Biochemistry **1997,** *36,* 2323−2331

Thermodynamics of Unfolding for Kazal-Type Serine Protease Inhibitors: Entropic Stabilization of Ovomucoid First Domain by Glycosylation[†]

Gregory T. DeKoster and Andrew D. Robertson*

Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242

Received October 15, 1996; Revised Manuscript Received December 12, 1996[⊗]

ABSTRACT: A synthetic gene for chicken ovomucoid first domain (OMCHI1) has been overexpressed in *Escherichia coli*. The resulting recombinant protein, rOMCHI1, is expressed and correctly folded without the use of fusion proteins or export secretion signal peptides incorporated into the gene. The thermostability of rOMCHI1 has been compared to that of the naturally occurring glycosylated OMCHI1 (gOMCHI1). The results of differential scanning calorimetry (DSC) studies show that the heat capacity change for unfolding, ΔC_p, for both rOMCHI1 and gOMCHI1 is approximately 600 cal/(mol·K). At any given pH, however, the presence of N-linked carbohydrate increases the T_m for thermal unfolding of gOMCHI1 over rOMCHI1 by 2−4 °C, without changing the enthalpy of unfolding, $\Delta H°_m$. This suggests that the increased thermal stability of gOMCHI1 is entropic. Comparison of the unfolding thermodynamics of rOMCHI1 with those of turkey ovomucoid third domain (OMTKY3), which is 36% identical to rOMCHI1, reveals similar ΔC_p values for both proteins, about 600 cal/(mol·K), but a reduction in $\Delta H°_m$ of about 5 kcal/mol for rOMCHI1 at all temperatures. Decreases in $\Delta H°_m$ for rOMCHI1 versus OMTKY3 may be explained by an overall less ordered native state in rOMCHI1. In the absence of a native structure for OMCHI1, the change in accessible surface area upon unfolding, ΔASA, was calculated using unfolding parameters and structural energetic relationships [Murphy & Freire (1992) *Adv. Protein Chem. 43*, 313−361; Murphy et al. (1993), *Proteins: Struct., Funct., Genet. 15*, 113−120]. These calculations suggest that the larger protein rOMCHI1 (M_r 7500) exposes less surface area than OMTKY3 (M_r 6100) upon thermal denaturation. Overall, structural energetic relationships may provide a useful framework for interpretation and comparison of thermodynamic data for structurally homologous proteins.

The aim of comparative studies of thermodynamic stability in proteins is a quantitative understanding of the relationship between structure and energetics. Chemical differences between the proteins in such studies have ranged from single amino acid substitutions (Sturtevant, 1994; Matthews, 1995) to a complete absence of homology in both sequence and structure (Murphy & Freire, 1992). One intermediate approach is investigation of a family of evolutionarily related, structurally homologous proteins. These studies should provide insights into how a given fold accommodates a wide variety of sequences, including insertions and deletions.

We are investigating the relationship between sequence, conformation, and stability in Kazal-type serine protease inhibitors, a large family of proteins with similar three-dimensional structures but widely varying degrees of sequence similarity. The long-term aim is to determine the extent to which interactions are conserved at equivalent positions in structurally homologous proteins. Sequenced variants of the Kazal inhibitor family now number in the hundreds (Laskowski et al., 1987, 1990; Apostol et al., 1993), and are found in animals ranging from sea anemones (Tschesche et al., 1987) and insects (Friedrich et al., 1993) to mammals. High-resolution X-ray and NMR structures have been determined for a number of Kazal-type inhibitors from a variety of organisms: turkey ovomucoid third domain (OMTKY3;[1] Read et al., 1983; Bode et al., 1986; Fujinaga et al., 1987; Hoogstraten et al., 1995), Japanese quail ovomucoid third domain (OMJPQ3; Papamakos et al., 1982), silver pheasant ovomucoid third domain (OMSVP3; Bode et al., 1985), bull seminal plasma inhibitor (BUSI IIA; Williamson et al., 1985), human pancreatic secretory trypsin inhibitor (HSTI; Klaus & Schomburg, 1993), porcine pancreatic secretory trypsin inhibitor (PSTI; Bolognesi et al., 1982), PEC-60 from pig intestine (Liepinsh et al., 1994), leech derived tryptase inhibitor (LDTI-C; Mühlhahn et al., 1994), and rhodniin, a thrombin inhibitor from the assassin bug (van de Locht et al., 1995). In general, the Kazal proteins and domains are cross-linked by 3 disulfides and contain a core structure consisting of a triple-stranded β-sheet, a single α-helix of 10−12 residues, and a loop of 7−8 residues between the second and third cystines which contains the inhibitory reactive site for serine proteases.

[†] This work was supported by the National Institutes of Health (GM 46869), by a grant from the Central Investment Fund for Research Enhancement at the University of Iowa, and by Eli Lilly & Co.

* Address correspondence to this author. Fax: (319) 335-9570. E-mail: andy-robertson@uiowa.edu.

[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1997.

[1] Abbreviations: ΔASA, change in the total solvent-accessible surface area; BUSI IIA, bull seminal plasma inhibitor; ΔC_p, change in heat capacity for protein unfolding; DSC, differential scanning calorimetry; GlcNAc, *N*-acetylglucosamine; gOMCHI1, glycosylated (native) chicken ovomucoid first domain; $\Delta H°_m$, enthalpy of protein unfolding; HSTI, human pancreatic secretory trypsin inhibitor; IPTG, isopropyl β-D-thiogalactopyranoside; LDTI-C, leech-derived tryptase inhibitor; NMR, nuclear magnetic resonance; OMCHI1, chicken ovomucoid first domain; OMCHI3, chicken ovomucoid third domain; OMJPQ3, Japanese quail ovomucoid third domain; OMSVP3, silver pheasant ovomucoid third domain; OMTKY3, turkey ovomucoid third domain; PSTI, porcine pancreatic secretory trypsin inhibitor; rms, root-mean-square; RNase, ribonuclease; rOMCHI1, recombinant chicken ovomucoid first domain; ΔS, entropy of protein unfolding; T_m, midpoint of thermal unfolding; TSP, sodium 3-(trimethylsilyl)propionate-*2,2,3,2*-d_4.

S0006-2960(96)02580-9 CCC: $14.00

Previous studies of OMTKY3 and chicken ovomucoid demonstrate that Kazal-type inhibitors are amenable to detailed analysis of stability (Griko & Privalov, 1984; Swint & Robertson, 1993; Swint-Kruse & Robertson, 1995, 1996). The focus of the present study is on the thermodynamics of unfolding for chicken ovomucoid first domain (OMCHI1). OMCHI1, the first of 3 domains in chicken ovomucoid, is a 68 residue glycoprotein containing 2 N-linked carbohydrate groups at asparagine residues 10 and 53 (Beeley, 1976; Kato et al., 1987). OMCHI1 and OMTKY3 share only 36% sequence identity and, relative to OMTKY3, OMCHI1 includes a nine residue insertion between the first two cystines.

To facilitate comparison of OMCHI1 stability with that of the nonglycosylated OMTKY3 and, eventually, other Kazal-type inhibitors, a gene for the recombinant form of OMCHI1 (rOMCHI1) has been designed and constructed. The design criteria were to create a DNA sequence that would be expressed well in *Escherichia coli* and easily amenable to mutagenesis at many sites simultaneously: the sequence variability between Kazal-type inhibitors can be very high, and the long-term aim is to overproduce new recombinant inhibitors readily. We wanted to increase the probability that a small, highly disulfide-bonded protein would be expressed. With *E. coli* codon optimization, rOMCHI1 is overexpressed efficiently without the help of a protein fusion system or the use of the bacterial cellular export machinery.

The thermodynamic unfolding parameters for both rOMCHI1 and glycosylated OMCHI1 (gOMCHI1) have been determined using differential scanning calorimetry (DSC). A comparison of these proteins allows us to assess the effects of glycosylation on protein stability. Furthermore, the large sequence differences between OMCHI1 and OMTKY3 will provide an opportunity to test the extent to which stabilizing interactions are conserved in structurally homologous proteins sharing little sequence identity.

MATERIALS AND METHODS

Gene Design and Construction. The gene for the recombinant form of chicken ovomucoid first domain, residues 1−68 (rOMCHI1), was assembled from 5 coding and 5 noncoding overlapping oligonucleotides consisting of 36−53 bases (Figure 1A). The cDNA sequence published by Catterall and co-workers (Catterall et al., 1980) was modified to insert new unique restriction sites and to optimize for codon usage in *E. coli*. The oligonucleotides were synthesized, 5′-phosphorylated, and purified by polyacrylamide gel electrophoresis by Bio-Synthesis, Inc. (Lewisville, TX).

Five hundred picomoles of each of the 10 oligonucleotides was combined, heated to 90 °C for 1 min, and allowed to anneal by cooling slowly to 16 °C (Figures 1A and 2A). T4 DNA ligase and ligase buffer (Promega, Madison, WI) were added, and ligation proceeded overnight at 16 °C. The ligated fragment was digested with *Bam*HI and *Hin*dIII, purified by agarose gel electrophoresis, and ligated into the plasmid pGEM3Z+ (Promega) to create the cloning plasmid pOMCHI1-C (Figure 2B); this was transformed into *E. coli* DH5α cells. Ampicillin-resistant colonies were picked, and plasmid DNA was isolated using the QIAprep Spin Plasmid Kit (Qiagen, Inc.). Plasmid DNA was sequenced by the University of Iowa College of Medicine DNA Facility.

The recombinant gene for rOMCHI1 was then subcloned into the expression plasmid pET3-d (Studier et al., 1990) using *Bam*HI and *Nco*I to create the plasmid pOMCHI1-X (Figure 2C). Plasmid pOMCHI1-X was amplified and sequenced as described above. This plasmid contains a copy of the rOMCHI1 gene under control of the phage T7 promoter ϕ10, and also contains the β-lactamase gene for ampicillin resistance (Figure 2C).

Recombinant Protein Purification. rOMCHI1 was expressed in cells grown using both flask culture and fermentation methods. For flask culture, *E. coli* BL21:DE3:pLysS cells (Studier et al., 1990) were transformed with the plasmid pOMCHI1-X and grown to saturation in 100 mL of LB liquid media (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) containing 100 mg/L ampicillin and 35 mg/L chloramphenicol at 37 °C overnight while rotating at 220 rpm. The saturated culture was used to inoculate 1.5 L of LB media containing antibiotics. Cells were then grown at 37 °C, rotating at 220 rpm to an OD_{600} of 0.9, at which point isopropyl β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM. The induced culture was allowed to grow for another 3 h before harvesting (see below).

For fermentation, *E. coli* BL21:DE3:pLysS cells transformed with the plasmid pOMCHI1-X were grown to saturation in 1 L of LB liquid media containing 200 mg/L ampicillin and 40 mg/L chloramphenicol at 37 °C overnight. Cells were pelleted by centrifugation at 5000*g* and resuspended in 50 mL of modified TB-rich media (11.8 g of casamino acids, 23.6 g of yeast extract, 9.4 g of potassium phosphate dibasic, and 2.2 g of potassium phosphate monobasic per liter). The resuspended cell pellet was added to a BioFlo II fermentor (New Brunswick Scientific Co. Inc., Edison, NJ) containing 5 L of modified TB media containing 200 mg/L ampicillin, 40 mg/L chloramphenicol, and 1 mL of antifoam A (Sigma Chemical Co., St. Louis, MO). During growth, the pH was maintained at 7.5 with additions of NaOH. When the OD_{600} was about 4, expression of rOMCHI1 was induced by addition of IPTG to a final concentration of 1 mM. Upon induction, the temperature was decreased to 27 °C, and 100 mg/L ampicillin and 20 mg/L chloramphenicol were added to maintain selective pressure on the plasmids. Cells were harvested 3 h after addition of IPTG.

Cells were harvested by centrifugation at 5000*g* in 1 L bottles for 10 min at 4 °C. Cell pellets were frozen at −80 °C overnight. Thawed pellets were resuspended in a minimal volume of 10 mM piperazine·HCl, pH 5.5. Cell walls were sheared using a French press. Cell membranes and unbroken cells were pelleted by ultracentrifugation at 4 °C for 90 min at 100000*g*. The supernatant (40 mL) was loaded onto a DE52 anion exchange column (2.5 × 15 cm) preequilibrated with 10 mM piperazine·HCl, pH 5.5. Protein was eluted from the column with a 500 mL linear gradient of buffer containing 0−0.5 M NaCl and collected in 4.5 mL fractions. Fractions were assayed for the presence of rOMCHI1 as indicated by a dark 7.5 kDa band on Coomassie-stained 17% SDS−polyacrylamide gels. Fractions containing rOMCHI1 were pooled and concentrated using an Amicon Diaflo Ultrafilter with a YM3 (3000 MW cutoff) membrane (Amicon, Beverly, MA). Concentrated samples were loaded onto a Sephadex G-50 column (2.5 × 42 cm) preequilibrated in 15 mM ammonium bicarbonate. Fractions (4.5 mL) were again assayed on SDS−polyacrylamide gels for the presence of the rOMCHI1 band. Fractions containing recombinant protein were pooled and lyophilized.

Final purification of rOMCHI1 was carried out by HPLC on a Hamilton reverse phase PRP-1 column (0.5 × 30 cm). Protein was loaded onto the column in 95% H_2O (with 0.1% TFA) and 5% organic phase (10% H_2O, 30% isopropyl alcohol, and 60% acetonitrile), and eluted from the column with a gradient of 5−60% organic phase over a period of 90 min at a flow rate of 1 mL/min. Elution was monitored by the absorbance at 276 nm, and rOMCHI1 eluted at about 60 min. Fractions containing rOMCHI1 were pooled, lyophilized, and stored at −20 °C. rOMCHI1 was judged ≥96% pure by amino acid analysis and amino-terminal sequencing (Protein Structure Facility, University of Iowa). The protein's identity was also confirmed with two-dimensional proton NMR spectroscopy.

Purification of Glycosylated OMCHI1. Whole chicken ovomucoid (OMCHI) was purified by the method of Kato et al. (1987). The glycosylated first domain of whole OMCHI (gOMCHI1) was purified using a modification of previously published protocols (Griko & Privalov, 1984; Kato et al., 1987). Cyanogen bromide (CNBr) cleavage was carried out in 70% formic acid with 10 mg/mL whole OMCHI and 10 mg/mL CNBr in the dark under N_2 gas atmosphere with slow stirring for 18 h. Nine volumes of water were then added, and the mixture was lyophilized.

The lyophilized product was dissolved in 4 M acetic acid and loaded onto a Sephadex G-50 size exclusion column (2.5 × 120 cm) which was preequilibrated in 4 M acetic acid. Protein was eluted with a flow rate of 20 mL/h, and 4.5 mL fractions were collected. Fractions were assayed for the presence of gOMCHI1 as indicated by a 10−12 kDa band on Coomassie-stained 17% SDS−polyacrylamide gels. Fractions containing the first domain were pooled and lyophilized. Lyophilized powder was dissolved in 20 mM sodium citrate buffer, pH 4.5, and loaded onto a CM-Sepharose cation exchange column (1.5 × 24 cm) preequilibrated in the same buffer. Pure gOMCHI1 was collected in the flow-through in 4 mL fractions and at a flow rate of 15 mL/h. Peak fractions containing gOMCHI1 were pooled and lyophilized. Lyophilized powder was dissolved in 15 mM ammonium bicarbonate, and loaded onto a Sephadex G-25 desalting column (1.5 × 34 cm) equilibrated in the same buffer. Protein was eluted at a flow rate of 35 mL/h. Fractions containing gOMCHI1 were pooled, lyophilized, and stored at −20 °C.

Nuclear Magnetic Resonance Spectroscopy. NMR samples were 2 mM in either rOMCHI1 or gOMCHI1 in 90% H_2O/10% D_2O containing 200 mM KCl and 0.02% sodium azide at pH 4. Samples also contained TSP as a chemical shift reference. NMR data were acquired on a Varian UNITY spectrometer located in the University of Iowa College of Medicine Nuclear Magnetic Resonance Facility. The instrument operates at a proton frequency of 500 MHz and is equipped with an IDT-500 probe from Nalorac Cryogenics Corp. (Martinez, CA). The spectrometer's variable temperature controller was calibrated using a methanol standard (van Geet, 1968).

During data acquisition, the carrier frequency of the NMR spectrometer was set on the water signal, and the spectral width was 6000 Hz. Eight steady-state pulses were applied at the beginning of each experiment. The relaxation delay in all experiments was 1.5 s. Data sets were the sum of 32 transients, each with 8196 time-domain points. Prior to Fourier transformation, data were multiplied by a Lorentzian function with a line broadening of 1 Hz.

Differential Scanning Calorimetry. Recombinant or glycosylated protein (1.5−2.5 mg/mL) was extensively dialyzed against 4 L of buffer containing 10 mM potassium acetate and 10 mM potassium phosphate at the desired pH. Protein solutions were then centrifuged to remove particulates and degassed immediately before transfer to the calorimeter. Sample pH was measured with an Orion digital pH meter, Model 611. Protein concentration was determined by the method of Edelhoch (1967) as modified by Pace and coworkers (Pace et al., 1995). The extinction coefficient for both glycosylated and recombinant proteins at 276 nm is 3412 M^{-1} cm^{-1}, with an estimated error of 5%.

Differential scanning calorimetry (DSC) was carried out on a DASM-1 microcalorimeter modified by The Johns Hopkins University Biocalorimetry Center. Blank scans consisted of the buffer without protein. Scans were carried out in the temperature range of 10−120 °C at a scan rate of 1 °C/min, where the protein was found to be in thermal equilibrium at all experimental pHs (data not shown). Outgassing of solutions during heating was prevented by applying 17 psi pressure to the sample and buffer cells.

DSC data were fit to a two-state model for unfolding using CPPLUS6 for the Macintosh (E. Freire, Johns Hopkins University, Baltimore, MD). After subtraction of the buffer base line, final base lines were established by subtracting both second-order polynomials and a sigmoidal curve from the data [modification of Swint-Kruse and Robertson (1995)]. Each data set was fit with a number of different base lines to determine ΔH°_m and T_m with the smallest variance. Errors of the fit were determined for ΔH°_m using F analysis, as described previously (Bevington & Robinson, 1992; Swint-Kruse & Robertson, 1995).

The solvent-accessible surface area, ASA, was evaluated with the Lee and Richards algorithm (Lee & Richards, 1971) with a 1.4 Å probe radius and a slice width of 0.25 Å using the program ACCESS written by Scott Presnell (University of California, San Francisco). The ASA of the unfolded state was taken as the sum of the residue areas in an extended tripeptide, Ala-Xaa-Ala. The buried surface area (changes in the accessible surface area, ΔASA) is the difference between the ASA of the unfolded state and the ASA of the native state as determined from the crystal structure. For these calculations, the crystallographic structure of the ovomucoid third domain from silver pheasant (OMSVP3), PDB file 2OVO, was used. OMSVP3 differs from OMTKY3 by having a methionine instead of leucine at residue 18, which is exposed to solvent.

RESULTS

Gene Design and Expression. OMCHI1 is N-glycosylated at two asparagine residues, Asn 10 and Asn 53. This heterogeneous carbohydrate adds, on average, approximately 4 kDa of complex glycan to the protein (Beeley, 1976; Kato et al., 1987; Yamashita et al., 1982, 1983; Yet et al., 1988). To examine the effect of glycosylation on OMCHI1 stability and to facilitate comparisons of OMCHI1 stability with other nonglycosylated Kazal-type serine protease inhibitors, a synthetic gene for recombinant OMCHI1 (rOMCHI1) has been constructed and expressed in *E. coli.* Removal of the

A

```
          Hind III            Nco I                           Pst I
                 10                  20                  30                  40                  50                  60
G C G C A/A G C T T C C C C/C A T G G C A G A A G T T G A C T G C A/G C A:G G T T C C C G A A C G C T A C T G A C A A A
C G C G T T C G A/A G G G G G T A C/C G T C T T C A A C T G/A C G T C G T C C A A G G G C T T G C G:A T G A C T G T T T
                               Met   Ala   Glu   Val   Asp   Cys   Ser   Arg   Phe   Pro   Asn   Ala   Thr   Asp   Lys

                              Spe I                                                              Kpn I
                 70                  80                  90                 100                 110                 120
G A A G G C A A A G A T G T A/C T A G T T T:G C A A C A A A G A C C T G C G T C C G A T C T G T G/G T A C C G A T G G C
C T T C C G T T T C T A C A T G A T C/A A A C G T T G T T T C T G G A C G C A G G C:T A G A C A C C A T G/G C T A C C G
 Glu   Gly   Lys   Asp   Val   Leu   Val   Cys   Asn   Lys   Asp   Leu   Arg   Pro   Ile   Cys   Gly   Thr   Asp   Gly

                                                                                      EcoR I
                130                 140                 150                 160                 170                 180
G T T A C T T A C A C T A A:C G A T T G C T T G C T G T G T G C T T A C A G C A T C G/A A T T C G G C A C C A A C A T C
C A A T G A A T G T G A T T G C T A A C G A A C G A C A C A C G:A A T G T C G T A G C T T A A/G C C G T G G T T G T A G
 Val   Thr   Tyr   Thr   Asn   Asp   Cys   Leu   Leu   Cys   Ala   Tyr   Ser   Ile   Glu   Phe   Gly   Thr   Asn   Ile

                                                                                            BamH I
                190                 200                 210                 220                 230
A:G C A A A G A G C A C G A T G G C G A A T G C A A A G A A A C T G T T C C G A T G T G A G/G A T C C G C G
T C G T T T C T C G T G C T A C C G:C T T A C G T T T C T T T G A C A A G G C T A C A C T C C T A G/G C G C
 Ser   Lys   Glu   His   Asp   Gly   Glu   Cys   Lys   Glu   Thr   Val   Pro   Met   ***
```

B

```
OMCHI1:     ⇩        20        30        40        50  ⇩     60
   AEVDCSRFPNATDKEGKDVLVCNKDLRPICGTDGVTYTNDCLLCAYSIEFGTNISKEHDGECKETVPM

OMTKY3:
LAAVSVDCSEYPKPA---------CTLEYRPLCGSDNKTYGNKCNFCNAVVESNGTLTLSHFGKC
```

FIGURE 1: (A) Optimized gene sequence for rOMCHI1. The original cDNA sequence from Catterall and co-workers (Catterall et al., 1980) was optimized for codon usage. Colons in the sequence indicate the lengths of synthetic oligonucleotides. Backslashes in the gene show unique sites where restriction endonucleases (boldface text) cut the sequence. (B) Alignment of the amino acid sequences of rOMCHI1 and OMTKY3. Hyphens indicate the site at which OMTKY3 differs by deletion relative to rOMCHI1. Arrows in the OMCHI1 sequence show sites of N-linked carbohydrate in gOMCHI1.

carbohydrate chains has also aided NMR assignments, now underway, by decreasing line widths and simplifying the proton NMR spectrum of the protein.

The OMCHI1 cDNA sequence, as determined by Catterall and co-workers (Catterall et al., 1980), was modified to facilitate future mutagenesis experiments and to take advantage of codons that are highly abundant in *E. coli* (Bulmer, 1987; Kane, 1995). Figure 1A shows the final optimized gene sequence for rOMCHI1. Thirty-five percent of the codons in the original cDNA sequence were changed to conform with efficient *E. coli* codon usage and to integrate 10 new unique restriction sites into the gene for future mutagenesis experiments.

The expression plasmid pOMCHI1-X was used to overexpress rOMCHI1 in *E. coli* cells upon addition of IPTG. The details of the purification scheme for rOMCHI1 are outlined under Materials and Methods. Protein expression from *E. coli* cultures grown in a fermentor have a final protein yield of about 40 mg/L while regular flask culture growths yield about 10 mg/L. Final purified rOMCHI1 appears as a single band (approximately 7.5 kDa) on silver-stained SDS−PAGE. rOMCHI1 was judged ≥96% pure by amino acid analysis, and amino-terminal sequencing gave the first five correct residues (Protein Structure Facility, University of Iowa). The remaining 4% consists of rOMCHI1 species whose sequences begin with alternatively cleaved amino termini whose sequences begin with either methionine −1, glutamate 2, or valine 3.

NMR Spectroscopy. Figure 3 shows the 1D ^{1}H NMR spectra of rOMCHI1 and gOMCHI1. Upfield-shifted resonances in the gOMCHI1 spectrum are also present in the spectrum of the recombinant protein. Similarly, downfield-shifted amides in the gOMCHI1 spectrum are also present in the rOMCHI1 spectrum. Two-dimensional NMR data show that resonances from methyl-containing amino acids in rOMCHI1 and gOMCHI1 have similar chemical shifts (data not shown). Taken together, the 1D and 2D NMR data for rOMCHI1 show that the purified recombinant protein is correctly folded and that glycosylation does not affect the chemical shifts of methyl-containing side chains.

The carbohydrate chains covalently attached to gOMCHI1 on Asn 10 and Asn 53 (Beely, 1976; Kato et al., 1987) have been extensively characterized (Yamashita et al., 1982, 1983; Parente et al., 1983; Yet et al., 1988). These studies have shown that the complex oligosaccharides attached to gOMCHI1 consist primarily of *N*-acetylglucosamine (GlcNAc), mannose, and galactose.

A qualitative comparison of the NMR spectra (Figure 3) shows that the major differences between rOMCHI1 and gOMCHI1 spectra are due to chemical shifts arising from sugar residues. In the gOMCHI1 NMR spectrum, H1 anomeric proton resonances fall between 5.2 and 4.6 ppm, sugar ring proton resonances are found between 4.6 and 3.5 ppm, and acetyl methyl proton resonances from GlcNAc sugars are around 2.0 ppm, while NH proton resonances from GlcNAc sugars fall between 8.0 and 9.0 ppm (Vliegenthart et al., 1983; Davis et al., 1994). The extra signal near 0 ppm in the gOMCHI1 spectrum is observed in multiple independent preparations and its origin is as yet unknown.

Calorimetry. Raw excess heat capacity data for rOMCHI1 and gOMCHI1 are plotted in Figure 4. OMCHI1 that has been subjected to very high temperatures at acidic pH shows multiple bands on SDS−polyacrylamide gels (data not shown), which is characteristic of irreversible processes such

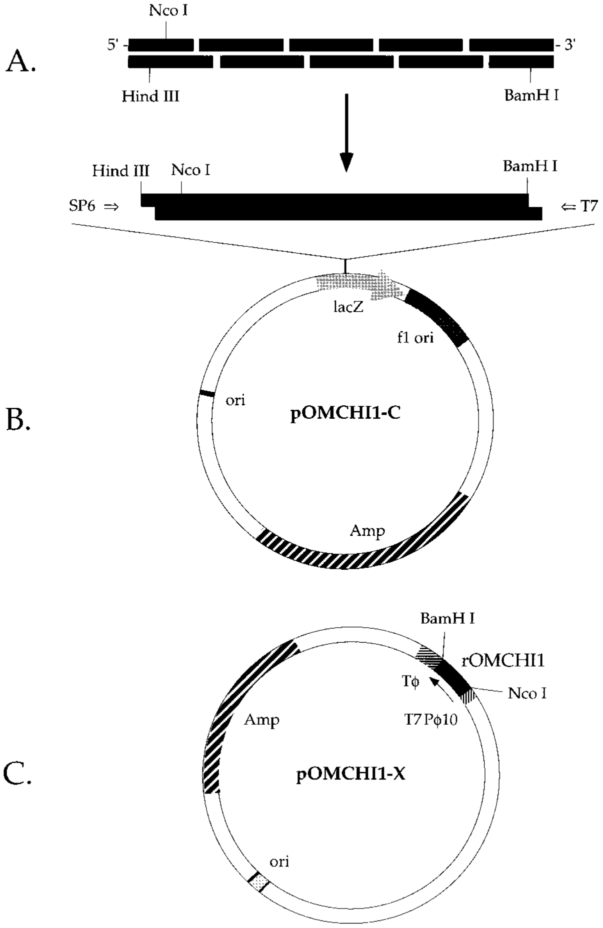

FIGURE 2: Construction and cloning of the rOMCHI1 gene. (A) Outline of strategy for annealing and ligating 10 oligonucleotides from Figure 1A into the full gene fragment. (B) The ligated fragment (from A) was inserted into the polylinker region of plasmid pGEM3Z+, creating the cloning plasmid pOMCHI1-C. (C) Subcloning the fragment between sites *Nco*I to *Bam*HI into plasmid pET3-d gave the expression plasmid pOMCHI1-X.

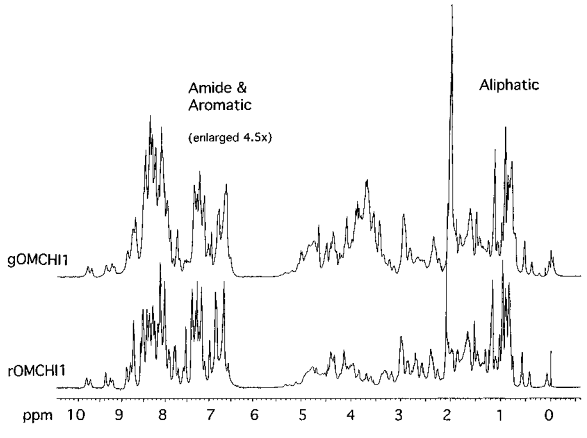

FIGURE 3: 1D ^{1}H-NMR spectra of gOMCHI1 and rOMCHI1 at 30 °C. Protein concentrations were 2 mM in 0.2 M KCl at pH 4. Samples also included 10% D_2O for a lock signal and 0.02% sodium azide as a bacterial inhibitor. Spectra were referenced to internal TSP at 0 ppm.

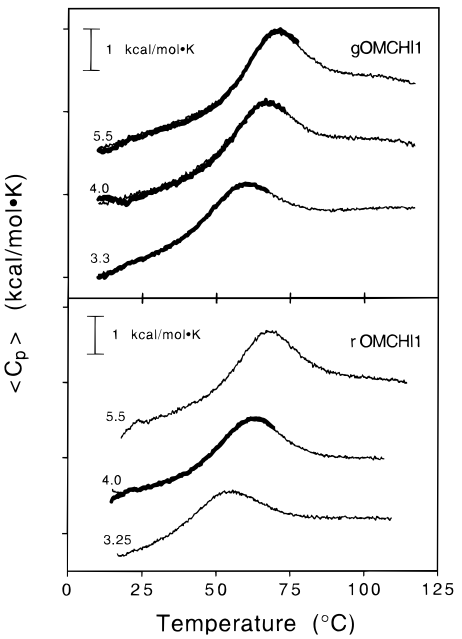

FIGURE 4: Excess heat capacity data for gOMCHI1 and rOMCHI1 after subtraction of a buffer scan and normalization to protein concentration. Protein samples were 1.5−2.5 mg/mL in 10 mM potassium acetate and 10 mM potassium phosphate at the indicated pH. Scan rate was 1 °C/min. Reversibility is shown for a few representative scans with thermal denaturation running just past the midpoint of unfolding (overlaid boldface lines). Data were offset vertically to aid inspection of the data.

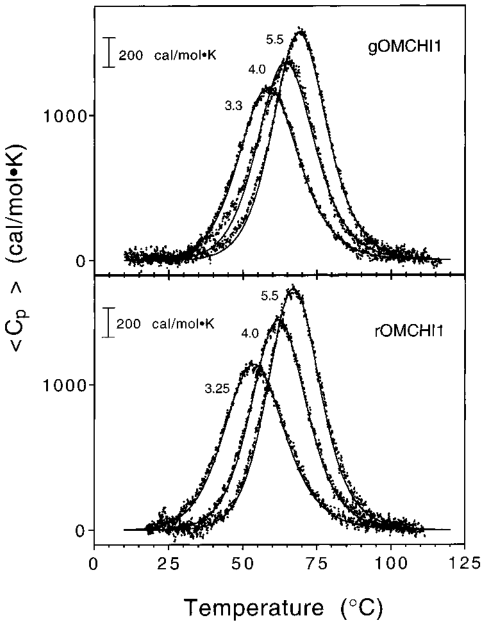

FIGURE 5: Excess transitional heat capacities for gOMCHI1 and rOMCHI1. Sample conditions were as in Figure 4 at the indicated pH. The continuous solid lines represent fits of the data to a two-state unfolding model.

as side chain chemical modifications and polypeptide hydrolysis (Zale & Klibanov, 1986). In order to minimize irreversible denaturation and assess reversibility through the thermal transition, both recombinant and glycosylated proteins were heated just past the midpoint of denaturation, cooled to low temperature, and then heated to high temperature in the second scan. The first and second scans superimpose well in all cases; representative data are shown in Figure 4. Thermal denaturation of both rOMCHI1 and gOMCHI1 is therefore highly reversible in the transition region.

The thermal unfolding transitions for both rOMCHI1 and gOMCHI1 are broad (Figure 4), and the variability in the pre- and post-transitional base lines precludes the direct determination of a small ΔC_p, the change in the heat capacity

Table 1: Thermodynamic Parameters of OMCHI1 Unfolding in 10 mM Potassium Acetate, 10 mM Potassium Phosphate

pH	$\Delta H°_{\mathrm{m}}$ (kcal/mol)[a]	T_{m} (°C)[b]	$\Delta H_{\mathrm{vH}}/\Delta H_{\mathrm{cal}}$
	gOMCHI1		
5.50	38.1 (0.3)	69.3	0.99
4.75	38.6 (0.3)	69.8	1.00
4.50	37.1 (0.3)	68.9	1.00
4.30	35.3 (0.3)	67.9	1.01
4.00	35.1 (0.5)	64.7	1.06
3.60	31.2 (0.4)	61.3	1.01
3.30	32.3 (0.3)	59.0	1.01
	rOMCHI1		
5.50	39.1 (0.4)	67.4	0.97
4.75	38.1 (0.4)	66.6	0.96
4.00	35.4 (0.4)	61.6	0.99
4.00	35.0 (0.3)	62.4	0.98
3.75	34.9 (0.4)	60.8	1.00
3.50	33.0 (0.3)	56.7	1.03
3.50	32.7 (0.3)	57.7	0.98
3.25	31.2 (0.3)	54.2	0.96

[a] Numbers in parentheses are fitting errors derived from *F* analysis (see Materials and Methods). [b] The fitting error in T_{m} is 0.2 °C.

upon unfolding, from a single scan. Since fitted values of ΔC_p are sensitive to the choice of base lines used, the data in Figure 4 were adjusted to remove ΔC_p as a fitting parameter: a second-order polynomial and a sigmoidal curve were subtracted from the data to yield the transition excess heat capacity (Figure 5; Freire, 1995). The values of $\Delta H°_{\mathrm{m}}$ and T_{m} for rOMCHI1 and gOMCHI1 were insensitive to the choice of base line used, as previously observed for other proteins (Privalov & Khechinashvili, 1974; Swint-Kruse & Robertson, 1995). The solid lines in Figure 5 represent the best fit of these data to a two-state unfolding model with ΔC_p fixed at zero. The data were also fit to a three-state unfolding model, but no significant improvement was observed in the minimized sum of the squared residuals over that obtained with the two-state model. Thermodynamic parameters characterizing the unfolding transitions of rOMCHI1 and gOMCHI1 are reported in Table 1. In the pH range 3−5.5, the midpoint of OMCHI1 thermal unfolding varied from about 54 to 70 °C, which resulted in calorimetric enthalpies, ΔH_{cal}, ranging from 31 to 39 kcal/mol.

The calorimetric enthalpies are in good agreement with van't Hoff enthalpies (Table 1) calculated using the equation (Privalov & Khechinashvili, 1974):

$$\Delta H_{\mathrm{vH}} = \frac{4RT_{\mathrm{m}}^{2}C_{\mathrm{p,max}}}{\Delta H_{\mathrm{cal}}} \qquad (1)$$

where R is the gas constant, $C_{p,\mathrm{max}}$ is the excess heat capacity at T_{m}, and ΔH_{cal} is the area under the transition heat capacity curve. Ratios of $\Delta H_{\mathrm{vH}}/\Delta H_{\mathrm{cal}}$ (Table 1) are close to 1, which is consistent with an absence of significantly populated intermediates in the thermal transition.

Figure 6A shows the dependence of $\Delta H°_{\mathrm{m}}$ on T_{m} for both rOMCHI1 and gOMCHI1. The slopes of these plots give ΔC_p (Privalov & Potekhin, 1986). Linear regression of the data for rOMCHI1 (●) and gOMCHI1 (□) yields essentially identical ΔC_p values of 582 ± 40 cal/(mol·K) and 619 ± 180 cal/(mol·K), respectively.

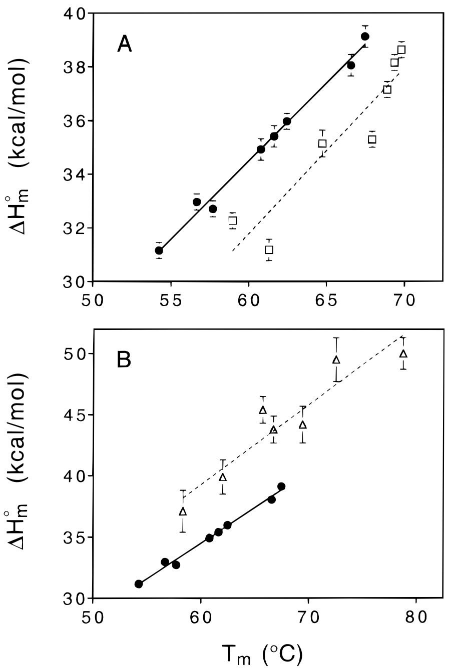

FIGURE 6: (A) Temperature dependence of $\Delta H°_{\mathrm{m}}$ for gOMCHI1 (□) and rOMCHI1 (●). The solid and dashed lines were determined by linear regression of data in Table 1. Error bars indicate fitting errors associated with $\Delta H°_{\mathrm{m}}$ values. (B) Temperature dependence of $\Delta H°_{\mathrm{m}}$ for rOMCHI1 (●) and OMTKY3 (△). The solid line representing the ΔC_p for OMTKY3 was determined previously (Swint-Kruse & Robertson, 1995). Fitting errors in $\Delta H°_{\mathrm{m}}$ for rOMCHI1 are masked by the symbol on this scale.

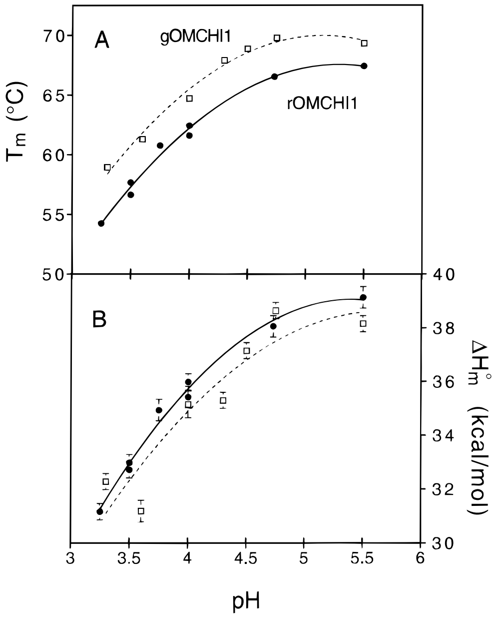

FIGURE 7: pH dependence of the unfolding parameters characterizing gOMCHI1 (□) and rOMCHI1 (●) stability: (A) T_{m} versus pH; (B) $\Delta H°_{\mathrm{m}}$ versus pH. The continuous lines represent fits of the data to a second-order polynomial and are to assist inspection of the data. Error bars in (A) represent estimated errors in determination of T_{m}, while error bars in (B) represent fitting errors in $\Delta H°_{\mathrm{m}}$.

In Figure 7, the parameters characterizing thermal unfolding of rOMCHI1 and gOMCHI1 are plotted versus pH. Figure 7A shows how T_{m} varies with pH while 7B shows how $\Delta H°_{\mathrm{m}}$ varies with pH. The data for rOMCHI1 (●) and gOMCHI1 (□) show that the $\Delta H°_{\mathrm{m}}$ for both proteins is the same at any given pH (Figure 7B). In contrast, the presence of carbohydrate increases the T_{m} for gOMCHI1 over rOMCHI1 at all pHs (Figure 7A). The difference in the midpoints of thermal unfolding, ΔT_{m}, between the two proteins is approximately 4 °C at low pH and decreases to 2 °C near pH 5.5. The increase in T_m amounts to an increase in the free energy of unfolding, $\Delta G°_u$, of about 0.5 kcal/mol for gOMCHI1 over rOMCHI1. Since the $\Delta H°_m$ of unfolding for both proteins is similar, the increase in $\Delta G°_u$ for gOMCHI1 is largely due to an entropic stabilization in the presence of carbohydrate.

DISCUSSION

The T_m values for gOMCHI1 are in good agreement with those previously reported (Griko & Privalov, 1984). However, the $\Delta H°_m$ values reported in the present study are 8−35% less than the values reported by the earlier study. The discrepancy in $\Delta H°_m$ increases with increasing T_m. One result is an apparent ΔC_p from the previous study of about 1.9 kcal/(mol·K), which is 34 cal/(mol·K) per residue. This value is high relative to the average ΔC_p value of 14 ± 2 cal/(mol·K) per residue (Privalov & Gill, 1988). We thus believe that the $\Delta H°_m$ and ΔC_p values from the present study are more accurate than those of the previous study. One simple explanation for the discrepancies between the two studies is differences in estimates of protein concentration: results of the two studies may be reconciled by increasing the estimated protein concentrations in the previous study by about 2-fold.

Effects of Glycosylation on OMCHI1 Stability. Increased thermal stability for proteins with N- and O-linked carbohydrate has been shown previously. Ribonuclease B (RNase B), the N-linked glycosylated form of RNase A, shows a 1 °C increase in T_m and is also stabilized against chemical denaturation (Grafl et al., 1987). A fucosylated proteinase inhibitor, the major peptide C from *Pars intercerebralis*, shows about a 20 °C increase in T_m (Mer et al., 1996). Wang and co-workers found that for three proteins with a variety of carbohydrate linkages, yeast external invertase, bovine serum fetuin, and glucoamylase, the presence of glycosylation increases T_m by about 1−3 °C (Wang et al., 1996).

Interestingly, Wang and co-workers found that the presence of heterogeneous carbohydrate does not alter the cooperativity of the unfolding transitions as evidenced by the ratio of ΔH_{vH} to ΔH_{cal}. Similarly, the oligosaccharides covalently attached to gOMCHI1 are heterogeneous (Yamashita et al., 1982; 1983; Yet et al., 1988), and do not show any effect on the cooperativity of unfolding (Figure 5, Table 1). This feature of the thermodynamics of glycoprotein unfolding may be attributable to (a) subtle differences among the various species of carbohydrate chains that are below the level of detection by DSC or (b) the entire effect of the attached carbohydrate being exercised by the first few sugar residues of the carbohydrate chain (Gerken et al., 1989; Wormald et al., 1991; Andreotti & Kahne, 1993; Liang et al., 1995; Wyss et al., 1995; Mer et al., 1996).

Proposed mechanisms of stabilization by glycosylation include solubilizing the denatured state and reducing irreversible denaturation (Wang et al., 1996), forming hydrogen bonds (Wyss et al., 1995; Mer et al., 1996), shielding or reinforcing hydrophobic patches (Blum et al., 1993; Mer et al., 1996), and stabilizing clusters of unfavorable charged residues (Wyss et al., 1995). Figure 7 shows that gOMCHI1 thermal stabilization appears to be entropic in nature: ΔC_p and $\Delta H°_m$ for both rOMCHI1 and gOMCHI1 are similar at all experimental pHs (Figures 6A and 7B). One way that an entropic stabilization may be manifested in gOMCHI1 is by reducing disorder in unfolded gOMCHI1 relative to rOMCHI1.

Several studies have shown that the addition of carbohydrate can lead to a reduction in disorder in polypeptide chains. Davis and co-workers (Davis et al., 1994) have studied the effects of glycosylation on a 10 residue glycopeptide from OMCHI1 which contains the Asn-linkage site, Asn 53. Their NMR study indicates that glycosylation significantly decreases the peptide's conformational mobility around Asn 53 but does not alter the peptide backbone conformation. Similar results were obtained by Wormald and co-workers (Wormald et al., 1991) on glycopeptides isolated from immunoglobulin M (IgM).

Kahne and co-workers used NMR to study small flexible peptides containing varying amounts of O-linked sugar residues in order to evaluate the effects of glycosylation on peptide backbone conformation (Andreotti & Kahne, 1993; Liang et al., 1995). Their results are consistent with unstructured peptides that may be driven into a β-turn conformation upon glycosylation. The authors suggested that conformational changes that arise by the addition of carbohydrate stem from a steric exclusion of many unstructured states and "...may influence protein thermal stability in a similar manner by restricting conformational space" (Andreotti & Kahne, 1993). Similar conclusions have been reached in more recent studies of glycopeptides containing N-linked carbohydrate (Imperali & Rickert, 1995; Live et al., 1996).

Our hypothesis to explain increased thermal stability in gOMCHI1 is that N-linked carbohydrate chains at asparagine residues 10 and 53 sterically restrict backbone mobility and the number of backbone conformations available in the denatured state of gOMCHI1. This decrease in disorder of the unfolded state of gOMCHI1, relative to rOMCHI1, thus stabilizes gOMCHI1 by reducing the entropy of unfolding.

Comparison of rOMCHI1 and OMTKY3 Stability. The present study is one in a series examining the relationship between structure and energetics in the Kazal family of serine protease inhibitors. OMTKY3 stability has been studied extensively in our laboratory (Swint & Robertson, 1993; Schaller & Robertson, 1995; Swint-Kruse & Robertson, 1995, 1996). OMTKY3 and rOMCHI1 share conserved cysteine residues and disulfide bonds, but are only 36% identical in sequence (Figure 1B; Kato et al., 1987). rOMCHI1 is a larger protein than OMTKY3 (68 versus 56 residues) and contains a 9 residue insertion in the first disulfide loop. Relative to OMTKY3, rOMCHI1 has three fewer residues at the amino-terminus and six additional residues beyond the last half-cystine in the sequence. Structural studies of OMTKY3 demonstrate that the amino-terminal residues are highly mobile, suggesting that they do not play a major role in stability. Similar information regarding the carboxy-terminal residues of rOMCHI1 is lacking, but we suspect that these residues are also mobile and not contributing to the stability of the protein. rOMCHI1 contains twice as many charged residues as OMTKY3 (20 versus 10), and most of this increase is due to a larger number of negatively charged residues.

The ΔC_p for rOMCHI1 unfolding, 582 ± 40 cal/(mol·K), is statistically identical to the ΔC_p of OMTKY3, 620 ± 20 cal/(mol·K) (Figure 6B; Swint-Kruse & Robertson, 1995). However, OMTKY3 is thermally stabilized over rOMCHI1: the midpoint of unfolding for OMTKY3 is almost

20 °C higher than that for rOMCHI1 at any given acidic pH. Furthermore, the $\Delta H°_m$ for rOMCHI1 at any given temperature is lower than that for OMTKY3 by about 5 kcal/mol. Calculations based on the ΔC_p and $\Delta H°_m$ for these two proteins show that a 20 °C difference in T_m, observed over the entire experimental pH range, corresponds to an approximately 2.5 kcal/mol greater free energy for OMTKY3. This net increase in free energy for OMTKY3 is primarily enthalpic: both the entropy of unfolding, ΔS_m, and $\Delta H°_m$ are greater in OMTKY3, but the increase in $\Delta H°_m$ is larger than the increase in $T\Delta S_m$.

The pH dependence of T_m can be used to calculate the number of protons, Δn, taken up by the protein upon unfolding (Privalov, 1979):

$$\Delta n = \frac{\Delta H°_m}{2.303RT_m}\frac{\partial T_m}{\partial \mathrm{pH}} \tag{2}$$

At pH values ranging from 3.25 to 4.00, Δn for rOMCHI1 ranges from 0.79 to 0.55 while Δn for OMTKY3 is significantly greater, 1.11−0.76. The pH dependence of OMTKY3 stability at acid pH is due primarily to the low pK_as of Asp 7 and Asp 27 in the native state relative to the denatured state (Schaller & Robertson, 1995; Swint-Kruse & Robertson, 1995). These residues are conserved in rOMCHI1, but the smaller Δn values for rOMCHI1 suggest that the pK_a values for these residues may not be perturbed to the extent seen in native OMTKY3. This is interesting in light of the large difference in the number of carboxyl groups in rOMCHI1 (14) versus OMTKY3 (6).

The relative magnitude of Δn for the two proteins changes above pH 4: Δn at pH 4.5 for rOMCHI1 and OMTKY3 is 0.34 and 0.07, respectively. The model compound pK_a values for glutamate cluster around 4.5 while those for aspartate are closer to 4.0. Proton uptake due to perturbed aspartate residues should thus be negligible at pH 4.5 (Yang & Honig, 1993). Hence, one explanation for a larger Δn in rOMCHI1 at pH 4.5 is a glutamate residue, not present in OMTKY3, with a low pK_a in the native state.

The structural basis for differences in unfolding thermodynamics for rOMCHI1 and OMTKY3 will be best understood when a structure for rOMCHI1 is available. In the interim, however, the structural energetic formalism of Freire and co-workers provides a framework for interpreting relationships between unfolding thermodynamics and protein structure (Murphy & Freire, 1992; Murphy et al., 1993). This formalism is based on parameterization of the unfolding thermodynamics for proteins of known structure with respect to changes in solvent-exposed polar and nonpolar surface area. The accuracy of the structural energetic approach is exemplified by the case of OMTKY3. Using the Lee and Richards algorithm (Lee & Richards, 1971), the change in the accessible surface area upon unfolding, ΔASA, for OMTKY3 is 4156 Å^2. This is similar to the ΔASA value for OMTKY3 derived from unfolding thermodynamics and the structural energetic formalism, 4380 ± 400 Å^2. Furthermore, ΔASA from apolar (ΔASA_{ap}) and polar (ΔASA_p) buried surface areas based on the X-ray structure give values of 2162 Å^2 and 1878 Å^2, respectively, which are also in reasonable agreement with values based on unfolding thermodynamics, 2480 ± 230 Å^2 and 1900 ± 170 Å^2, respectively. Errors in ΔASA are propagated from estimated uncertainties in both the unfolding thermodynamics and the structural energetic coefficients (Murphy & Freire, 1992).

Using this approach for rOMCHI1, the unfolding thermodynamics suggests that the protein exposes a smaller amount of buried surface area, 3910 ± 400 Å^2, than OMTKY3 upon unfolding even though rOMCHI1 is a larger protein. The decrease in ΔASA for rOMCHI1 relative to OMTKY3 is evenly distributed between ΔASA_{ap} and ΔASA_p. While none of the differences in ΔASA is statistically significant, the trend of a relatively small ΔASA for rOMCHI1 is consistent with arguments, presented below, based on extrapolation of thermodynamic parameters to convergence temperatures.

Unfolding thermodynamics for rOMCHI1 can be compared to other small globular proteins through extrapolation of the enthalpies and entropies of unfolding to the convergence temperatures T_H* and T_S*, when ΔC_p is assumed to be temperature-independent (Privalov & Khechinashvili, 1974; Privalov, 1979; Privalov & Gill, 1988). T_H* and T_S* are temperatures where it has been hypothesized that apolar contributions to ΔH and ΔS are zero (Murphy et al., 1990; Murphy & Gill, 1990). The extrapolated value of $\Delta H°_m$, ΔH*, reflects the polar contributions to the enthalpy of unfolding while the extrapolated value of ΔS_m, ΔS*, is a measure of the configurational contribution to the entropy of unfolding. For a set of 12 globular proteins, T_H* is 100.5 °C and ΔH* is 1.35 ± 0.11 kcal/(mol•res), while T_S* is 112 °C and ΔS* is 4.30 ± 0.12 cal/(mol•K•res) (Murphy & Gill, 1990). More recent observations suggest that ΔH* and ΔS* are generally smaller for small globular proteins (McCrary et al., 1996).

The value of ΔH* for rOMCHI1 is 0.9 kcal/(mol•res), which is at the low end of values for other small globular proteins: 1.1 for ubiquitin (76 residues), 1.3 for protein G (56 residues), 1.0 for Sac7d (66 residues), and 1.2 for OMTKY3 (56 residues). The value of ΔS* for rOMCHI1 is 2.8 cal/(mol•K•res), which is much smaller than values from other small globular proteins: 3.2 for ubiquitin, 3.9 for protein G, 3.3 for Sac7d, and 3.7 for OMTKY3. The fact that ΔH* and ΔS* for rOMCHI1 are less than values for other small proteins is consistent with the picture derived from structural energetic calculations: relative to other proteins, a smaller fraction of the residues in rOMCHI1 participate in stabilizing the native structure. In other words, rOMCHI1 appears to have a more disordered native state relative to OMTKY3 and other small proteins.

One hypothesis to explain a more disordered native state is that the nine residue insertion in rOMCHI1 does not adopt a well-defined conformation (Figure 1B). This insertion is in the first disulfide loop of the protein. In the Kazal family of proteins, this region is the most variable with respect to the number of residues, ranging from 1 to 16 amino acids (Kato et al., 1987; Mühlhahn et al., 1994; van de Locht et al., 1995). This region generally does not adopt a well-defined secondary structure, and crystallographic thermal factors for the peptide backbone tend to be greater than the average values for the entire molecule (Bode et al., 1986; Klaus & Schomburg, 1993; Liepinsh et al., 1994; Hoogstraten et al., 1995). These observations are consistent with a statistical analysis of insertions in homologous proteins of known structure: short insertions of 1−15 residues usually adopt turn or random coil structures (Pascarella & Argos, 1992). Nevertheless, other explanations for energetic differences between rOMCHI1 and OMTKY3 are certainly plausible, such as a rearrangement of the hydrophobic core or effects from the overall increase in the number of charged residues in rOMCHI1.

ACKNOWLEDGMENT

We thank Eric Vincent for advice on cloning the rOMCHI1 gene, Dr. John Shriver for communicating results prior to publication, and Dr. Kenneth Murphy for valuable advice and discussions in carrying out and interpreting DSC experiments. We also thank Dr. David Gibson and Juan Parales for generous assistance with fermentation.

REFERENCES

Andreotti, A. H., & Kahne, D. (1993) *J. Am. Chem. Soc. 115*, 3352−3353.

Apostol, I., Giletto, A., Komiyama, T., Zhang, W. L., & Laskowski, M., Jr. (1993) *J. Protein Chem. 12*, 419−433.

Beeley, J. G. (1976) *Biochem. J. 159*, 335−345.

Bevington, P. R., & Robinson, D. K. (1992) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill Inc., New York.

Blum, M. L., Down, J. A., Gurnett, A. M., Carrington, M., Turner, M. J., & Wiley, D. C. (1993) *Nature 362*, 603−609.

Bode, W., Epp, O., Huber, R., Laskowski, M., Jr., & Ardelt, W. (1985) *Eur. J. Biochem. 147*, 387−395.

Bode, W., Wei, A.-Z., Huber, R., Meyer, E., Travis, J., & Neumann, S. (1986) *EMBO J. 5*, 2453−2458.

Bolognesi, M., Gatti, G., Menegatti, E., Guarneri, M., Marquart, M., Papamokos, E., & Huber, R. (1982) *J. Mol. Biol. 162*, 839−868.

Bulmer, M. (1987) *Nature 325*, 728−730.

Catterall, J. F., Stein, J. P., Kristo, P., Means, A. R., & O'Malley, B. W. (1980) *J. Cell Biol. 87*, 480−487.

Davis, J. T., Hirani, S., Bartlett, C., & Reid, B. R. (1994) *J. Biol. Chem. 269*, 3331−3338.

Edelhoch, H. (1967) *Biochemistry 6*, 1948−1954.

Freire, E. (1995) in *Methods in Molecular Biology, Vol. 40: Protein Stability and Folding, Theory and Practice* (Shirley B. A., Ed.) pp 191−218, Humana Press Inc., Totowa, NJ.

Friedrich, T., Kröger, B., Bialojan, S., Lemaire, H. G., Höffken, H. W., Reuschenbach, P., Otte, M., & Dodt, J. (1993) *J. Biol. Chem. 268*, 16216−16222.

Fujinaga, M., Sielecki, A. R., Read, R. J., Ardelt, W., Laskowski, M., Jr., & James, M. N. G. (1987) *J. Mol. Biol. 195*, 397−418.

Gerken, T. A., Butenhof, K. J., & Shogren, R. (1989) *Biochemistry 28*, 5536−5543.

Grafl, R., Lang, K., Vogl, H., & Schmid, F. X. (1987) *J. Biol. Chem. 262*, 10624−10629.

Griko, Y. V., & Privalov, P. L. (1984) *Biophysics 29*, 211−216.

Hoogstraten, C. G., Choe, S., Westler, W. M., & Markley, J. L. (1995) *Protein Sci. 4*, 2289−2299.

Imperiali, B., & Rickert, K. W. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 97−101.

Kane, J. F. (1995) *Curr. Opin. Biotechnol. 6*, 494−500.

Kato, I., Schrode, J., Kohr, W. J., & Laskowski, M., Jr. (1987) *Biochemistry 26*, 193−201.

Klaus, W., & Schomburg, D. (1993) *J. Mol. Biol. 229*, 695−706.

Laskowski, M., Jr., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, M. W., Kohr, W. J., Park, S. J., Parks, K., Schatzley, B. L., Schoenberger, O. L., Tashiro, M., Vichot, G., Whatley, H. E., Wieczorek, A., & Wieczorek, M. (1987) *Biochemistry 26*, 202−221.

Laskowski, M., Jr., Apostol, I., Ardelt, W., Cook, J., Giletto, A., Kelly, C. A., Lu, W., Park, S. J., Qasim, M. A., Whatley, H. E., Wieczorek, M., & Wynn, R. (1990) *J. Protein Chem. 9*, 715−725.

Lee, B., & Richards, F. M. (1971) *J. Mol. Biol. 55*, 379−400.

Liang, R., Andreotti, A. H., & Kahne, D. (1995) *J. Am. Chem. Soc. 117*, 10395−10396.

Liepinsh, E., Berndt, K. D., Sillard, R., Mutt, V., & Otting, G. (1994) *J. Mol. Biol. 239*, 137−153.

Live, D. H., Kumar, A. R., Beebe, X., & Danishefsky, S. J. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 12759−12761.

Matthews, B. W. (1995) *Adv. Protein Chem. 46*, 249−278.

McCrary, B. S., Edmondson, S. P., & Shriver, J. W. (1996) *J. Mol. Biol. 264*, 784−805.

Mer, G., Hietter, H., & Lefèvre, J. F. (1996) *Nat. Struct. Biol. 3*, 45−53.

Mühlhahn, P., Czisch, M., Morenweiser, R., Habermann, B., Engh, R. A., Sommerhoff, C. P., Auerswald, E. A., & Holak, T. A. (1994) *FEBS Lett. 355*, 290−296.

Murphy, K. P., & Gill, S. J. (1990) *Thermochim. Acta 172*, 11−20.

Murphy, K. P., & Freire, E. (1992) *Adv. Protein Chem. 43*, 313−361.

Murphy, K. P., Privalov, P. L., & Gill, S. J. (1990) *Science 247*, 559−561.

Murphy, K. P., Xie, D., Garcia, K. C., Amzel, L. M., & Freire, E. (1993) *Proteins: Struct., Funct., Genet. 15*, 113−120.

Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., & Gray, T. (1995) *Protein Sci. 4*, 2411−2423.

Papamakos, E., Weber, E., Bode, W., Huber, R., Empie, M. W., Kato, I., & Laskowski, M., Jr. (1982) *J. Mol. Biol. 158*, 515−537.

Parente, J. P., Strecker, G., Leroy, Y., Montreuil, J., Fournet, B., van Halbeek, H., Dorland, L., & Vliegenthart, J. F. G. (1983) *FEBS Lett. 152*, 145−152.

Pascarella, S., & Argos, P. (1992) *J. Mol. Biol. 224*, 461−471.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167−241.

Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665−684.

Privalov, P. L., & Potekhin, S. A. (1986) *Methods Enzymol. 131*, 4−51.

Privalov, P. L., & Gill, S. J. (1988) *Adv. Protein Chem. 39*, 191−234.

Read, R. J., Fujinaga, M., Sielecki, A. R., & James, M. N. G. (1983) *Biochemistry 22*, 4420−4433.

Schaller, W., & Robertson, A. D. (1995) *Biochemistry 34*, 4714−4723.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−89.

Sturtevant, J. M. (1994) *Curr. Opin. Struct. Biol. 4*, 69−78.

Swint, L., & Robertson, A. D. (1993) *Protein Sci. 2*, 2037−2049.

Swint-Kruse, L., & Robertson, A. D. (1995) *Biochemistry 34*, 4724−4732.

Swint-Kruse, L., & Robertson, A. D. (1996) *Biochemistry 35*, 171−180.

Tschesche, H., Kolkenbrock, H., & Bode, W. (1987) *Biol. Chem. Hoppe-Seyler 368*, 1297−1304.

van de Locht, A., Lamba, D., Bauer, M., Huber, R., Friedrich, T., Kröger, B., Höffken, W., & Bode, W. (1995) *EMBO J. 14*, 5149−5157.

van Geet, A. L. (1968) *Anal. Chem. 40*, 2227−2229.

Vliegenthart, J. F. G., Dorland, L., & van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209−374.

Wang, C., Eufemi, M., Turano, C., & Giartosio, A. (1996) *Biochemistry 35*, 7299−7307.

Williamson, M. P., Havel, T. F., & Wüthrich, K. (1985) *J. Mol. Biol. 182*, 295−315.

Wormald, M. R., Wooten, E. W., Bazzo, R., Edge, C. J., Feinstein, A., Rademacher, T. W., & Dwek, R. A. (1991) *Eur. J. Biochem. 198*, 131−139.

Wyss, D. F., Choi, J. S., Li, J., Knoppers, M. H., Willis, K. J., Arulanandam, A. R. N., Smolyar, A., Reinherz, E. L., & Wagner, G. (1995) *Science 269*, 1273−1278.

Yamashita, K., Kamerling, J. P., & Kobata, A. (1982) *J. Biol. Chem. 257*, 12809−12814.

Yamashita, K., Kamerling, J. P., & Kobata, A. (1983) *J. Biol. Chem. 258*, 3099−3106.

Yang, A.-S., & Honig, B. (1993) *J. Mol. Biol. 231*, 459−474.

Yet, M.-G., Chin, C. C. Q., & Wold, F. (1988) *J. Biol. Chem. 263*, 111−117.

Zale, S. E., & Klibanov, A. M. (1986) *Biochemistry 25*, 5432−5444.

BI962580B