Two Stable Unfolding Intermediates of the Disease-Causing L68Q Variant of Human Cystatin C[†]

Bernd Gerhartz,[‡] Irena Ekiel,[§] and Magnus Abrahamson*,[‡]

Department of Clinical Chemistry, Institute of Laboratory Medicine, University of Lund, University Hospital, S-221 85 Lund, Sweden, and Biomolecular NMR Group, Pharmaceutical Biotechnology Sector, Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montreal, Quebec, H4P 2R2, Canada

Received April 17, 1998; Revised Manuscript Received August 25, 1998

ABSTRACT: In hereditary cystatin C amyloid angiopathy (HCCAA), presence of the Leu68 → Gln substitution in cystatin C is coupled to a decreased concentration of this major cysteine proteinase inhibitor in cerebrospinal fluid and leads to its amyloid deposition in the brain. We established a high-yield expression system for L68Q cystatin C in *Escherichia coli* resulting in inclusion body accumulation at a level of 40% of the total cellular protein. Refolding of protein from purified inclusion bodies yielded a pure, almost completely monomeric and active inhibitor. CD and NMR spectroscopy demonstrated that so produced L68Q cystatin C is folded, conformationally homogeneous, and structurally very similar to wild-type cystatin C. Incubation at pH 7.0−5.5 caused the cystatin C variant to dimerize rapidly. The molecular form present at pH 6.0 displayed a slightly increased amount of hydrophobic parts on the surface as measured by 1-anilinonaphthalene-8-sulfonic acid (ANS) binding. NMR results showed that the dimer has a structure similar to that of the wild-type cystatin C dimer formed as a result of slight denaturation. Under more acidic conditions, at pH 4.5, another stable unfolding intermediate of L68Q cystatin C was identified. This molecular form exists in a monomeric state, is characterized by changes in secondary structure according to far UV CD spectroscopy, and shows an altered ANS binding resembling that of a molten globule state. The acidic pH also caused an almost complete monomerization of preformed dimers. The state of denaturation of L68Q cystatin C in vivo is thus a critical factor for the concentration of active cysteine proteinase inhibitor in cerebrospinal fluid and likely also for the development of amyloidosis, in HCCAA patients.

Aggregation of soluble, monomeric proteins into insoluble amyloid fibrils is associated with a number of serious disorders such as Alzheimer's disease and prion-related diseases (*1*, *2*). In hereditary cystatin C amyloid angiopathy (HCCAA),[1] a disease leading to massive brain hemorrhages in young adults (*3*), the amyloid fibril-forming protein is a L68Q variant of cystatin C (*4*). Like other amyloid plaques, L68Q cystatin C plaques can be stained by Congo Red and give a characteristic green birefringence under polarized light (*5*). In patients expressing the L68Q variant, amyloid plaques have been found mainly in brain vessels, but also in lymph nodes, spleen, salivary glands, seminal vesicles, and skin (*5*−*7*). In contrast to the L68Q variant, cystatin C in its wild-type form has not been reported to form amyloid in vivo.

Cystatin C is a small cysteine proteinase inhibitor (120 amino acids) present in all human body fluids at physiologically relevant concentrations (*8*). The physiological role of cystatin C is likely to regulate extracellular cysteine proteinase activity, which results from microbial invasion (*9*) or release of lysosomal proteinases from dying or diseased cells (*10*−*12*). In HCCAA patients, the cerebrospinal fluid concentration of cystatin C is drastically decreased compared to healthy individuals (*5*, *13*). Injection of chymopapain, a cysteine proteinase, in cerebrospinal fluid (*14*) or in the subarachnoid space (*15*) has been reported to cause cerebral hemorrhage, strongly indicating a physiological role of the balance between cysteine proteinases and their major extracellular inhibitor, cystatin C, in the brain. The mechanism behind the brain hemorrhages in HCCAA thus still remains unclear, as both the presence of amyloid fibrils and the low levels of cystatin C in cerebrospinal fluid can be causing events.

In addition to amyloid fibril formation leading to decreased total levels of soluble inhibitor, a low level of functional L68Q cystatin C in the cerebrospinal fluid of HCCAA patients can be caused by dimerisation according to previous in vitro results (*16*). In contrast to the L68Q variant, wild-

[†] The present study was supported by a grant from the European Commission Training and Mobility of Researchers Program (ERB4001GT953156), by the Swedish Medical Research Council (Project 09915), and by the Canadian National Research Council (no. 41466).

* Author to whom correspondence should be addressed.

[‡] University of Lund.

[§] Biotechnology Research Institute.

[1] Abbreviations: PBS, phosphate-buffered saline (10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl); IPTG, isopropyl-β-D-thiogalactopyranoside; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; Z, *N*-α-carbobenzoxy; NHMec, 7-amido-4-methylcoumarin; SEC, size-exclusion chromatography; HSQC, heteronuclear single quantum coherence spectroscopy; HCCAA, hereditary cystatin C amyloid angiopathy; NMR, nuclear magnetic resonance; CD, circular dichroism, ANS, 8-anilino-1-naphthalene-sulfonic acid.

10.1021/bi980873u CCC: $15.00
Published on Web 11/18/1998

type cystatin C does not dimerize under physiological conditions. Nevertheless, it has been shown that mildly denaturing conditions cause dimerization of the wild-type protein (*17*). Two different structural models of this dimer have been proposed (*18*). In one model, two cystatin molecules associate directly via hydrophobic loops. In the other model, there is a crossing over of β-strands from one monomer to the other. Both models have in common that the structural parts needed for cysteine proteinase inhibition are no longer exposed in the dimer. Similar associations may occur for the amyloidogenic L68Q cystatin C, the dimer of which is also inhibitory inactive (*17*) but further investigations have so far been limited by insufficient expression systems.

In the present study, we have developed a new expression system yielding larger amounts of L68Q cystatin C. This enabled a detailed characterization of the properties of L68Q cystatin C with the aim to define the molecular background to HCCAA. We have thereby identified two stable intermediates of the variant on the path toward total denaturation by lowering pH, which are different in the degree of unfolding and tendency to dimerize.

EXPERIMENTAL PROCEDURES

Expression of L68Q Cystatin C. Coding sequences for wild-type and L68Q cystatin C were cloned into pET3a (Novagen) that had been cleaved with *Nde*I/*Bam*HI (Gibco) to remove the N-tag encoding sequence. Plasmids pHD313 and pCmutH5 (*16*, *19*) containing cystatin C encoding sequences (wild-type and L68Q cystatin C, respectively) were used as templates to produce PCR-derived inserts. The upstream primers contained besides a *Nde*I cleavage site (which includes a start ATG codon) either the beginning of the OmpA signal sequence (5′-GCCATATGAAAAAAACT-GCTATCGCTA-3′) for periplasmatic expression or the beginning of the native cystatin C sequence (5′-TGCATAT-GTCTTCTCCGGGTAAACCG-3′) for cytoplasmatic expression. The downstream primer, 5′-TAGGATCCAGG-GGTGGGAATACAG-3′, with a tailing *Bam*HI cleavage site, was directed to a segment 60 basepairs after the stop codon. Plasmid DNA from clones of transformed *E. coli* JM109 (pET3a-cystatin C, pET3a-L68Q cystatin C, pET3a-OmpA-cystatin C, pET3a-OmpA-L68Q cystatin C) was isolated, verified, and transformed into *E. coli* BL21(DE3) (Novagen). DNA sequencing was used to verify the correct coding sequence (ABI Prism 310 Genetic Analyzer, and reagents from Perkin-Elmer). The expected N-terminal amino acid sequences of the expressed recombinant proteins were confirmed using an Applied Biosystems 477A sequencer (*20*).

For cystatin production, cultures of the BL21(DE3) clones were grown in LB medium with ampicillin at 33 °C, induced with 0.5 mM IPTG for 4 h (at $OD_{555} = 0.6$), and harvested by centrifugation. Periplasmic cystatin expression in isolated bacterial subclones was analyzed by extraction of the periplasmic fraction from 10 mL cultures (*21*). Cytoplasmatic expression was monitored by analyzing total cell protein, soluble protein, and insoluble protein by 15% SDS−PAGE. To assess total cell protein, the bacteria were heated (100 °C, 10 min) in SDS sample buffer [containing 2% (w/v) SDS; (*22*)]. The soluble protein fraction was obtained after sonification of the cells, followed by centrifugation to collect the supernatant. The remaining pellet was heated (100 °C, 10 min) in SDS sample buffer to obtain the insoluble protein fraction.

Isolation and Refolding of Recombinant Cystatins. Purified inclusion bodies (*23*) from 1 L cultures of expression-induced bacteria were resolved in 5 mL of 6 M guadinium hydrochloride. The protein was refolded by SEC (Superdex 75 HR 10/30; Pharmacia Biotech) in 50 mM ammonium bicarbonate, pH 7.8, as described earlier (*16*).

Protein Analyses. Analytical electrophoreses in agarose and SDS−polyacrylamide gels were performed as described by Jeppsson et al. (*24*) and Laemmli (*22*) with separation gels containing 15% acrylamide. Quantitative densitometric scanning of analytical gel electropherograms was performed using a Bio-Rad scanning system. The concentration of cystatin C was determined by A_{280} measurements [$\epsilon_{280} = 0.83$ l g^{-1} cm^{-1}; (*25*)]. The biological activity of refolded cystatin C preparations was controlled in activity assays against papain (EC 3.4.22.2) as described earlier (*16*). Papain was purified from the Sigma type III preparation by affinity chromatography using Gly-Gly-Tyr-Arg−Sepharose (*26*). Inhibition of cathepsin B (EC 3.4.22.1) (bovine; Sigma) by cystatin C was measured in enzyme assays with Z-Arg-NHMec (Bachem) as substrate at pH 6.5 (100 mM phosphate buffer, containing 1 mM DTT and 1 mM EDTA) or pH 4.5 (100 mM acetate buffer, containing 1 mM DTT and 1 mM EDTA). Liberated NHMec was measured in a Perkin-Elmer LS50 fluorimeter at excitation and emission wavelengths of 360 and 460 nm, respectively. To correctly assess the active concentration of cystatin C by such assays, the enzyme concentration used was ∼20 nM, i.e., >100−fold over the K_i value for the cystatin C−cathepsin B complex (*16*).

Analysis of Dimerization. Determination of dimer and monomer amounts in recombinant cystatin samples was performed by SEC (Superdex 75 HR 10/30) using an HPLC system (Waters) with the help of integration software (Waters 990). As chromatography buffer, 50 mM ammonium bicarbonate, pH 7.8, containing 100 mM NaCl was used. Cystatins were concentrated by pressure ultrafiltration on an Amicon cell (filter with cutoff 1 kDa) to a concentration of 0.3 mg/mL. The buffer was changed on a desalting column (High-trap, Pharmacia) by applying 0.5 mL of sample and collecting 1.5 mL of the eluate. The samples were incubated at 25 °C, and aliquots were taken immediately and at different time points for oligomerization analysis. Dimers of L68Q and wild-type cystatin C were produced by heating for 2 h at 42 and 70 °C, respectively. Monomers remaining in the heated samples were removed by SEC using the Superdex 75 column. The solution conditions in experiments to convert dimers into monomers were changed by dialysis (Spectra/Por membranes, cutoff 3500 Da) for 20 h at 4 °C.

Circular Dichroism Spectroscopy. Far-UV CD spectra were collected at 1 nm intervals using a Jasco J700 spectropolarimeter with 1 mm path length quartz cuvettes in a temperature-controlled housing at 25 °C over the wavelength range 190−240 nm. The protein samples analyzed were at 0.15 mg/mL concentration in 5 mM acetate buffer, pH 4.5, or 5 mM Tris buffer, pH 8.5. The amount of secondary structure was assessed from the spectra using the JSSE software (Jasco).

NMR Experiments. ^{15}N−Enriched cystatin C was produced by growth of transformed *E. coli* BL834(DE3) in defined medium [0.1 M phosphate buffer, pH 7.5, 20 mM NaCl, 7.5 mM $(NH_4)_2SO_4$, 0.2% (w/v) glucose, 2 mM $MgSO_4$, 300 mM methionine, 120 mM $CaCl_2$, 75 mM thiamine, 15 mM $FeSO_4$] containing ^{15}N-ammonium sulfate (Cambridge Isotope Laboratories) as nitrogen source. The cultures were induced with 0.5 mM IPTG for 20 h when cell density reached an OD_{555} of 0.2. The protein was purified as described above and concentrated to approximately 0.1−0.2 mM. Buffer was exchanged using Sephadex G15 columns. ^{1}H−^{15}N HSQC (*27*) spectra were acquired at 500 MHz using a Bruker DRX500 spectrometer equipped with a 5 mm triple resonance (^{1}H/^{13}C/^{15}N) actively shielded *x,y,z* gradient probehead and Bruker pulse field gradient accessory. Water suppression was achieved using the WATERGATE sequence (*28*), employing typically 1 ms gradient pulses of 15 G/cm and a 3-9-19 binomial sequence. The carrier frequency was shifted to the middle of the NH frequencies (7.3 ppm) to increase digital resolution in the proton dimension. NMR spectra were acquired at 20 °C, with the acquisition time varying between 4 and 14 h/spectrum. A wide pH region was initially covered (8.5, 7.8, 7.0, and 6.0) to identify conditions giving the best NMR results. Because of an exchange broadening of amide signals, especially seen for the dimeric form at high pH values, experiments carried out at pH above 7.0 were generally not very successful. The final choice, pH 7.0 (50 mM phosphate buffer, containing 0.15 M NaCl), was a compromise which involved partial dimerization of the monomeric sample.

ANS Binding and Fluorescence. Fluorescence emission spectra were recorded at 25 °C in a Perkin-Elmer LS50 luminescence spectrophotometer at an excitation wavelength of 385 nm and total fluorescence emission monitored between 400 and 600 nm. The concentration of completely monomeric or dimeric cystatin C samples, isolated by SEC (above), was adjusted by ultrafiltration to 0.22 mg/mL in 5 mM Tris-HCl buffer, pH 8.5. Different pH values were accomplished by adding 20 μL of 1 M buffer stock solution to 180 μL of monomeric cystatin C solution, so that a final concentration of 0.20 mg/mL cystatin C in 100 mM buffer was achieved. ANS (Sigma) was added from a 10 mg/mL stock solution to a final concentration of 0.05 mg/mL. After adjusting the pH and adding ANS, the spectra were immediately recorded.

RESULTS

Expression and Refolding of L68Q Cystatin C. In a previous study, we found that periplasmatic expression yields in *E. coli* of L68Q cystatin C relied on the temperature during expression (*16*). The ability to modulate the temperature to increase the yields was, however, limited to the 38−42 °C range by our routinely used temperature-controlled expression vector. To overcome this limitation, the coding sequences for human wild-type and L68Q cystatin C, alone or preceded by the *E. coli* OmpA signal sequence (*19*), were cloned into the phage T7 based expression vector pET3a. A comparison of the periplasmatic expression levels using the two OmpA containing constructs at different temperatures (25, 30, and 37 °C) revealed that only low amounts (<0.5 mg/L) of L68Q cystatin C were properly secreted to the periplasm, whereas the wild-type cystatin C construct yielded high amounts of secreted protein (>10 mg/L). In contrast, high expression yields (>30 mg/L) with similar amounts produced for both cystatin variants were obtained using the two cytoplasmatic expression vector constructs (Figure 1); L68Q and wild-type cystatin C were both expressed at a level corresponding to 40% of the total *E. coli* protein. The cytoplasmatic expression yields did not depend on temperature and both expressed cystatin variants were found in the insoluble fraction after sonification, indicating aggregation and accumulation in inclusion bodies. The obtained yields of wild-type and L68Q cystatin C were similar under all conditions supporting inclusion body formation. By contrast, growing of the cells in minimal medium and inducing expression with lactose lead to expression of soluble wild-type cystatin C found in the cytoplasm but resulted in no detectable expression of the L68Q variant.

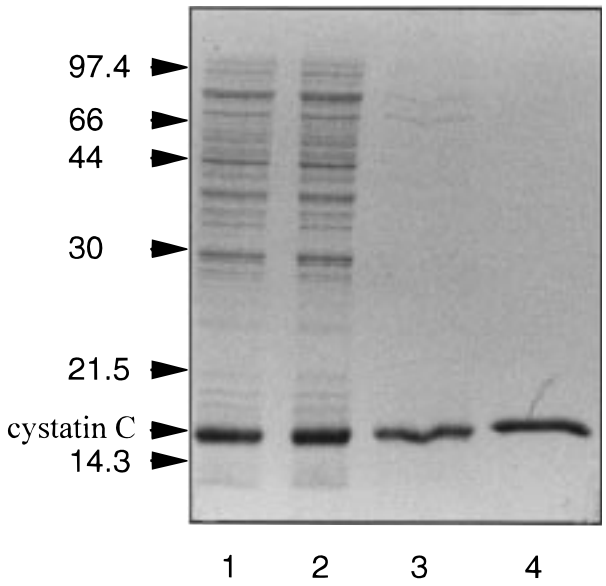

FIGURE 1: SDS−PAGE analysis of L68Q and wild-type cystatin C produced by cytoplasmatic *E. coli* expression. Samples were loaded on a 15% polyacrylamide gel under reducing conditions and stained by Serva Blue following electrophoresis: lane 1, total cell extract of *E. coli* BL21(DE3) expressing wild-type cystatin C (construct pET3a-cystatin C); lane 2, total cell extract of bacteria expressing L68Q cystatin C (construct pET3a−L68Q cystatin C); lane 3, purified inclusion bodies from L68Q cystatin C expressing bacteria resolved in 6 M guanidinium hydrochloride; lane 4, refolded L68Q cystatin C.

The inclusion bodies formed at expression using the cytoplasmatic constructs were purified and resolved in 6 M guadinium hydrochloride. The highly enriched (>80%), denatured inhibitor (Figure 1) was refolded by buffer exchange on a SEC column. The fractions containing refolded, monomeric protein were collected. Both wild-type and L68Q cystatin C displayed a single sharp band at SDS−PAGE (Figure 1) and at agarose gel electrophoresis. The obtained yields after refolding were approximately 2 mg/L bacterial culture. N-Terminal protein sequencing demonstrated that the first 10 amino acids of the purified proteins were SSPGKPPRLV, i.e., coinciding with the native cystatin C sequence (*29*). The efficient cleavage of the methionine residue encoded by the expression constructs from cystatin C is consistent with the subsequent residue being Ser (*30*). Both refolded cystatin variants were inhibitory active against papain, indicating a correct folding. The refolded L68Q

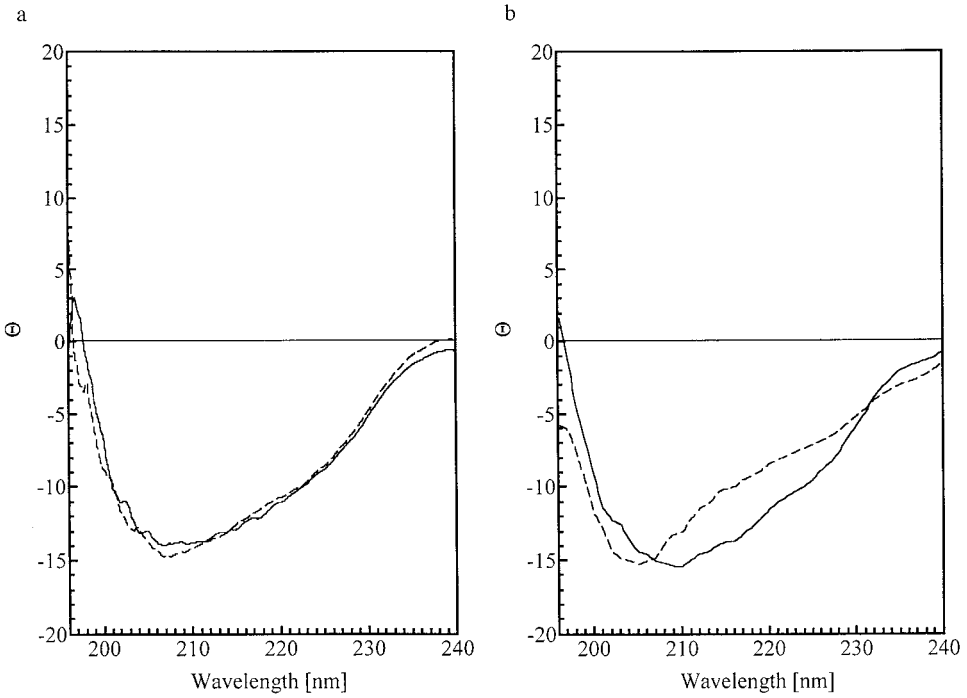

FIGURE 2: Circular dichroism spectra of L68Q and wild-type cystatin C under different pH conditions. (A) Far UV CD spectra were collected for the isolated cystatin variants in 5 mM Tris buffer, pH 8.5. (B) Far UV CD spectra obtained in 5 mM acetate buffer, pH 4.5. Dashed line, L68Q cystatin C; solid line, wild-type cystatin C.

cystatin C was studied by far-UV CD spectroscopy and the obtained spectra were compared with those for wild-type cystatin C produced by periplasmic expression. The spectra were almost identical (Figure 2a) and in full agreement with a properly folded protein with proportions of α-helical structure, β-strands, turns, and random coiled structure similar to what has been determined for recombinant wild-type cystatin C by NMR spectroscopy (*17*). Additional strong support for homogeneity and a fold similar to that of wild-type cystatin C was given by NMR data (see below).

L68Q Cystatin C Dimerization. To investigate dimerization, analytical HPLC-SEC was used as a method with capacity to detect monomeric cystatin C and its oligomers under nondenaturing, nonaffecting conditions. The chromatography buffer, 50 mM ammonium bicarbonate, pH 7.8, containing 100 mM NaCl, had no effect on dimerization at 25 °C up to at least 2 h incubation. Because the starting monomer concentration was critical for a proper interpretation of our experiment, the cystatin preparation was concentrated by ultafiltration directly after refolding, followed by rapid buffer exchange on a desalting column. The amount of dimer formed under these conditions was reproducibly less than 10%. In Figure 3, the influence of pH on L68Q cystatin C dimer formation is shown. The monomeric cystatin variant was stable between pH 7.5 and 9.0. At higher pH, a dimerization event was evident. Lowering the pH similarly resulted in dimerization. At pH 6.0, the dimerization rate was maximally accelerated and lead to almost complete dimerization after 4 h of incubation at 25 °C. At pH 4.5 and 4.0, the amount of dimer present directly after buffer exchange even decreased during 24 h of incubation. To confirm this observation, we dialyzed a 100% dimer containing sample (produced as described under Experimetal Procedures) against acetate buffer at pH 4.5. The amount of monomer formed after 20 h dialysis was 85% (Figure 4). The newly formed monomer revealed a stability

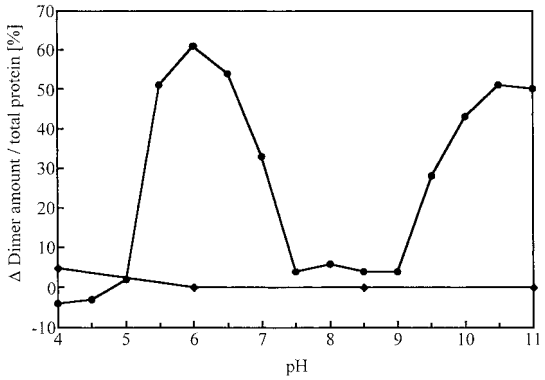

FIGURE 3: Influence of pH on the dimerization of L68Q and wild-type cystatin C. The buffers used were acetate, phosphate, Tris-HCl, or carbonate, adjusted to pH values from 4 to 11 in a concentration of 20 mM. Buffer exchange was performed on a desalting column prior to incubation. The samples were incubated at 25 °C for 24 h at a concentration of 0.1 mg/mL. Amounts of dimer and monomer were determined by HPLC-SEC. L68Q cystatin C (●); wild-type cystatin C (◆).

similar to that of the native monomeric form of L68Q cystatin C present at pH 7.5−9.0. We never observed aggregation or substantial loss of protein during incubation at the low pH values.

We have earlier demonstrated that pH values 3.0−4.4 achieve mild denaturation and result in dimerization of wild-type cystatin C (*17*). In agreement with this but in contrast to what was observed for L68Q cystatin C, a low degree of dimerization of wild-type cystatin C was seen only for the measurement at pH 4.0 (Figure 3). Similar to what we observed for the L68Q cystatin C dimer, the wild-type cystatin C dimer could be converted into a monomeric form by dialysis against acetate buffer, pH 4.5. This monomerization process reached 38% after 20 h of incubation (Table 1).

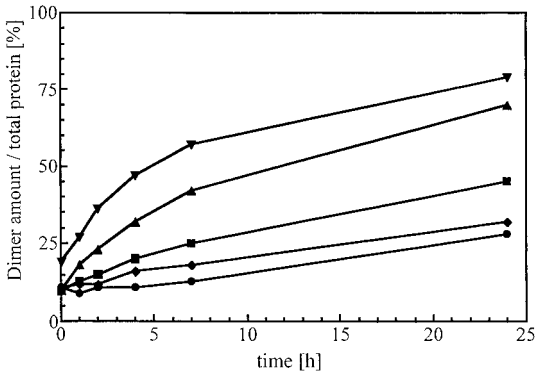

FIGURE 4: Influence of ionic strength on the dimerization of L68Q cystatin C. Cystatin C was concentrated by ultrafiltration to 0.3 mg/mL, followed by buffer exchange on a desalting column. The samples were incubated at 25 °C for 24 h at a concentration of 0.1 mg/mL. Amounts of dimer and monomer were estimated by HPLC-SEC; 0 M NaCl (●); 0.1 M NaCl (◆); 0.2 M NaCl (■); 0.5 M NaCl (▲); 1 M NaCl (▼).

Table 1: Conversion of L68Q and Wild-Type Cystatin C Dimers into Monomers[a]

	monomer/total amount of protein (%)	
buffer	wild-type cystatin C	L68Q cystatin C
NH_4HCO_3, pH 7.8, 0.1 M NaCl	8	17
PBS	10	18
H_2O	12	33
20% ethanol/H_2O	10	30
acetate, pH 4.5	38	85

[a] L68Q and wild-type cystatin C dimers were produced by 2 h of incubation at 42 and 70 °C, respectively. To obtain samples containing 100% dimers, the monomer fraction remaining after heating was removed by SEC. The samples were dialyzed against different buffers for 20 h at 4 °C at a concentration of 0.2 mg/mL. Amounts of dimers and monomers were assessed by HPLC-SEC.

To investigate the role of hydrophobic interactions for dimer formation, we incubated monomeric L68Q cystatin C in refolding buffer (ammonium bicarbonate buffer, pH 7.8) at different NaCl concentrations for 24 h (Figure 4). During the first 4 h of incubation, no increased amounts of dimers could be observed under salt-free conditions. After 24 h of incubation, the amount of dimer was still below 20%. A clear correlation between ionic strength and degree of dimerization could be observed for all salt concentrations analyzed. At 1 M NaCl, the final dimer amount was approximately 80%. In control experiments, no influence of up to 1 M NaCl on wild-type cystatin C dimerization could be observed. Additionally, an effect of ionic strength on the reconversion of L68Q cystatin C dimers could be shown. Dialysis of a L68Q cystatin C dimer sample against H_2O resulted in approximately 33% remonomerization after 20 h incubation (Table 1). But lowering the ionic strength even more by dialyzing against 20% ethanol in distillated water, we were not able to increase the amount of reconverted monomers. In contrast to what was observed for L68Q cystatin C, the reconversion to monomers from wild-type cystatin C dimers was not affected by ionic strength, which is in agreement with previous results for the wild−type cystatin C dimer, concluding that the protein is trapped energetically in the dimer (*17*).

Equilibrium between Monomeric and Dimeric L68Q Cystatin C. It was observed in the experiments described above that cystatin dimerization never proceeded to completion (Figures 3 and 4). Under conditions strongly supporting dimerization of L68Q cystatin C, like pH 6.0 or 1 M NaCl, the amount of dimer reached approximately 80% at equilibrium. Similar to the experiments conducted at low pH and by decreasing the ionic strength, we investigated the reconversion of L68Q cystatin C dimers in physiological buffer (PBS; pH 7.2). The amount of monomer formed from a 100% dimerized sample after dialysis for 20 h against this buffer was 18% (Table 1). The amount obtained after dialysis against 50 mM ammonium bicarbonate buffer, pH 7.8, the buffer chosen for dimer production by heating, was similar (17%). Thus, an equilibrium between monomer and dimer is likely at hand for L68Q cystatin C under physiological conditions.

Structure of L68Q Cystatin C. NMR spectroscopy was used to obtain structural information for L68Q cystatin C and to study the dimerization process at amino acid resolution. The signals in a $^1H-^{15}N$ HSQC spectrum of monomeric wild−type cystatin C at pH 7.0 (Figure 5a) were assigned by comparison with published results for cystatin C at pH 6.0 (*18*), to allow interpretation of pH 7.0 spectra of L68Q cystatin C (Figure 5, panels b and c). The spectra of wild−type and L68Q cystatin C showed similar, good dispersion of the amide signals with no evidence for an unfolded or misfolded form of the L68Q variant, confirming successful refolding of the L68Q cystatin C samples from inclusion bodies. Amide signals for 25 amino acids and the indole NH of Trp106 in the L68Q variant were assigned in an unambiguous way, as they were only minimally shifted between the two cystatin C variants and many additional signals were found at exactly the same positions for the two variants. As chemical shifts are extremely sensitive to both global and local conformational changes, the close match of the $^1H-^{15}N$ HSQC spectra is supportive for similar conformations of wild-type and L68Q cystatin C. Some few signals of wild−type cystatin C were strongly shifted in the spectra of the L68Q variant, including the signals for Leu/Gln68, Leu47, and Gly69. These shifts represent local influence on chemical shifts by the Leu/Gln68 substitution.

From a comparison of the $^1H-^{15}N$ HSQC spectra of partially (50%) and completely dimerized L68Q cystatin C samples (Figure 5, panels b and c, respectively), it is clear that the chemical shifts of nearly all amide signals of L68Q cystatin C are the same, demonstrating that dimerization does not cause major global changes in the protein as it does not affect the majority of amino acids in the molecule. Only a few signals, including those of the indole NH of Trp106 and the amides of Val57, Gln107, and Thr109 appear as doublets on the HSQC spectrum of the mixture of both forms (Figure 5b), representing different environments in the dimeric and the monomeric form. These are the same signals as the ones which were significantly affected in wild-type cystatin C upon dimer formation and correspond to locations on the hydrophobic, proteinase-binding interface participating in the dimerization of the inhibitor (*18*). Thus, the NMR results provided evidence that the L68Q cystatin C dimer is structurally very similar to the wild−type cystatin C dimer.

pH-Dependent Structural Changes in L68Q Cystatin C. The binding of the hydrophobic dye ANS is a widely used tool to identify and characterize partly folded states of proteins, including molten globule states (*31*). ANS binds

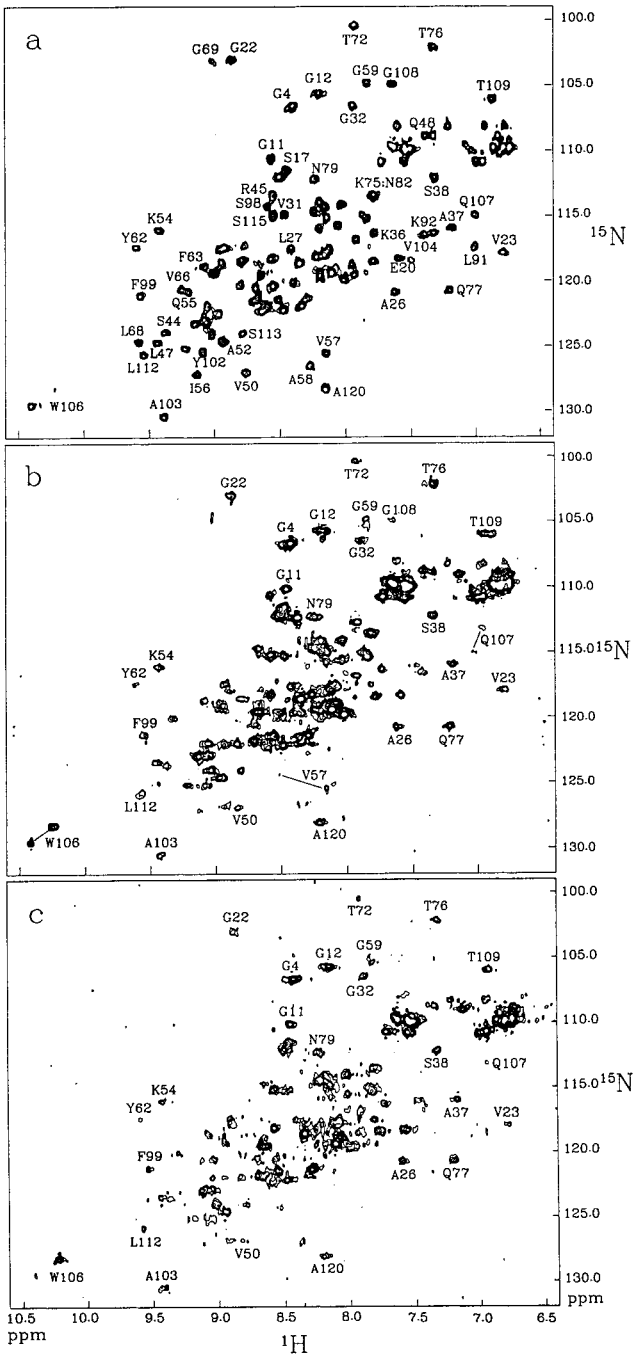

FIGURE 5: ^{1}H-^{15}N HSQC spectra of uniformly ^{15}N-enriched cystatins. (A) monomeric wild-type cystatin C; (B) L68Q cystatin C, a sample containing similar amounts of the monomeric and dimeric form; (C) L68Q cystatin C in its dimeric form. Backbone amide resonances are labeled according to sequential assignments performed for the wild-type cystatin C. Lines indicate shifts resulting from dimer formation. All spectra were acquired at 20 °C in 50 mM sodium phosphate buffer containing 0.15 M NaCl at pH 7.0.

to hydrophobic regions of proteins with an increase of fluorescence and with a blue-shift of λ_{max}, reflecting the burial of the ANS molecule in a more hydrophobic environment. We compared the ANS binding to L68Q and wild-type cystatin C in their monomeric and dimeric forms at pH 8.5, the pH value where the L68Q variant is most stably monomeric (Figure 3). The obtained spectra of L68Q and wild−type cystatin C comparing the respective form (monomer or dimer) are very similar in fluorescence intensity and

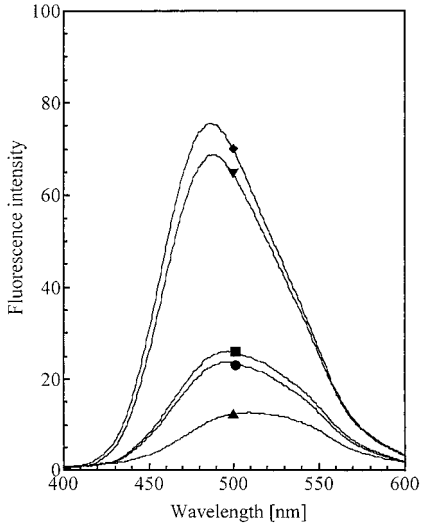

FIGURE 6: Fluorescence spectra showing ANS binding to cystatin C monomers and dimers. Aliquots of cystatin C monomer and dimer preparations with a final protein concentration of 0.2 mg/mL in 50 mM Tris buffer, pH 8.5, were mixed with ANS (final concentration, 0.1 mg/mL). Fluorescence emission spectra were taken exciting the samples at 385 nm immediately after mixing. Wild-type cystatin C dimer (◆); L68Q cystatin C dimer (▼); L68Q cystatin C monomer (■); wild-type cystatin C monomer (●); buffer (▲).

λ_{max} (Figure 6), in total agreement with the NMR and CD results (Figures 2a and 5). The increased fluorescence intensities and the shifts of λ_{max} of the spectra obtained with the dimeric forms indicate a larger hydrophobic area on the surface of the dimer than on the surface of the monomer, for both cystatin C variants.

To establish a possible correlation between dimerization caused by lowering pH (shown in Figure 3) and the concomitant exposure of hydrophobic groups, the ANS fluorescence emission at 480 nm of freshly refolded L68Q cystatin C was recorded as a function of pH. Because of the high emission of the dimeric form, the spectra were recorded before a substantial amount of dimer had been formed. Following this approach, the amount of dimer could be kept below 10% during the recording of spectra, as seen by parallel analysis of samples by SEC. Additionally, the influence of ANS on the conformation of the protein (*34*) was kept as low as possible this way. The fluorescence emission intensity obtained for wild-type cystatin C was rather constant from pH 8.5 to 5.0 and increased below pH 4.5 (Figure 6), seemingly in parallel to the formation of dimers shown in Figure 3 and recorded in the literature (*17*). The ANS binding to L68Q cystatin C was also increased by lowering the pH (Figure 7). In the range between pH 8.5 and 7.5 where L68Q cystatin C dimerization is low, the ANS fluorescence intensity was unchanged and low. Between pH 7.0 and 6.0, the emission intensity was only slightly increased (Figure 8) whereas the tendency to dimerize is drastically increased (Figure 3). This shows that the L68Q cystatin C dimers are formed under conditions where little extra exposure of hydrophobic surface is on hand. That is in excellent agreement with the NMR results, which demonstrate structural conservation of the cystatin fold in the dimeric form. Acidic pH (4.5 and below) caused a dramatic effect on the binding of ANS. At those pH values, dimerization is prevented and highly reversible. The spectra obtained at this low pH resemble spectra obtained from

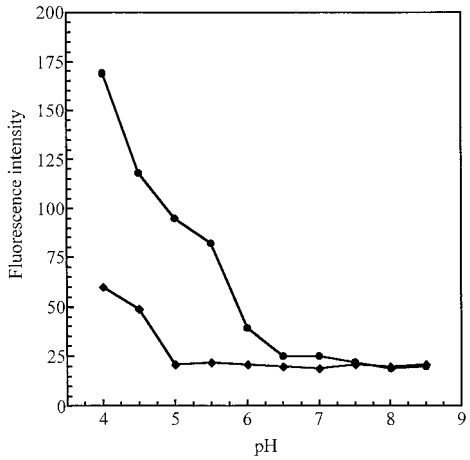

FIGURE 7: ANS fluorescence intensity as a function of pH. Aliquots of cystatin C were pH adjusted by adding concentrated buffer (acetate, phosphate, or Tris buffer) to a final concentration of 100 mM. The fluorescence intensity was measured at an emission wavelength of 480 nm and an excitation wavelength of 385 nm. L68Q cystatin C (●); wild-type cystatin C (◆).

molten globule-like intermediates of lysozyme and β-lactamase (*32*, *33*) and differ drastically from obtained spectra of totally heat-denatured cystatin C.

Low pH was also found to influence the secondary structure of L68Q cystatin C. The CD spectra obtained at pH 4.5 with the two cystatin C variants differ significantly (Figure 2b). The spectrum of L68Q cystatin C at this low pH value agrees with a substantial amount of retained secondary structure, but with an increased amount of random coil structure compared to wild-type cystatin C as indicated by secondary structure predictions.

To investigate the influence of the observed structural changes on the inhibitory activity of cystatin C, we measured inhibition of cathepsin B activity under titrating conditions (i.e., at an enzyme concentration 100-fold higher than the K_i value for the cystatin C−cathepsin B complex, 0.3 nM). Cathepsin B is enzymatically active at pH 4.5, but unstable above neutral pH (*16*). At pH 6.5, cathepsin B remained stable during a 30 min measurement and was efficiently inhibited by both wild-type and L68Q cystatin C. The dimerization of L68Q cystatin C occurring at pH 6.5 is slow under the conditions which, as in the case of the ANS binding, were chosen to minimize dimerization. Consequently, during continuous recording of the inhibition rate, we never observed a recovered cathepsin B activity which would have resulted from formation of the inhibitory inactive dimeric cystatin form in the equilibrium assay (*16*). Nevertheless, the conditions caused the structural changes observed by ANS-binding and CD experiments. Inhibition of cathepsin B at pH 6.5 (taken for 100% inhibitory activity) decreased to 28% remaining inhibitory activity at pH 4.5 in case of L68Q cystatin C, whereas wild-type cystatin C retained 76% of its inhibitory activity at pH 6.5 when assayed at pH 4.5. Preincubation of L68Q cystatin C at pH 4.5 for 30 min, which results in formation of the monomeric intermediate (Figures 2b and 7), followed by activity measurement at pH 6.5, resulted in 79% of the inhibitory activity displayed by the preparation originally (97% activity for wild−type cystatin C in a parallel control experiment). Thus, the monomeric form present at low pH is a stable intermediate with partially conserved inhibitory activity, which may be stabilized by binding to the proteinase, and rapidly regains most of its activity after transference back to more neutral pH values. The easy and direct conversion to the native monomeric form is further confirmed by the observation that the monomeric form present at low pH, when analyzed by SEC, never showed the typical chromatogram obtained at refolding of cystatin C, which typically displays significant amounts of misfolded protein.

DISCUSSION

Efficient production of properly folded wild-type cystatin C and several variants of the inhibitor in a secretory, temperature-controlled *E. coli* expression system has been reported (*19*, *34*, *35*). Earlier attempts to use this system for the production of L68Q cystatin C resulted in insufficient yields of soluble protein recovered from the periplasmic space (*16*). This indicated that the temperature necessary to induce the expression system (37−42 °C) is in conflict with stability of the expressed L68Q cystatin C, which tends to dimerize under these conditions (*16*). The results we obtained in the present study using a temperature independent T7 expression system, which allowed expression of an OmpA signal peptide−L68Q cystatin C construct at lower temperatures, demonstrate that the earlier low yields of L68Q cystatin C are not mainly due to induction temperatures conflicting with stability of the cystatin C variant in the periplasm. High amounts of L68Q cystatin C could be obtained using a signal peptide-lacking construct under conditions where the expressed protein accumulates in the form of inclusion bodies in the T7 system. But under conditions where inclusion body formation is avoided, the T7 system resulted in soluble protein in the cytoplasm for wild-type cystatin C and was unsuccessful for L68Q cystatin C. This suggests that an intracellular protein folding/stability problem, rather than limitations in the periplasmic secretory system, is the main cause for the previous low yields. On the other hand, similar in vitro refolding rates from inclusion bodies were observed for wild-type cystatin C and the amyloidogenic L68Q variant. It thus seems that the in vivo picoenviromental conditions are important either for the intracellular stability of readily folded cystatin C or for the folding itself, which results in an impeded transport out of the cell. This may be one reason for the lower cystatin C levels found in body fluids of HCCAA patients, who are heterozygous for L68Q cystatin C (*5*).

It is known that various hereditary amyloidosis syndromes are characterized by point mutations leading to the production of amyloidogenic protein variants (*2*). It has been shown for some of these proteins that the amyloidogenic variants can form native folds (*32*, *36*). As shown by our present CD, NMR, and ANS-binding results, the secondary and tertiary structures of L68Q cystatin C are similar to those of wild-type cystatin C, although the native form for which this is valid is stable under much more narrow conditions. The native form of L68Q cystatin C is further confirmed by the conserved function of the variant as a proteinase inhibitor (*16*), which requires the correct arrangement of three different structural parts of the inhibitor (*37*). By structural alignment of human cystatin C (*18*) with chicken cystatin (*38*, *39*) the Leu/Gln 68 residue appears to be located in the hydrophobic core of the protein. As known from other proteins, mutations in the hydrophobic core can decrease protein stability (*40*−

42), and in some cases, such destabilization results in amyloid formation (*43*−*45*). In the case of cystatin C, the Leu68 → Gln substitution seems to destabilize the protein to the degree that the mild denaturation required for dimerization occurs under close to physiological conditions, and could be triggered, e.g., by an increase of the body temperature from 37 to 40 °C (*16*) or by small pH changes. Therefore, the inactive dimeric form is most likely the prevalent state of L68Q cystatin C in HCCAA patients' cerebrospinal fluid. The resulting lowered cystatin C activity may result in an enhanced proteolytic activity of cysteine proteinases, which are known to have capacity to cause cerebral hemorrhage (*14*, *15*) in the brain at HCCAA.

Unlike the L68Q variant, wild-type cystatin C does not have the tendency to dimerize at close to physiological conditions. Nevertheless, wild-type cystatin C dimers can be formed by heating (to 70 °C), allowing studies of the conformational changes occurring during cystatin C dimerization (*17*, *18*). NMR studies on such dimers have shown that the three-dimensional structure of cystatin C remains largely unchanged in the molecular form present in the dimer (*18*). The two molecules in the dimer are symmetrically mirrored at the three regions involved in proteinase inhibition, viz., the N-terminal segment and the two hairpin loops. But, it is still unknown whether association occurs directly via the hydrophobic loops or if there is a crossing-over of β-strands from one monomer to the other (domain swapping) (*18*). Our present results clearly speak in favor of the domain-swapping model, as they demonstrate enhanced dynamic properties for the L68Q variant, which should support domain swapping. The present NMR, CD, and ANS-binding data strongly indicate that L68Q cystatin C is structurally similar to wild-type cystatin C, in both the native monomeric and dimeric form. However, the native monomeric cystatin fold is stable only in a very narrow range of conditions in case of the L68Q variant. Outside of this range, the dynamics of the structure differs drastically between the wild-type protein and the amyloidogenic variant, which is indicated by an alterated amount of hydrophobic parts on the protein surface, an alterated dimerization behavior at different pH values, an alterated influence of ionic strength on the dimerization, and an alterated remonomerization. One can assume that this alterated dynamic behavior is caused by a missing contribution of the hydrophobic Leu68 side chain to the stability of the hydrophobic core in L68Q cystatin C.

We found that the cystatin C dimer is not the only intermediate formed on the pathway toward total denaturation. By lowering the pH to below 5.0, a pH value that corresponds to the pH in lysosomes, another monomeric form of L68Q cystatin C could be observed. This monomer is characterized by a molten globule-like binding of ANS, changes in secondary structure, reduced inhibitory activity, and no inheritant tendency to dimerize. Additionally, it could be shown that this monomeric form readily can be converted into the native monomeric form. The newly discovered monomeric form clearly displays the characteristic behavior of a stable intermediate on the path toward total denaturation. Such changes in the cystatin fold as observed for this intermediate are similar to the reported conformational changes which the amyloidogenic lysozyme variants undergo under conditions where fibril formation occurs (*32*). A molten globule-like intermediate would also support a structural rearrangement resulting in β-sheet-based aggregation (*31*), as has been reported for Aβ peptide (*46*) and prion fibril formation (*47*).

ACKNOWLEDGMENT

The technical assistance of Mrs. Inger Nilsson is gratefully acknowledged.

REFERENCES

1. Sipe, J. D. (1992) *Annu. Rev. Biochem. 61*, 947−975.
2. Tan, S. Y., and Pepys, M. B. (1994) *Histopathology 25*, 403−414.
3. Gudmundsson, G., Hallgrimsson, J., Jonasson, T. A., and Bjarnason, O. (1972) *Brain 95*, 387−404.
4. Ghiso, J., Jensson, O., and Frangione, B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2974−2978.
5. Löfberg, H., Grubb, A. O., Nilsson, E. K., Jensson, O., Gudmundsson, G., Blöndal, H., Arnason, A., and Thorsteinsson, L. (1987) *Stroke 18*, 431−440.
6. Thorsteinsson, L., Georgsson, G., Asgeirsson, B., Bjarnadottir, M., Olafsson, I., Jensson, O., and Gudmundsson, G. (1992) *J. Neurol. Sci. 108*, 121−128.
7. Benedikz, E., Blöndal, H., and Gudmundsson, G. (1990) *Virchows Arch. A Pathol. Anat. Histopathol. 417*, 325−331.
8. Abrahamson, M., Barrett, A. J., Salvesen, G., and Grubb, A. (1986) *J. Biol. Chem. 261*, 11282−11289.
9. Stoka, V., Nycander, M., Lenarcic, B., Labriola, C., Cazzulo, J. J., Björk, I., and Turk, V. (1995) *FEBS Lett. 370*, 101−104.
10. Heidtmann, H. H., Salge, U., Abrahamson, M., Bencina, M., Kastelic, L., Kopitar-Jerala, N., Turk, V., and Lah, T. T. (1997) *Clin. Exp. Metastasis 15*, 368−381.
11. Burnett, D., Abrahamson, M., Devalia, J. L., Sapsford, R. J., Davies, R. J., and Buttle, D. J. (1995) *Arch. Biochem. Biophys. 317*, 305−310.
12. Heidtmann, H. H., Salge, U., Havemann, K., Kirschke, H., and Wiederanders, B. (1993) *Oncol. Res. 5*, 441−451.
13. Grubb, A., Jensson, O., Gudmundsson, G., Arnason, A., Lofberg, H., and Malm, J. (1984) *N. Engl. J. Med. 311*, 1547−1549.
14. Garvin, P. J., Jennings, R. B., Smith, L., and Gesler, R. M. (1965) *Clin. Orthop. 41*, 204−223.
15. Davis, R. J., North, R. B., Campbell, J. N., and Suss, R. A. (1984) *J. Neurosurg. 61*, 169−171.
16. Abrahamson, M., and Grubb, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1416−1420.
17. Ekiel, I., and Abrahamson, M. (1996) *J. Biol. Chem. 271*, 1314−1321.
18. Ekiel, I., Abrahamson, M., Fulton, D. B., Lindahl, P., Storer, A. C., Levadoux, W., Lafrance, M., Labelle, S., Pomerleau, Y., Groleau, D., LeSauteur, L., and Gehring, K. (1997) *J. Mol. Biol. 271*, 266−277.
19. Abrahamson, M., Dalbøge, H., Olafsson, I., Carlsen, S., and Grubb, A. (1988) *FEBS Lett. 236*, 14−18.
20. Olafsson, I., Gudmundsson, G., Abrahamson, M., Jensson, O., and Grubb, A. (1990) *Scand. J. Clin. Lab. Invest. 50*, 85−93.
21. Dalbøge, H., Jensen, E. B., Tottrup, H., Grubb, A., Abrahamson, M., Olafsson, I., and Carlsen, S. (1989) *Gene 79*, 325−332.
22. Laemmli, U. K. (1970) *Nature 227*, 680−685.
23. Lin, K. H., and Cheng, S. Y. (1991) *Biotechniques 11*, 748.
24. Jeppsson, J. O., Laurell, C. B., and Franzen, B. (1979) *Clin. Chem. 25*, 629−638.
25. Lindahl, P., Abrahamson, M., and Bjork, I. (1992) *Biochem J. 281*, 49−55.
26. Funk, M. O., Nakagawa, Y., Skochdopole, J., and Kaiser, E. T. (1979) *Int. J. Pept. Protein Res. 13*, 296−303.
27. Bodenhausen, G., and Ruben, D. J. (1980) *Chem. Phys. Lett. 69*, 185−189.

28. Piotto, M., Saudek, V., and Sklenar, V. (1992) *J. Biomol. NMR. 2*, 661−665.
29. Grubb, A., and Löfberg, H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3024−3027.
30. Hirel, P. H., Schmitter, M. J., Dessen, P., Fayat, G., and Blanquet, S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8247−8251.
31. Dobson, C. M. (1995) *Nat. Struct. Biol. 2*, 513−517.
32. Booth, D. R., Sunde, M., Bellotti, V., Robinson, C. V., Hutchinson, W. L., Fraser, P. E., Hawkins, P. N., Dobson, C. M., Radford, S. E., Blake, C. C., and Pepys, M. B. (1997) *Nature 385*, 787−793.
33. Goto, Y., and Fink, A. L. (1989) *Biochemistry 28*, 945−952.
34. Hall, A., Dalbøge, H., Grubb, A., and Abrahamson, M. (1993) *Biochem J. 291*, 123−129.
35. Hall, A., Hakansson, K., Mason, R. W., Grubb, A., and Abrahamson, M. (1995) *J. Biol. Chem. 270*, 5115−5121.
36. Kelly, J. W. (1996) *Curr. Opin. Struct. Biol. 6*, 11−17.
37. Stubbs, M. T., Laber, B., Bode, W., Huber, R., Jerala, R., Lenarcic, B., and Turk, V. (1990) *EMBO J. 9*, 1939−1947.
38. Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J., and Turk, V. (1988) *EMBO J. 7*, 2593−2599.
39. Dieckmann, T., Mitschang, L., Hofmann, M., Kos, J., Turk, V., Auerswald, E. A., Jaenicke, R., and Oschkinat, H. (1993) *J. Mol. Biol. 234*, 1048−1059.
40. Eigenbrot, C., and Kossiakoff, A. A. (1992) *Curr. Opin. Biotechnol. 3*, 333−337.
41. Gilis, D., and Rooman, M. (1997) *J. Mol. Biol. 272*, 276−290.
42. Munson, M., Balasubramanian, S., Fleming, K. G., Nagi, A. D., O'Brien, R., Sturtevant, J. M., and Regan, L. (1996) *Protein Sci. 5*, 1584−1593.
43. Riek, R., Hornemann, S., Wider, G., Billeter, M., Glockshuber, R., and Wuthrich, K. (1996) *Nature 382*, 180−182.
44. Jones, L. A., Skare, J. C., Cohen, A. S., Harding, J. A., Milunsky, A., and Skinner, M. (1992) *Clin. Genet. 41*, 70−73.
45. Pepys, M. B., Hawkins, P. N., Booth, D. R., Vigushin, D. M., Tennent, G. A., Soutar, A. K., Totty, N., Nguyen, O., Blake, C. C., and Terry, C. J. (1993) *Nature 362*, 553−557.
46. Zagorski, M. G., and Barrow, C. J. (1992) *Biochemistry 31*, 5621−5631.
47. Nguyen, J., Baldwin, M. A., Cohen, F. E., and Prusiner, S. B. (1995) *Biochemistry 34*, 4186−4192.

BI980873U