Characterization of Human Calcitonin Fibrillation in Aqueous Urea Solution by ^{1}H NMR Spectroscopy

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ABSTRACT: The inhibitory effects of urea on the normally rapid fibrillation of human calcitonin (hCT) were investigated by ^{1}H NMR. From subtle differences in the chemical shift of hCT in the presence and absence of urea, the occurrence of weak interactions between urea and hCT was confirmed. The chemical shifts on the NH and Cα protons of residues in the C-terminal (Gln^{24}−Pro^{32}) region were unaffected by the urea interactions, while the chemical shifts of the N-terminal (Cys^1−Cys^7) and central (Met^8−Pro^{23}) residues were observed to move significantly downfield with increasing urea concentrations. These findings suggest that urea serves to stabilize the monomeric form of hCT and to promote the concentration of the extended hCT conformer. However, it was also found that even by storing hCT in urea the fibrillation process cannot be circumvented, as the gradual carbamylation of the N-terminus takes place. The time course of 1D and 2D spectra of carbamylated hCT showed that cross peaks of residues in the N-terminal and central regions disappear faster than those in the C-terminal regions, indicating that the fibrillation of carbamylated hCT is initiated in the N-terminal and central regions. It is postulated that carbamylation increases the hydrophobicity of the N-terminal region and hence fosters fibrillation, even in urea solution.

Calcitonin (CT)[1] is a polypeptide hormone which is known to regulate calcium−phosphorus metabolism (Copp et al., 1962; Kumar et al., 1963; Austin & Heath, 1981) and is used as a drug to treat various diseases, most notably osteoporosis. However, human CT (hCT) readily associates and precipitates as insoluble fibrils upon storing in aqueous solution (Sieber et al., 1970; Arvinte et al., 1993), thereby presenting a serious disadvantage for its practical therapeutic use (Arvinte & Ryman, 1992).

The phenomenon of peptide aggregation and precipitation in biological systems is widespread, with commonly cited examples of biomedical complications, arising from their occurrence, being Down's syndrome and Alzheimer's disease (Glenner & Wong, 1984; Masters et al., 1985). The structural aspects of the above medical conditions have been examined on the molecular level by ^{1}H NMR (Zagorski & Barrow, 1992; Jarvis et al., 1994); however, little is known about the physical forces involved in the mechanisms that lead to peptide aggregation or the forms of the secondary or tertiary structure of the peptide in the aggregate.

CT itself has been widely studied and is known to consist of 32 amino acids with an N-terminal disulfide bridge between positions 1 and 7 and a C-terminal proline amide residue. In various structure-promoting solvents, CT takes on an amphiphilic α-helical conformation, mostly in the residue range 8−22 (Doi et al., 1990; Meyer et al., 1991; Motta et al., 1991; Meadows et al., 1991). However, in aqueous solution it does not assume a rigid conformation. Recently, we applied 2D NMR techniques to investigate the mechanism of the hCT fibrillation process in aqueous solution and revealed that the molecular association of hCT is initiated by the intermolecular hydrophobic interaction of the N-terminal (Cys^1−Cys^7) and central regions (Met^8−Pro^{23}) and that the C-terminal region (Gln^{24}−Pro^{32}) subsequently becomes involved in the fibrillation (Kanaori & Nosaka, 1995a). Furthermore, we also demonstrate that the structure of the fibril is considered to be stabilized by inter- and/or intramolecular hydrogen bonds and that the amphiphilicity of the peptide plays an important role in the association of the hCT molecules (Kanaori & Nosaka, 1995a).

It has been established that urea has an important function to play in influencing the solubility or aggregation behavior of protein molecules. The effects of urea on protein structure and protein−urea interaction have been investigated by X-ray crystallography (Pike & Acharya, 1994) and NMR spectroscopy (Lumb & Dobson, 1992; Kim & Woodward, 1993; Liepinsh & Otting, 1994).

After the formation of hCT fibril in aqueous solution (80 mg/mL, 3 h storage at room temperature), the lyophilization of the solution gave a powder of the hCT fibrils (fhCT), which was characterized by ^{1}H NMR (Kanaori & Nosaka, 1995b). Although fhCT fibrillates much more easily than hCT in aqueous solution, the dissolution of fhCT in 6 M urea solution showed no fibrillation, indicating that the fibril core becomes dissociated to afford the monomer molecules (Kanaori & Nosaka, 1995b). In order to investigate the influence of urea on the fibrillation behavior of hCT in aqueous urea media and how hCT fibrillation can be inhibited by the presence of urea, ^{1}H NMR spectra of hCT in aqueous urea solution were measured. On the other hand, caution must be preserved as carbamylation of the N-terminal amino group of hCT by urea is also found to occur, which substantially promotes the fibrillation process.

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[1] Abbreviations: CT, calcitonin; hCT, human calcitonin; 2D, two dimensional; 1D, one dimensional; DQFCOSY, double-quantum-filtered correlated spectroscopy; HOHAHA, homonuclear Hartmann−Hahn spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; TPPI, time-proportional phase incrementation method; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; BPTI, bovine pancreatic trypsin inhibitor.

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EXPERIMENTAL PROCEDURES

Materials. Synthetic hCT was provided by Ciba-Geigy Pharmaceuticals, Basel, Switzerland. Urea-d_4 was purchased from Sigma.

Sample Preparation. Peptide concentration was determined by weight. For resonance assignment, the peptide was dissolved in a freshly prepared 90% H_2O/10% D_2O (v/v) solution containing 6 M urea-d_4 and 0.015 M CD_3COOD (pH 2.9). The final concentration of peptide was 20 mg/mL (5.9 mM).

For time dependence experiments, the peptide (80 mg/mL) was dissolved in 100% D_2O solution containing 6 M urea-d_4 and 0.015 M CD_3COOD (pH 2.9), and a series of spectra on the same sample were recorded; the first spectrum was acquired about 6 min after the dissolution in D_2O.

NMR Measurements. All the NMR spectra were recorded on a Bruker AMX600 spectrometer at 300 K. For resonance assignments, DQFCOSY (Piantini et al., 1982; Rance et al., 1983), HOHAHA (Braunschweiler & Ernst, 1983; Davis & Bax, 1985), and NOESY (Jeener et al., 1979; Macura et al., 1981) were run according to the time-proportional phase incrementation method (TPPI) (Marion & Wüthrich, 1983). NOESY spectra were recorded with a mixing time of 300 ms, and HOHAHA experiments were acquired with mixing times of 40 and 80 ms. FELIX (Biosym inc.) was used for data processing and signal assignment on a Silicon Graphics workstation. Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$.

For rapid 2D measurements, HOHAHA (mixing time 80 ms) and ROESY (Bax & Davis, 1985) (mixing time 200 ms) data were collected by the States−TPPI method (Marion et al., 1989) without phase cycling. Applying this method, it took about 5 min to measure the 256 (t_1) × 512 (t_2) data matrix. Shifted sine-bell-squared weight functions were applied in both dimensions, and the data were zero filled to a final size of 1K × 1K.

RESULTS AND DISCUSSION

^{1}H NMR Spectra of hCT in Urea Solution. 1D NMR spectra of hCT in a freshly prepared 6 M urea solution were measured at various concentrations of the peptide (20−80 mg/mL). There were no differences observed in the chemical shift values and the line widths of nonlabile and labile protons in all the recorded spectra (data not shown). In order to compare the fibrillation features of hCT in the presence and absence of urea, the time course of ^{1}H NMR spectra of hCT was obtained at the peptide concentration of 80 mg/mL. In the absence of urea, all the peptide signals were significantly broadened within 1 h under identical conditions (Kanaori & Nosaka, 1995a). Conversely, in urea solution, the results showed no change in the peptide signals over 24 h, in terms of both chemical shift and appearance of new peaks. Figure 1 shows the time dependence of the peak intensities for aqueous solutions of hCT in the presence and absence of urea. From the above findings, it can be readily concluded that urea actively functions in preventing the fibrillation of hCT. However, careful examination of the time dependence behavior of peak intensities revealed that urea does not completely inhibit fibrillation, as the process continues to occur, albeit at a much reduced rate. The reasons for this behavior will be addressed later in the paper.

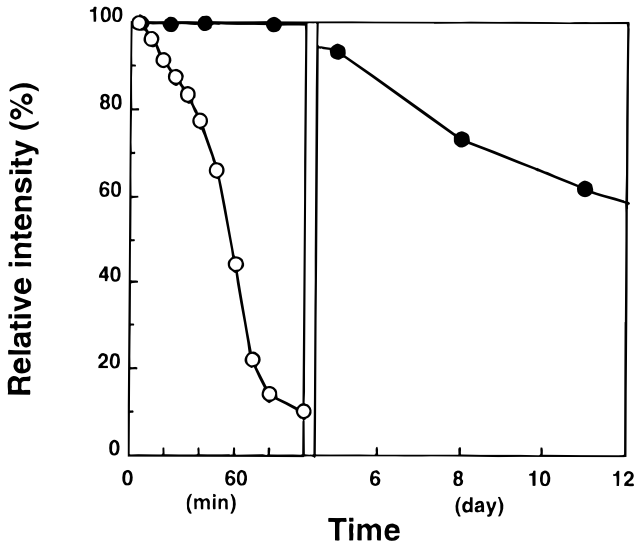

FIGURE 1: Time dependence of peak intensities of hCT in the absence of urea (open circle) (Kanaori & Nosaka, 1995a) and in the presence of 6 M urea (closed circle). The averaged relative peak heights of resolved nonexchangeable protons are plotted. Other conditions, hCT concentration 80 mg/mL, temperature 300 K, pH 2.9, were the same for both of the solutions. No changes in chemical shifts were observed over time in the presence of urea.

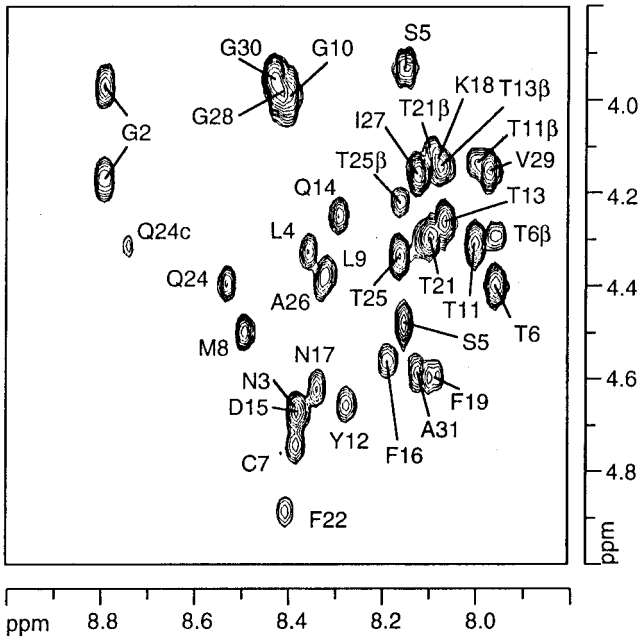

FIGURE 2: Backbone fingerprint region of the HOHAHA (80 ms) of hCT (20 mg/mL) in a freshly prepared 6 M urea and 90% H_2O/10% D_2O solution at pH 2.9 and 300 K. All cross peaks are labeled with their amino acid residue name (single letter code) and sequence numbers. Q24c means a *cis* isomer of Pro^{23}. A table of chemical shifts of hCT in 6 M urea is available as Supporting Information.

Assignments of the hCT signals in urea solution were performed by using the well-established sequential approach (Billeter et al., 1982; Wüthrich et al., 1982), based on DQFCOSY, HOHAHA (mixing time 40 and 80 ms), and NOESY (mixing time 300 ms) spectra. Figure 2 displays the NH−α fingerprint region of the HOHAHA spectrum of hCT in the urea solution and yields all the expected cross peaks except for Cys^1, Pro^{23}, and Pro^{32}. Some additional peaks in the δ-proton region for the prolyl residues were assigned to isomers of Pro^{23} and Pro^{32} (Kern et al., 1993; Kanaori & Nosaka, 1995a). The ratio of the isomers in the

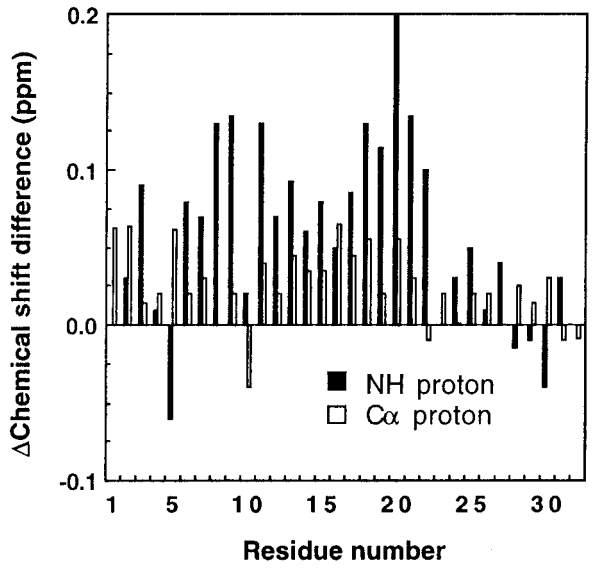

FIGURE 3: Differences in the chemical shifts of the NH (solid bars) and Cα (open bars) protons of hCT in freshly prepared 6 M urea solution at 300 K and pH 2.9 from the values in aqueous solution under the same conditions (Kanaori & Nosaka, 1995a).

absence of urea is 67%, 25%, and 8% for (*trans,trans*)-, (*cis,-trans*)-, and (*trans,cis*)-(Pro[23],Pro[32]) conformers, respectively (Kern et al., 1993), whereas in urea solution, the ratio was changed to 75%, 16%, and 9% for these three conformers.

Compared to the chemical shift values of hCT in aqueous solution assigned by 2D NMR (Kanaori & Nosaka, 1995a), all nonlabile protons of side chains showed only a very small chemical shift change ($\leq$0.02 ppm) with urea concentrations up to 8 M. As mentioned before, the peptide signals in the absence of urea were completely broadened 1 h after the dissolution. This suggests that conformational details of the amino acid side chains appear to be preserved in the presence of urea although the fibrillation did not proceed. On the other hand, resonances of the amide protons were significantly shifted downfield (solid bars in Figure 3). The magnitudes of the downfield shifts were large in the N-terminal and central regions, whereas those in the C-terminal region were small. Hence, the environment around the N-terminal and central regions changes significantly through interactions with urea molecules.

The maximum observed shift was 0.2 ppm for the amide proton of His[20], and the average value was 0.07 ppm, which was almost equivalent to that observed in bovine pancreatic trypsin inhibitor (BPTI) and the acidic protein PEC-60 in urea solutions (Liepinsh & Otting, 1994). Liepinsh and Otting defined the binding constants of urea molecules to proteins to be less than 1, by measuring the chemical shift of the protein resonance as a function of urea concentration. The similar changes of the NH chemical shifts observed in the case of hCT indicate that the binding constants of urea with hCT are small and that urea molecules interact weakly with hCT monomer, possibly through hydrogen bonding to the amino and carbonyl groups of the peptide. This is supported by the negative binding enthalpies for urea−protein interactions, which suggest the existence of hydrogen bonding between urea and exposed polar groups on the protein (Makhatadze & Privalov, 1992), most notably with the backbone amide and carbonyl groups (Robinson & Jencks, 1965; Nozaki & Tanford, 1970).

The chemical shifts of the Cα protons also showed downfield shifts in urea solution, although the magnitude of the shifts was smaller than those of the NH protons (open bars in Figure 3). From previous studies that have used the chemical shift of Cα protons as an indicator of secondary structure (Wishart et al., 1991), the downfield shifts in the urea solution usually suggest a decrease in the α-helicity of the N-terminal and central regions. Since hCT molecules are considered to exist in a rapidly interconverting mixture of several conformers, the proposed decrease in the α-helicity indicates an increase in concentration of conformer with extended structure for urea solutions of hCT. It is hypothesized that the fibril nucleus consists of a helical bundle held together by hydrophobic interactions in the N-terminal and central regions and is stabilized by inter- and/or intramolecular hydrogen bonds (Arvinte et al., 1993; Kanaori & Nosaka, 1995a). It is considered that urea, a well-known denaturant for proteins, would not initiate protein unfolding by actively disrupting the native protein conformation but would stabilize spontaneously formed, unfolded protein, because of the weak interaction of urea with proteins (Liepinsh & Otting, 1994). Since the interaction between urea and hCT is similarly weak, it would be unlikely that the fibril core disaggregates by direct interaction with urea. It has been reported in the literature that there is an equilibrium between associated and monomeric hCT, conforming to the double nucleation mechanism (Ferrone et al., 1980, 1985; Samuel et al., 1990). The increase of the extended conformations would be unfavorable to form the helical bundle which is required to initiate the formation of fibril nucleus. In this regard, the stabilization of the extended form of the hCT molecule is considered central to the prevention of the fibrillation. The equilibrium may be shifted to the dissociation of the aggregates to the monomers through the interaction with urea molecules, which could explain why the fibril nucleus of hCT, which remains after lyophilization of the hCT fibril, disentangles to form monomers in urea solution (Kanaori & Nosaka, 1995b).

The existence of CT receptors that distinguish between conformational features of CT molecules was reported: CT receptors on bones in mammals were suggested to selectively recognize CT molecules that do not easily adopt an α-helical structure, i.e., hCT, whereas helical CT, like sCT, seems to bind specifically to kidney (Nakamuta et al., 1990). Since a strong α-helix was not required for the kind of the hCT receptors, a rather extended form might also be a biologically active conformation.

Carbamylation at an N-Terminal Amino Group of hCT in Urea Solution. As shown in Figure 1, the peak intensities of hCT show a gradual decrease, indicating that fibrillation occurs even in the urea solution, despite the fact that urea can prevent fibrillation. After the hCT urea solution (40 mg/mL) was heated for 15 min at 343 K, 1D and 2D ^{1}H NMR spectra were measured at 300 K and pH 2.9. As a consequence, all peptide peaks broadened significantly within 1 h, accompanied by gelation of the solution. Furthermore, besides the original peaks of hCT in urea solution before heating, new NH−Cα cross peaks appeared before gelation. The proportion of the new peaks to the corresponding original peak depends on the heating time at 343 K. It was also confirmed that there was no chemical exchange between the original and new peaks by changing temperature and pH.

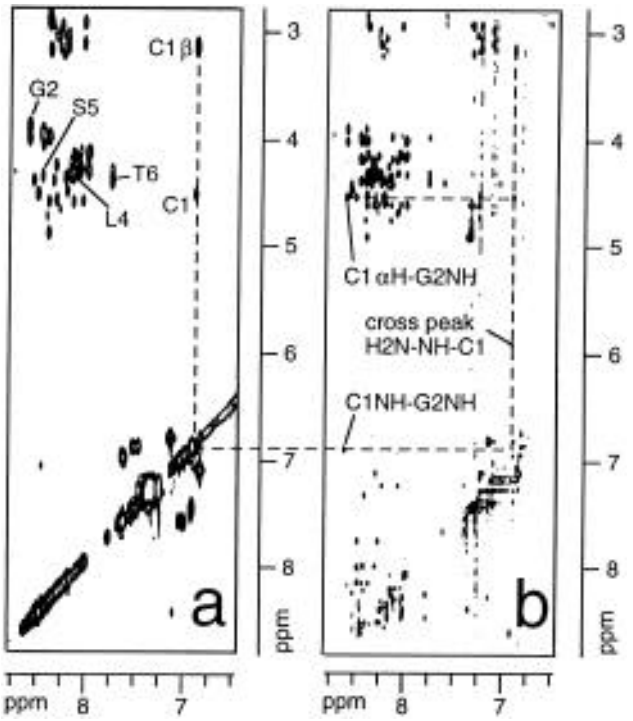

FIGURE 4: HOHAHA (80 ms) (a) and ROESY (200 ms) (b) spectra of carbamylated hCT in 6 M urea solution at 300 K. Some cross peaks which were mentioned in the text are labeled with their amino acid residue name (single letter code) and sequence number. A table of chemical shifts of carbamylated hCT in 6 M urea is available as Supporting Information.

Some of the new NH protons (Gly[2], Leu[4], Ser[5], and Thr[6]) shifted more than 0.2 ppm, as compared to the original peaks (Figure 4a).

An explanation for the above phenomenon can be seen from the scheme below (Stark et al., 1960), where urea breaks down to form the cyanate, which in turn reacts with the α-amino group to form carbamyl derivatives.

$$H_2NCONH_2 \rightarrow CNO^- + NH_4^+$$

$$CNO^- + H_3N^+-\text{peptide} \rightarrow H_2NCONH-\text{peptide}$$

These reactions proceed much faster in the case where the urea solution is heated, but even at room temperature it gradually occurs (Stark et al., 1960).

It would also follow that the α-amino group of the N-terminus and ϵ-NH_3^+ group of Lys[18] of hCT should undergo carbamylation. From HOHAHA and ROESY spectra of heat-treated hCT, sequential NOEs ($d_{\alpha N}$ and d_{NN}) between Cys[1] and Gly[2] were observed, as well as a cross peak between the carbamyl NH_2 group and NH of Cys[1] (Figure 4b). However, new peaks of ϵ-CH_2 belonging to Lys[18] were not detected, which would suggest that the ϵ-amino group of Lys[18] is not carbamylated. This may be consistent with the fact that an α-amino group is carbamylated much faster than an ϵ-amino group. In short, the above findings clearly indicate that only the N-terminal α-amino group of hCT becomes carbamylated in heated urea solution. In the case of the aggregation and precipitation of apomyoglobin in aqueous urea solution, the concentration of the protein decreased approximately 6% a day and is considered to arise from the covalent modification of lysine groups (De Young et al., 1993). The fibrillation of noncarbamylated

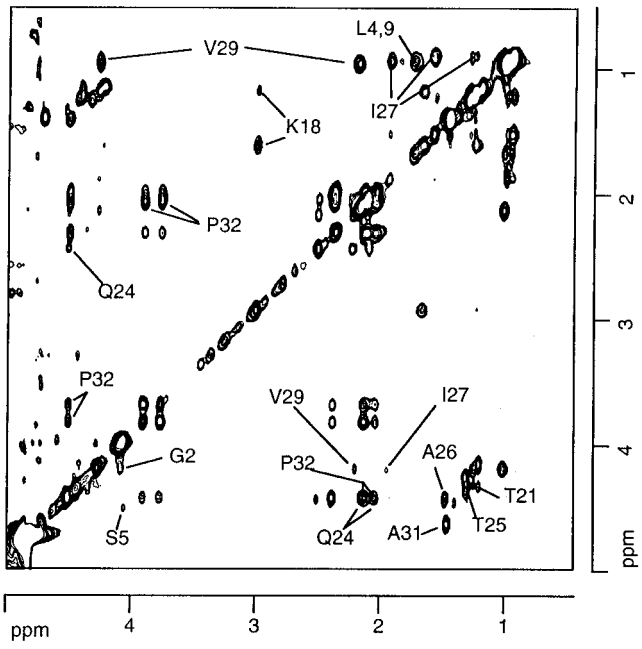

FIGURE 5: HOHAHA (80 ms) spectrum of carbamylated hCT in 6 M urea solution 1 h after hCT is carbamylated. Cross peaks are labeled with their amino acid residue name (single letter code) and sequence number.

hCT would not proceed in urea solution since fhCT, which fibrillates much more easily in aqueous solution than noncarbamylated hCT, showed no fibrillation in 6 M urea solution: The fibril nucleus would be dissociated to the monomers (Kanaori & Nosaka, 1995b). Thus, occurrence of the carbamylation at the N-terminal α-amino group can explain the gradual fibrillation of hCT in urea solution after several days at 300 K although there might be the other factor responsible for the gradual fibrillation, which could not be identified by the present experiments.

Carbamylated hCT molecules also possess the ability to fibrillate readily, even in urea solution. The time course of the 1D and 2D HOHAHA spectra of the carbamylated hCT in urea solution showed features similar to those in aqueous solution; i.e., peaks in the N-terminal and central regions disappeared more rapidly than those in the C-terminal region (Figure 5). This serves to strengthen the argument that association of carbamylated hCT is initiated in the N-terminal and central regions, with the C-terminal region subsequently becoming involved in the actual fibrillation as well as that of noncarbamylated hCT. Since the hydrophobic interactions in the N-terminal and central regions contribute to initiating the association of hCT (Kanaori & Nosaka, 1995a), the association of carbamylated hCT, which is more hydrophobic, would be conducted by hydrophobic interactions in these regions. It is also interesting to note that the large shifts in the NH and Cα protons, of all residues in the N-terminal region of carbamylated hCT, from those of hCT (Figure 6) suggest a change in the binding affinity of urea molecules to the peptide, as well as an alteration of the peptide conformation in the N-terminal region.

The loss of the N-terminal charge by carbamylation increases the hydrophobicity in the N-terminal region and hence reduces the electrostatic repulsion between hCT molecules. It was reported that hCT derivatives whose N-terminal charge has been eliminated fibrillate at very low

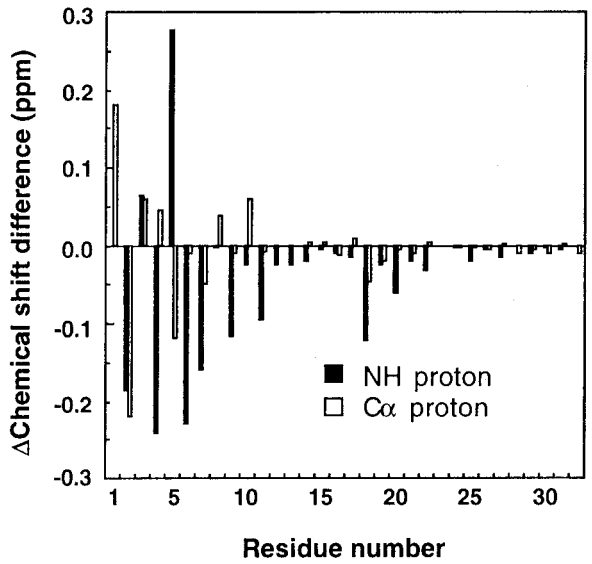

FIGURE 6: Differences in the chemical shifts of the NH (solid bars) and Cα (open bars) protons of carbamylated hCT from the values of hCT in 6 M urea solution at 300 K and pH 2.9.

concentrations, as compared to hCT (Arvinte et al., 1992). The strong hydrophobic interaction in the N-terminal and central regions causes the fibrillation even in urea solution, indicating that the fibril core formed by the strong interaction is unable to disaggregate to the monomeric form. This phenomenon is considered analogous to the mechanism that a stable protein, BPTI, is unable to unfold even in highly concentrated urea solution (Kim & Woodward, 1993; Liepinsh & Otting, 1994). In another theoretical study on protein aggregation (Fields et al., 1992), hydrophobic interactions drive protein to aggregate as an entangled network of denatured chains, and the denaturant-mediated disaggregation is predicted to be very sensitive to amino acid composition. Our results demonstrate that the hCT fibrillation is also sensitive to slight changes in the hydrophobicity in the N-terminal region. Bearing in mind the previous study on salmon CT, which is slightly hydrophilic in the N-terminal and central regions and is highly stable in aqueous solution (Arvinte et al., 1993), it can be hypothesized that, by decreasing the hydrophobicity in the N-terminal and central regions of hCT, the prevention of hCT fibrillation can be attained.

CONCLUSIONS

Effects of urea on the peptide aggregation−disaggregation are somewhat similar to those of urea on protein folding−unfolding. Urea molecules interact weakly with hCT monomer, possibly through hydrogen bonding to the amino and carbonyl groups of the peptide, and serve to stabilize the extended conformations of hCT, which lead to the disentanglement of the fibril core and inhibition of fibrillation. However, the strong hydrophobic interaction in the N-terminal and central regions resulting from carbamylation of the N-terminal amino group, by decomposition of an urea molecule, significantly promotes the fibrillation in urea solution. All these results suggest that the problems of hCT fibrillation in therapeutic uses may be circumvented by the addition of compounds which form hydrogen bonds with the peptide molecule or induce a slight decrease of the hydrophobicity in the N-terminal and central regions of the peptide.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Two tables showing chemical shift data of hCT (20 mg/mL) and of carbamylated hCT (20 mg/mL) in 6 M urea solution, pH 2.9, 300 K (2 pages). Ordering information is given on any current masthead page.

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