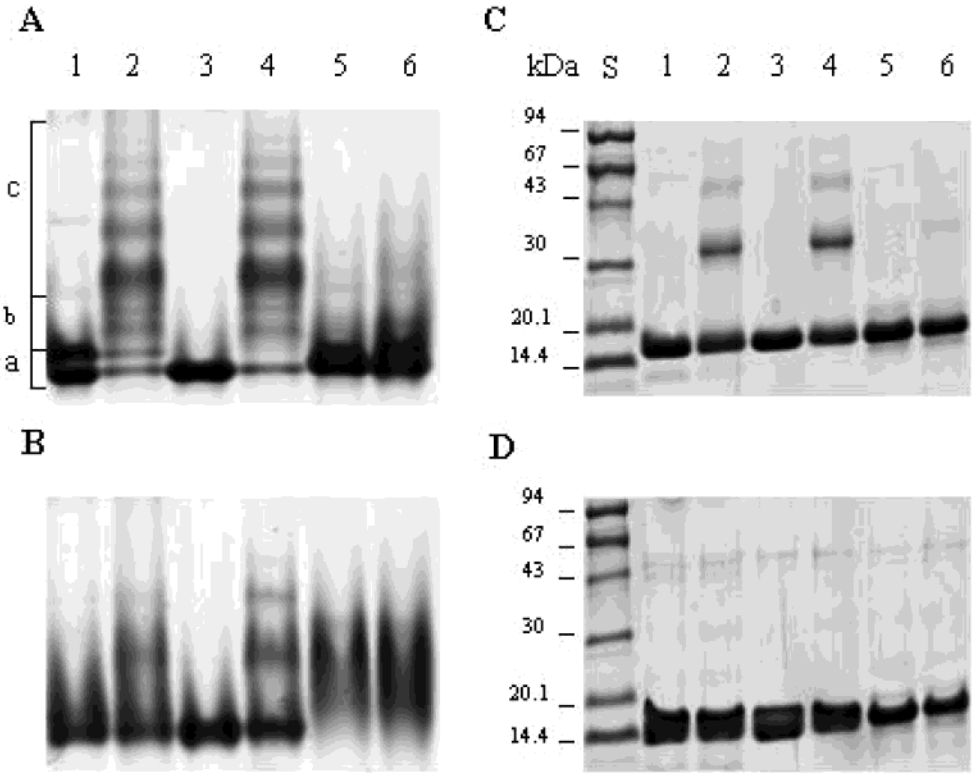

FIGURE 3: Electrophoresis of nonheated and heated rBLGs. Left: Alkaline PAGE without (A) or with (B) β-mercaptoethanol. Right: SDS−PAGE without (C) or with (D) β-mercaptoethanol. Lanes: 1, standard BLG; 2, heated standard BLG; 3, WT-rBLG; 4, heated WT-rBLG; 5, C121S; 6, heated C121S; S, molecular mass standards. For an explanation of zones a, b, and c, see main text.

(*8*, *41*). They migrate between the monomer and dimer forms, because partial unfolding of the protein occurs simultaneously with thiol−disulfide interchange (*41*, *42*).

The heat-treated C121S showed the same profile as the nonheated protein (Figure 3A, lanes 5 and 6), without bands corresponding to protein aggregates or to denaturation intermediates. This result was confirmed by the GP-HPLC profiles, which also showed that no aggregation had occurred (data not shown). Thus, the absence of a cysteine completely prevented BLG from undergoing irreversible heat-induced aggregation and thiol−disulfide exchange. Formation of covalent and noncovalent aggregates, as well as of denaturation intermediates, was inhibited, as previously demonstrated in the case of chemically blocked C121 (*4*, *5*, *10*).

Addition of 6% β-ME to the samples before native PAGE eliminated most bands in the heat-treated standard and WT profiles (Figure 3B, lanes 2 and 4), indicating that most of the aggregates observed in Figure 3A were linked by disulfide bonds. The remaining bands may represent aggregates stabilized by strong hydrophobic interactions. The disappearance of these aggregate bands, as well as the two bands corresponding to variants A and B in the standard (Figure 3A,B, lane 1), indicates that β-ME allowed almost complete reduction of the proteins.

In the presence of β-ME, both unheated and heated C121S showed a large and diffuse band (Figure 3B, lanes 5 and 6). In contrast, both standard and WT migrated as a single band corresponding to the reduced monomer (Figure 3B, lanes 1 and 3). This indicates that in the presence of a reducing agent the C121S mutant unfolded during the electrophoresis.

Heat-treated standard and WT SDS−PAGE profiles (Figure 3C, lanes 2 and 4) clearly showed the presence of intermolecular disulfide bonds, leading to the formation of covalently linked dimers, trimers, and tetramers of 36, 54, and 72 kDa, respectively. The heat-treated C121S profile was free of such aggregates (Figure 3C, lane 6). Most of the aggregates disappeared following addition of SDS to the samples (Figure 3A,C), and the further addition of β-ME led to the disappearance of the remaining aggregate bands (Figure 3C,D). Thus, the irreversible heat-induced aggregation of the native BLG, under the conditions used here, is only due to intermonomer disulfide bonding.

Reversibility of Unfolding after Heat Treatment. The reversibility of rBLG denaturation was also investigated by measuring their near-UV CD spectra before and after heat treatment (Figure 4). The CD minima at 285 and 293 nm, as well as all other tertiary structure signals in the spectra of the unheated WT (Figure 4, dashed lines) and standard BLG (not shown) disappeared after the heat treatment, showing that the loss of structure was irreversible. In contrast, after heating at 90 °C and cooling, C121S still showed the typical CD spectrum of the native BLG, with minima at 285 and 293 nm. Thus, the heat-induced denaturation of BLG appears to become almost completely reversible in the absence of a free thiol moiety.

Effect of Temperature on the Tertiary Structure. The change of ellipticity at 293 nm was measured as a function of temperature (Figure 5). Both standard and WT showed the same behavior and were stable until almost 60 °C. Both proteins lost 50% of their ellipticity at 293 nm at around 77 °C. This result is in agreement with those of Qi et al. (*43*), who showed that BLG unfolding occurs mostly above 65 °C. In the case of C121S, the 50% ellipticity loss was reached at only 58 °C, nearly 20 °C below the temperature observed

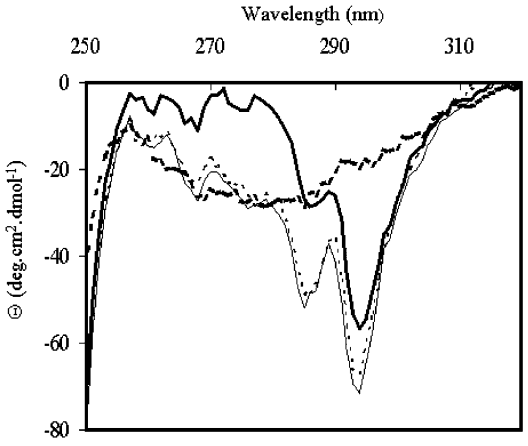

FIGURE 4: Circular dichroism spectra of nonheated and heated (90 °C, 10 min) rBLGs in 10 mM sodium phosphate, pH 6.7, in the near-UV. Dashed line: nonheated (thin) or heated (thick) WT-rBLG. Solid line: nonheated (thin) or heated (thick) C121S mutant.

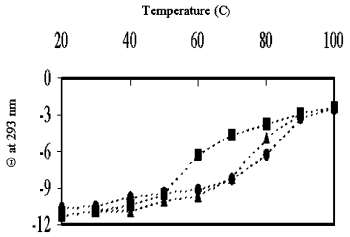

FIGURE 5: Ellipticity at 293 nm of the rBLGs as a function of temperature. Triangles: standard BLG. Circles: WT-rBLG. Squares: C121S mutant. Buffer solution was 10 mM sodium phosphate, pH 6.7.

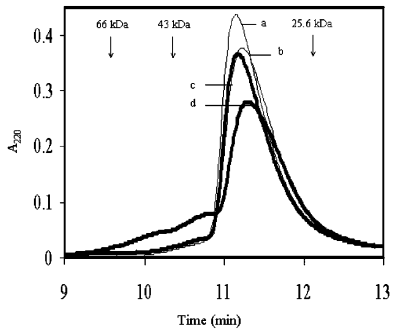

FIGURE 6: Gel permeation HPLC (TSK G3000 SKWL, 300 × 7.8 mm i.d.) of rBLGs before and after incubation in 10 mM sodium phosphate and 5 mM dithiotreitol, pH 6.7, for 90 min. Elution was performed with 50 mM sodium phosphate and 0.1 M NaCl, pH 7.0, at a flow rate of 0.8 mL/min. Solid line: a, WT-rBLG − DTT, and b, WT-rBLG + DTT. Thick line: c, C121S mutant − DTT, and d, C121S + DTT. Standards were chymotrypsinogen (25.6 kDa), ovalbumin (43 kDa), and bovine serum albumin (66 kDa).

with the WT and standard, giving evidence that the C121S mutant is less stable to heating than the native proteins.

Effect of Reduction with Dithiothreitol. The effect of the reduction of the disulfide bonds on C121S stability was further investigated by incubating the rBLGs (1 mg/mL) at 30 °C for 90 min in the presence of a 10-fold molar excess of dithiothreitol (DTT) and submitted to near-UV CD (not shown) and GP-HPLC (Figure 6). After incubation with DTT, WT had the same CD spectrum as the native form, whereas a slight loss of signal was observed with the C121S mutant, indicating some loss of tertiary structure (data not shown). Whereas WT shows no difference in GP-HPLC

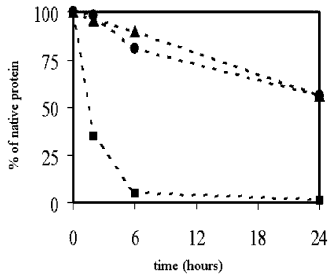

FIGURE 7: Disappearance in time of standard and recombinant BLG during proteolysis with pepsin. Triangles: standard BLG. Circles: WT-rBLG. Squares: C121S mutant.

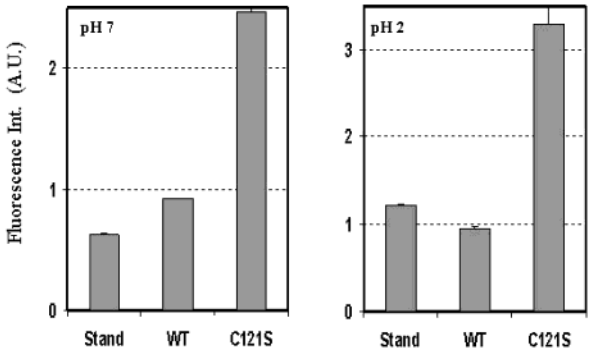

FIGURE 8: Fluorescence intensity for ANS−rBLG complexes at pH 7.0 and 2.0. Stand: standard BLG. Excitation and emission wavelengths were set at 390 and 485 nm, respectively. Solution buffers were H_2O−HCl, pH 7.0 or 2.0. ANS was prepared in dimethylformamide (DMF).

elution time after reduction, C121S has a more complex profile, exhibiting a gradual baseline increase and a slightly increased retention time of the dimer peak (Figure 6). This profile fits well with the diffuse pattern observed in electrophoresis (Figure 3B) and confirms that the C121S mutant is less stable than the WT after reduction.

Effect of Peptic Digestion. Due to its stable structure at acid pH, BLG is resistant to pepsin, a protease secreted in the stomach and active at pH 2 (*44*). The rBLGs were therefore submitted to pepsinolysis, and the extent of reaction was followed by RP-HPLC (Figure 7). Both the native and the WT BLG are resistant to pepsin: more than 80% of the protein is undigested after 6 h incubation (Figure 7). In contrast, only 35% of the C121S mutant is still undigested after 2 h, and the protein has almost completely disappeared after 6 h (Figure 7). This clearly shows that, despite maintaining its structure at acid pH, the C121S mutant BLG is highly susceptible to pepsinolysis.

ANS Fluorescence. ANS fluorescence enhancement was measured upon binding to BLG, at pH 7.0 and 2.0 (Figure 8). ANS is a probe showing an increase of fluorescence when bound to hydrophobic patches of proteins. At both neutral and acid pH, ANS fluorescence has the same intensity for standard and WT, indicating a similar surface hydrophobicity in these proteins. In contrast, ANS fluorescence upon binding to the C121S mutant is 2.5 and 3 times higher at neutral and acid pH, respectively. This clearly shows that the C121S mutant has an increased surface hydrophobicity as monitored by ANS fluorescence. This result demonstrates that the C121S mutant has a more mobile structure, in which the hydrophobic sites are more accessible to ANS.

Crystal Structure. The crystal structure of the C121S mutant protein (Figure 9) reveals little difference from the

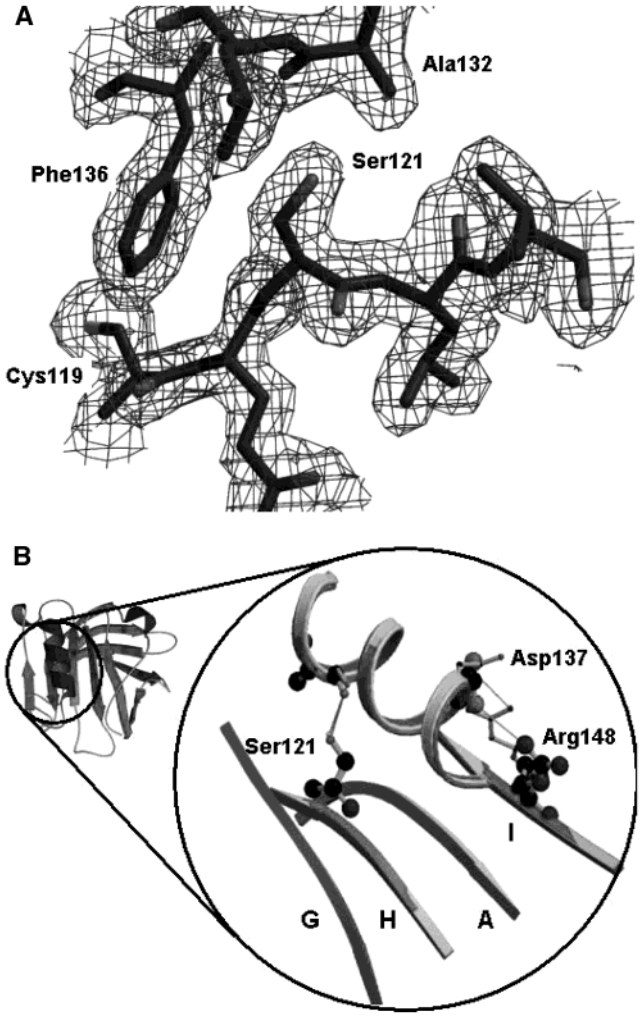

FIGURE 9: Refined crystal structure of the C121S mutant of BLG. (A) Final $2|F_o| - |F_c|$ electron density map around mutated residue 121. The map is contoured at 1.0σ and was drawn with the program BOBSCRIPT (*33*, *34*). (B) Superposition of the native A-variant of BLG (1qg5) and the C121S mutant showing the interactions between residue 121, the α-helix, and β-strand I. The superposition was performed in the CCP4 program suite using the CA atoms of strands F, G, and H. The rms deviation was 0.115 Å. Black sticks indicate the native BLG, balls and sticks show the C121S BLG, except for the O of Ala132 which is drawn so as not to hide the carbonyl of the native structure. Notice the slight movement of the helix and the I strand and the significant repositioning of the Arg148 side chain.

native protein (and indeed the WT-rBLG; data not shown). The reduction in the atomic number lowers the electron density somewhat, and the shortening of the Cβ−Oγ bond is entirely consistent with the expected seryl residue. Refinement proceeded in a straightforward manner with the same, highly mobile sections observed in previous work remaining indistinct in the current structure: the sections at 61−66, 86−88, and 111−115 and the C-terminal section from 153 (*3*, *28*, *45*). However, only the last four residues are omitted from the final model. A pairwise superposition of the CA atoms of strands F, G, and H of the four A-variant structures obtained at pH values above 7 (1bsy, 2blg, 1qg5, and the present data) gave an rms deviation of 0.279 ± 0.090 Å. The value for the three comparisons of the mutant data was 0.254 ± 0.122 Å. It is clear that there are significant changes at the dimer interface of the mutant structure (Figure 9B), but it is not obvious that these arise only as a result of the mutation. However, the burying of the more polar seryl OG in place of the cysteinyl SG has been cited as the basis of the observed destabilization (*20*).

DISCUSSION

Effect of the C121S Mutation on the Conformation of the Protein. Our results show that mutation of the BLG Cys121 into Ser affects neither the BLG structure nor the equilibrium between its monomer and dimer forms to a significant extent, confirming the results from Yagi et al. (*20*). However, these results contrast with previous studies that showed that chemical blocking of C121 perturbed the BLG fold (*12*−*14*). The absence of structural modifications observed in the present study and in Yagi et al. (*20*) with a genetically modified BLG strongly suggests that most of the perturbations previously observed should be interpreted as a consequence of the denaturing conditions used during the chemical treatment and/or to the bulkiness of the blocking group, rather than to a specific role for C121 in maintaining the protein fold.

Disulfide Interchange during Denaturation. The absence of the free C121 in BLG completely eliminates the irreversible heat-induced aggregation, confirming the central role played by C121 in the early steps of BLG denaturation and polymerization (*10*, *46*−*48*).

Previous studies have pointed out that, during denaturation induced by heat (*8*, *41*), urea (*49*), Gu-HCl (*50*), or high pressure (*51*), BLG unfolds via a multistep process, with formation of a stable intermediate, containing, in some cases, non-native disulfide bonds (*41*, *51*). Thus, C121 might be expected to play an important role in many, if not all, of these BLG denaturation pathways. The availability of a mutation at this residue will allow detailed studies on the role of the thiol−disulfide exchange mechanism during BLG unfolding.

Stability of the C121S Mutant. We have shown that the C121S mutant BLG is less resistant to denaturation by heating and reducing agents and is highly sensitive to proteolysis by pepsin. These results confirm and extend what was reported previously by Yagi et al. (*20*) on the urea-induced denaturation of BLG mutants. The CD, peptic digestion, and ANS binding experiments presented in our study are consistent with results from Dalgalarrondo et al. (*52*) which showed that in 25% aqueous ethanol BLG had little structural change but was more sensitive to pepsinolysis. This was interpreted as a consequence of the increased exposure of BLG hydrophobic regions to the hydroethanolic solvent. In light of our results, we suggest that the increased exposure of hydrophobic regions of the C121S mutant is better considered as a shift in the equilibrium of the folded protein structure toward a more mobile and, hence, more accessible state.

C121 lies on the outer surface of the β-barrel, with its side chain in the interface between the barrel and the α-helix (*3*). This interface is hydrophobic, stabilizing the interactions of the helix and the barrel (*39*). As shown in Figures 9 and 10, C121 is central to this hydrophobic patch and is occluded by the α-helix. There is a long hydrogen bond (3.01 Å) between the serine side chain and the carbonyl O of residue 132 in the first turn of the α-helix. The helix in thereby subtly repositioned relative to β-strands F, G, and H, and this in

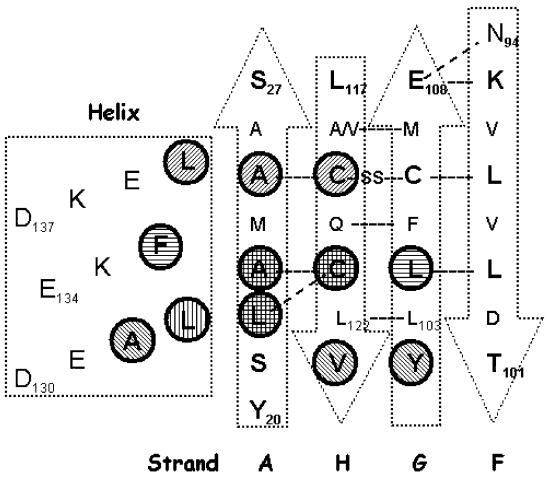

FIGURE 10: Representation of the helix−sheet interface opened out to show the hydrophobic interactions involving residues on the α-helix and β-strands A, H, G, and F on the outer surface of the β-barrel. Large letters represent side chains on the outer surface with the hydrophobic ones in bold; the smaller capitals represent residues with side chains on the inner surface of the barrel. Circled residues are within 4 Å of those similarly shaded. Thus atoms in F136 on the helix are close to atoms in A23, C121, and L104; L133 is close to L22, A23, and C121; and A132 approaches Y102, C121, and V123. Notice that the side chain of L22 is also able to interact with the helix because of the severe bend in strand A at that point to allow the strand to participate in both the lower and the upper β-sheets. Dashed lines indicate sites of main chain H-bonding. It is clear that C121 is buried at the center of a significant, hydrophobic patch under the helix on the protein surface.

turn appears to result in a repositioning of the side chains of Asp137 and Arg148, the latter residue being central to β-strand I in the dimer contact. This repositioning is only observed in the C121S BLG structure reported here. Thus, the C121S mutation may be expected to lead to subtle perturbations of the neighborhood of C121, particularly this helix−barrel interface, that are transmitted to the dimer interface. However, the OG−O distance above compares with 3.62 Å for the SG−O distance in 1qg5, which is in the same crystal form as the C121S mutant BLG; the value rises to ∼4 Å in the trigonal crystal structures 1bsy and 2blg, indicating that crystal packing forces are also involved.

Previous studies on BLG reacted with mercaptopropionic acid have shown that thiol blocking had a destabilizing effect that was interpreted as the consequence of the loss of water-mediated H-bonds (*13*). However, examination of the BLG structure reveals that C121 is inaccessible to H_2O between pH 6.2 and pH 8.0 (*3*, *45*). Further, examination of all of the deposited coordinate sets reveals no ordered H_2O closer than 7.5 Å from the sulfhydryl group of C121.

Our hypothesis is that the C121S mutant is less stable than the native protein because a seryl residue prefers the hydrophilic environment of an unfolded structure to a greater extent than does a cysteinyl residue. This is the essence of the hydrophobic effect whereby the folding of a protein is driven, to a certain degree, by the desolvation of residues that then form the hydrophobic core in the globular, folded state. An indication of the relative preference of cysteinyl over seryl residues for the hydrophobic interior can be gained from the partition coefficients of ethanol and ethanethiol between octanol and water. For ethanol the partition coefficient is 2.29, and for ethanethiol it is 70.31. In terms of free energy difference, the preference amounts to 8.62 kJ/mol (calculated from the web site http://www.pirika.com/chem/TCPEE/LOGKOW/ourlogKow.htm). The experimental transfer free energies of methanol (−14.6 kJ/mol) and methanethiol (5.2 kJ/mol) from cyclohexane to water also reflect this same trend (*53*).

Weaker stabilization of the helix alongside the barrel induced by the perturbation of this hydrophobic effect may drive the protein folding equilibrium toward a partially unfolded structure, leading to the decreased stability observed in our experiments. The urea denaturation experiments of Yagi et al. (*20*) have shown that the C121S, C121V, and C121A mutants of BLG are destabilized to different degrees relative to the native protein. The greatest perturbation arises from the introduction of the more polar Ser. The extra bulk of Val is somewhat less destabilizing, while the "hole" introduced by Ala causes only a small reduction in stability relative to the native BLG. These results support our hypothesis that the destabilizing effect of C121S mutation in BLG is mainly due to the perturbation of the hydrophobic effect in the helix−barrel interface.

BLG Folding Pathway. The presence of a stable intermediate containing non-native α-helix in the folding pathway of BLG is well documented (*54*−*59*), as well as similarities between the unfolding and folding pathways of BLG. In particular, the hydrophobic core made of F, G, and H strands has been shown to be the first to fold and the last to unfold (*58*, *60*−*62*). Moreover, non-native SS bridges are known to be often involved in globular proteins folding pathways (*55*, *63*−*65*), and BLG can unfold via formation of an intermediate with a non-native disulfide bridge involving C121 (*8*, *41*). It is thus tempting to speculate that a similar intermediate may also be formed during BLG folding. As the crystal structure of the C121S mutant is closely similar to that of the native protein, the mutant protein is clearly able to fold correctly. Thus, any requirement in the folding pathway for the formation of a non-native C106−C121 disulfide bridge cannot be absolute, but its absence may contribute to the destabilizing effect of the C121S mutation of BLG.

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