Fibrillation of a human calcitonin mutant (hCT) at the position of Asp$^{15}$ (D15N-hCT) was examined to reveal the effect of the electrostatic interaction of Asp$^{15}$ with charged side chains. The secondary structures of fibrils and soluble monomers in the site-specific $^{13}$C-labeled D15N-hCTs were determined using $^{13}$C cross-polarization magic angle spinning and dipolar decoupled magic angle spinning NMR approaches, sensitive to detect $^{13}$C signals from the fibril and the soluble monomer, respectively. The local conformations and structures of D15N-hCT fibrils at pH 7.5 and 3.2 were found to be similar to each other and those of hCT at pH 3.3 and were interpreted as a mixture of antiparallel and parallel $\beta$-sheets, whereas they were different from the hCT fibril at pH 7.5 whose structure is proposed to be antiparallel $\beta$-sheets. Thus the negatively charged Asp$^{15}$ in the hCT molecule turned out to play an essential role in determining the structures and orientations of the hCT molecules. Fibrillation kinetics of D15N-hCT was analyzed using a two-step autocatalytic reaction mechanism. The results indicated that the replacement of Asp$^{15}$ with Asn$^{15}$ did not reduce the rate constants of the fibrillation formation but rather increased the rate constants at neutral pH.

Calcitonin (CT) is a peptide hormone consisting of 32 amino acid residues, which contains an intrachain disulfide bridge between Cys$^1$ and Cys$^7$ and a proline amide at the C terminus. In mammals CT plays a central role in calcium-phosphorus metabolism as a thyroid hormone (1–3). In concentrated aqueous solution, human calcitonin (hCT), however, has a tendency to form a fibril precipitate known as a disease-associated amyloidosis (Alzheimer’s disease, type II diabetes, Creutzfeldt-Jakob disease, etc.). Resulting fibers of 8 nm in diameter (4) as revealed by electron microscopic studies on hCT fibrils gave rise to a common ultrastructure with clinically and biochemically diverse amyloid fibrils (5–8). Analogous to $\beta$-amyloid, calcitonin is another model peptide associated with medullary carcinoma of the thyroid (9, 10). However, the detailed molecular mechanism of fibril formation has not yet been well understood. It is therefore important to clarify the fibrillation process and mechanism in hCT not only to contribute to further improvement of aqueous therapeutic formations with a long term stability but also to gain insight into a general picture as to molecular mechanism of amyloid formation.

It has been shown that an amphiphilic $\alpha$-helical structure is formed in the central region of hCT in aqueous acidic solution (12). Subsequently, local conformational transitions from an $\alpha$-helix to a $\beta$-sheet structure at the central region and from a random coil to a $\beta$-sheet at the C terminus region were induced simultaneously during the fibril formation in the acetic acid solution (pH 3.3) (13). We further noticed that a two-step reaction model can be used to analyze the fibrillation kinetics by the observation of a certain delay time followed by a simultaneous decrease and increase from the DD- and CP-MAS NMR signals, sensitive to detection of $^{13}$C NMR signals from the soluble monomer and fibril component, respectively. The first step is a homogeneous association to form the nucleus of fibril, and the second step is an autocatalytic heterogeneous fibrillation to mature the fibril; the kinetics parameters for the first ($k_1$) and second ($k_2$) steps were determined individually (13). As a mechanism of the molecular association in the first nucleation process, it was proposed that the $\alpha$-helices are bundled together by hydrophobic interaction among the side chains of the amphiphilic $\alpha$-helices in the central region of hCT (14).

It was reported that the rate of fibril formation at neutral pH is much faster than that at acidic pH, because hCT monomer is more stable in an acidic medium in the absence of salts and buffer than in physiological saline solution. Local molecular structures and macroscopic features of the hCT fibrils formed in the sodium acetate solution (pH 7.5) were different from those at pH 3.3 (13). We proposed that the fibril at pH 7.5 is comprised of antiparallel $\beta$-sheets because of the favorable electrostatic interaction between side chains of Asp$^{15}$ and Lys$^{18}$ (at pH 7.5). On the contrary, the mixture of antiparallel and parallel $\beta$-sheet structures is formed at pH 3.3, because the side chains of Lys$^{18}$ and His$^{20}$ and the amino group in the N terminus were positively charged and there is no favorable direction among the molecules to associate. Therefore, it is worthwhile to perform further examination using mutant hCT having different local charges whether fibril formation of hCT...
is affected not only by the hydrophobic interaction but also by the electrostatic interaction among charged side chains (13). 

13C chemical shifts of carbonyl and Cα and Cβ carbons in amino acid residues obtained from high resolution solid state 13C NMR spectroscopy have been established to correlate with local secondary structures of polypeptides (15, 16). These conformation-dependent 13C chemical shifts were proven to provide reliable secondary structures for fibrous and membrane proteins (17, 18). Use of site-specific 13C-labeled carbonyl groups for Gly⁴⁴ and Phe²² or methyl carbons at Ala²⁶ and Ala⁶¹ in hCT provided a clue as to the local conformations at the specific sites over the entire molecule, together with simultaneous observation of local conformational changes of hCT from solution to fibril state by the DD-MAS and CP-MAS NMR spectra, respectively, during the fibril formation (13).

In the present study, we focused on the effect of the electrostatic interaction between the charged side chains in the fibril formation of hCT. In the hCT molecule, there is only one amino acid that charges negatively, Asp¹⁵, and there are two amino acids with positive charges (Lys¹⁸ and His²⁰ (see Scheme I)) by chemical synthesis to examine the effect of electrostatic interaction in the structure and kinetics of the fibril formation. The hydrophilic residue at position 15 of hCT is not required for biological activity (19), with the expectation of a slow fibrillation rate as a prerequisite for a therapeutic agent. In contrast to this expectation, there is anticipation that the most common type of aging-related damage arising from the neutralization of aspartate leads to an increased β-sheet content to cause an increased propensity for fibril formation (20). Thus the artificial mutation of hCT from Asp¹⁵ to Asn¹⁵ can serve as a suitable model of the aging-related damage in the β-amyloid formation, too.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—We prepared three types of isotopically labeled D15N-hCT preparations, I–III (Scheme I) by chemical synthesis (13). Note that the Pro residue was not contained for I and II as manifested by matrix-associated laser desorption time-of-flight mass spectrometry (MALDI MS) analysis. For the 13C solid state NMR experiments, the lyophilized preparations were dissolved in 15 mM aqueous acetic acid solution and 5 mM phosphate buffer. Immediately after D15N-hCTs were dissolved in the solution, a portion of the solution (80 μl) was placed in a 5-mm outer diameter zirconia rotor and sealed with Araldite® (Vantico) to prevent evaporation of the mother liquor throughout the NMR measurements. The same solution stood in the test tubes at the same temperature as used in the NMR measurements to check the turbidity or viscosity of the solution by visual observation.

**NMR Measurements**—13C NMR spectra were recorded on a Chemagnetics CMX 400 NMR spectrometer at the resonance frequency of 100.6 MHz. The 13C NMR spectra were recorded alternatively by means of 13C CP-MAS and DD-MAS techniques because solid fibril and soluble monomer were preferentially recorded by the former and latter methods, respectively, in view of their cross-polarization times (τCP) and spin-lattice relaxation times (τl). The 13C chemical shifts were calibrated by using the external carboxyl peak of crystalline glycine at 176.03 ppm from tetramethylsilane. The lengths of π/2 pulse for the carbon and proton nuclei were 5.0 μs, and the recycle delay times were 4 and 5 s for the CP- and DD-MAS studies, respectively. All NMR measurements were performed at 20 °C. Acquisition of the 13C DD- and CP-MAS NMR spectra was started at 20 °C after 6 h from dissolution, because a time delay was necessary to achieve tight enough sealing of the sample rotor by glue. The number of accumulations for the DD- and CP-MAS signals were 1000 and 2000, respectively, in the time course studies.

**Electron Microscope Observation**—For electron microscopic analyses, D15N-hCT fibrils grown from the solution at pH 3.2 and 7.5 (80 mg/ml), respectively, at room temperature were diluted with each buffer solution after 4 h and 3 days, respectively, from the dissolution and vortexed to reduce their viscosity. 5 μl of the suspension was placed on a Formvar-covered grid and stained with 5 μl of uranyl acetate. The negative stained samples were viewed in a JEOL 1200 EX II electron microscope at 80 kV.

**Circular Dichroism Measurements**—CD measurements were performed on an Aviv model 62DS using quartz cuvettes with the path length of 0.2 cm. CD spectra were recorded in the wavelength range of 250–200 nm. The concentration of D15N-hCT was 0.2 mg/ml (58.5 μM), and 20 mM phosphate buffer (pH 7.2) and 15 mM acetic acid solution (pH 3.2) were used. The temperature was controlled to 25 °C using a thermostatted cell holder throughout the CD measurements.

**RESULTS**

Fig. 1 illustrates the electron micrographs for the two preparations, which clearly indicate the presence of fibrils from D15N-hCT (II) in pH 7.5 and 3.2 solutions (80 mg/ml). The D15N-hCT fibrils at pH 7.5 (Fig. 1A) were much shorter than those at pH 3.2 (Fig. 1B). Although the distinct differences in the length of the fibrils were observed between the two samples, the diameters of the fibrils at pH 7.5 were about 15 nm, which was almost the same as those at pH 3.2. The fact that fibrils at the neutral condition are shorter than those at acidic ones is the same as the case of hCT (13).

The local secondary structures were determined by observing DD-MAS and CP-MAS 13C NMR spectra in [1-13C]Phe¹⁶-[3-13C]Ala²⁶-labeled D15N-hCT (I) and [1-13C]Gly¹⁸,[3-13C]Ala²⁶-labeled D15N-hCT (II) for soluble monomer and fibril, respectively. In the 13C DD-MAS spectra of I at pH 7.5, the carbonyl signal of Phe¹⁶ at 173.4 ppm and the methyl signal of Ala³¹ at 17.2 ppm (Fig. 2A) of liquid-like component were ascribed to species taking a random coil, with reference to the conformation-dependent 13C chemical shifts; α-helix, random coil, and β-sheet are 171.6, 170.9, and 168.5 ppm for [1-13C]Gly, 175.2, 173.2, and 169.0 ppm for [1-13C]Phe, and 14.9, 16.9, and 20.0 ppm for [3-13C]Ala residues (15–18). The [1-13C]Gly¹⁸-[3-13C]Ala²⁶-[Ala³¹] signals of I and III at pH 7.5 and 2.6, respectively, observed in DD-MAS spectra are shown in Fig. 2 (A and D) and Fig. 3A, and the 13C chemical shifts are also summarized in Table I with their assignments. In the case of Ala³¹ of III, we took into account the additional upfield displacement of the peaks by 1.0 ppm arising from the carboxyl carbon directly bonded to the amide nitrogen of a Pro residue (21, 22). Thus, the secondary structures of D15N-hCT monomers were exactly the same among the preparations at different pH values. The 13C CP-MAS NMR signals of the fibrils (Fig. 2, B, C, E, and F, and Fig. 3, B and C) were relatively broader than the corresponding 13C DD-MAS NMR signals of the soluble monomers (Fig. 2, A and D, and Fig. 3A) because of the shorter spin-spin relaxation times and the conformational heterogeneity. The peak positions of CP-MAS spectra of the most intense one as

**Scheme I.** Amino acid sequence of hCT and D15N-hCTs.
the most populated conformation are summarized in Table I for D15N fibrils (I–III) at pH 7.5 and 3.2 or 2.6 (for III) together with their assignments. Obviously, the conformational transition from the random coil to β-sheet around Phe 16 occurred during the fibril formation, whereas a part of the α-helix and the random coil changed to the β-sheet forms around Gly 10, Ala26, and Ala31 residues, respectively. We found that the presence and absence of Pro32 at D15N-hCT does not affect the change of secondary structures of monomer and fibril as for the central region.

The secondary structure of D15N-hCT (II) monomer was also examined using CD measurements (0.2 mg/ml) at pH 7.2 and 3.3 (Fig. 4). They showed similar spectra as those of hCT in the wavelength between 200 and 250 nm (23, 24). This means that the D15N-hCT monomer takes mainly the random coil form as confirmed by the CD data of the hCT solution (23, 24).

Fig. 5 shows the time courses of the variation of the 13C NMR signal intensities at 20 °C for D15N-hCT (III) with the concentration of 25 mg/ml at pH 2.9, because the fibrils were formed in a few minutes under the higher concentration (80 mg/ml) at both pH 7.5 and 3.2. The aqueous solution of III at pH 2.9 became a gel after 8 h from the dissolution. The Gly10 C=O signal in the 13C DD-MAS spectra decreases gradually during the first 40 h (Fig. 5A). On the contrary, the Gly10 C=O signal (170.1 ppm) in the CP-MAS NMR spectra was visible after 20 h from the dissolution and increased rapidly during the next 20 h. It turned out that the fibrillation mechanism for D15N-hCT was interpreted as a two-step reaction model that is the same as that of wild type hCT (13) on account of the rate constants, \( k_1 \) and \( k_2 \), obtained from the fitting to the data points (Fig. 5B, Table II).

The time course of the fibril formation of D15N-hCT (III) at pH 7.5 was also examined by CD measurements. The monomeric component assigned to random coil (205 nm) was decreased gradually as a result of fibril formation after 2 h from dissolution (Fig. 6A). The fitting plot is shown in Fig. 6B, and the rate constants obtained by fitting to the data points are summarized in Table II. The characters of \( k_1 \) and \( k_2 \) will be discussed later.
Electrostatic Interaction of hCT during Fibrillation

Table I

| Samples | pH 3.2 (2.6 for III) | pH 7.5 | Standarda |
|---------|---------------------|--------|-----------|
| Gly15O |                     |        |           |
| II     | Monomer             | 171.9 α-helix | 171.9 α-helix | 171.6 α-helix |
|        | Fibril              | 169.7 β-sheet | 169.8 β-sheet | 170.9 random coil |
|        |                     | 171.5 α-helix | 171.7 α-helix | 168.5 β-sheet |
| III    | Monomer             | 171.9 α-helix | 171.9 α-helix |           |
|        | Fibril              | 170.0 β-sheet | 169.9 β-sheet |           |
|        |                     | 171.4 α-helix | 171.5 α-helix |           |
| Phe16  | Monomer             | 173.4 random coil | 173.4 random coil | 175.2 α-helix |
|        | Fibril              | 171.8 β-sheet | 171.7 β-sheet | 173.2 random coil |
|        |                     |           | 169.0 β-sheet |           |
| CH3Ala |                     |        |           |
| II     | Monomer             | 17.0 random coil | 16.9 random coil | 14.9 α-helix |
|        | Fibril              | 17.0 random coil | 16.9 random coil | 16.9 random coil |
|        |                     | 19.5 β-sheet | 18.9 β-sheet | 20.0 β-sheet |
| Ala31  | Monomer             | 17.2 random coilb | 17.2 random coilb |           |
|        | Fibril              | 17.2 random coilb | 17.2 random coilb |           |
|        |                     | 19.1 β-sheet | 19.4 β-sheet |           |
| III    | Monomer             | 15.9 random coil | 15.9 random coil | 13.9' α-helix |
|        | Fibril              | 15.9 random coil | 15.9 random coil | 15.9' random coil |
|        |                     | 18.9' β-sheet | 19.9' β-sheet | 19.9' β-sheet |

a Typical chemical shifts corresponding to the secondary structures determined in the model peptides (15–17).
b Or looplike structure (18).
c Took into account of the effect of neighboring Pro32 (21, 22).
d Minor peaks.

Fig. 4. Far-UV circular dichroism spectra of D15N-hCT (III) in 20 mM phosphate buffer (pH 7.2, A) and 15 mM acetic acid solution (pH 3.2, B). The concentration of D15N-hCT (III) was 0.2 mg/ml (58.5 µM). The spectra were measured in the first 5 min from dissolution.

Discussion

Conformation of D15N-hCT Monomer and Fibril—It was found that the D15N-hCT (I, II) monomer forms the local α-helical structure in the vicinity of Gly15 and random coil around Phe16, Ala26, and Ala31 residues in the neutral and acidic solutions, showing the same structures as those of hCT in view of their conformation-dependent 13C chemical shift data (13). The CD spectra of the D15N-hCT (III) solution (Fig. 4) also showed similarities to that of hCT, which indicated disordered secondary structure in solution (24, 25). Although the chemical shift values indicate that the vicinity of Phe16 forms a random coil, the central region of D15N-hCT monomer might be in equilibrium between the α-helix structure and random coil in solution as suggested for hCT that could form partially an α-helix in the central region (Thr12-Phe19) and undergo exchange with another structure to show the random coil form (12). The methyl signal from Ala31 (I) was observed at 17.2 ppm, which is shifted from the standard value of random coil (16.9 ppm). This discrepancy indicates that the C terminus may form a turn structure as suggested by a solution NMR study (12), because the chemical shift is similar to that of loop structure of bacteriorhodopsin (18).

During fibril formation, it was shown that the local random coil formed around the Phe16 residue in D15N-hCT was converted into the β-sheet structure (Figs. 2 and 3). Although 13C chemical shifts of [1-13C]Phe16 can be assigned to β-sheet structure, the deviation from the standard value is 2.7 ppm. This large discrepancy may be because of the formation of β-turn/β-sheet structure in the Asn17-Thr21 region (26). Because this structure is crucial for bioactivity, it is expected that the bioactivity of D15N-hCT will be the same as that of hCT. A part of the α-helical component around Gly15 residue is also changed to the β-sheet structure, although the α-helical structure remains as the major component around Gly15 in the D15N-hCT fibril. The chemical shifts of 169.7–170.0 ppm and 18.9–19.9 ppm for [1-13C]Gly and [3-13C]Ala, respectively, in D15N-hCT fibrils are quite similar to those of silk fibroin (type II), distinguished from the polymorphic form, silk I (27, 28), and of the native dragline silk fibroin (29).

Interestingly, two conformations exist also in the C terminus region as viewed from the 13C NMR peaks of [3-13C]Ala26 and [3-13C]Ala31 in the D15N-hCT fibril except for those of D15N-
Electrostatic Interaction of hCT during Fibrillation

Table II

| Method | pH conc. | k_1 \( s^{-1} \) | k_2 \( s^{-1} M^{-1} \) | aR_2 \( s^{-1} \) |
|--------|---------|------------------|-------------------|----------------|
| NMR Gly_{10} C=O | pH 2.9, 25 mg/ml | 6.17 (± 1.35) \( \times 10^{-7} \) | 5.36 (± 1.22) \( \times 10^{-3} \) | 3.92 (± 0.69) \( \times 10^{-5} \) |
| CD | pH 7.2, 0.2 mg/ml | 1.55 (± 0.02) \( \times 10^{-5} \) | 5.46 (± 1.18) | 3.19 (± 0.69) \( \times 10^{-4} \) |

Fig. 5. A, plots of peak intensities for \([1-^{13}C]Gly^{10}\) in \(^{13}C\) DD-MAS and CP-MAS spectra of D15N-hCT (III) at pH 2.9 (25 mg/ml) against the elapsed time. Acquisition was started after 4.8 h from dissolution. Closed diamond, DD-MAS signal; open circle, CP-MAS signal. The time of dissolution was regarded as 0 h. Acquisition was started after 6 h from dissolution by alternatively accumulating 1000 scans for DD-MAS and 2000 scans for CP-MAS experiments. B, the same plot as the intensity of CP-MAS signal (A) presenting the vertical line with the fraction of fibril after normalizing the intensity observed 60 h after dissolution as unity. Dotted line is the best fit to the equation representing the two-step reaction mechanism (13).

Fig. 6. A, time course of CD spectra of D15N-hCT (III) at pH 7.2 (0.2 mg/ml). B, plot of peak heights at 205 nm against the elapsed times. Vertical line presents the normalized fraction of the peak height with that observed after 5 h from dissolution.

hCT fibril (III) at pH 7.5. These conformations are similar to the case of hCT fibrils at pH 3.3 (13). These results led to a model of the fibril formation of D15N-hCT that indicates the fibrils form the mixture of antiparallel and parallel \(\beta\)-sheet structures at pH 7.5 and 3.3 as depicted in Fig. 7. It is emphasized that the conformation of D15N-hCT fibrils is almost the same as those grown at pH 3.2 and 7.5 and those of hCT at pH 3.0 except for the existence of the \(\alpha\)-helix component around Gly^{10} in the D15N-hCT fibrils. These structures, however, were quite different in the case of hCT at pH 7.5 (13).

Influence of Local Charges to Molecular Structure of the hCT Fibrils—Artificially mutated \(\beta\)-amyloid peptides changed from an ionic side chain to a neutral one have been investigated by means of electron microscopy, Fourier transform infrared spectroscopy, fiber x-ray diffraction, and NMR spectroscopy (30–32). The previous studies in the formation of amyloid fibrils from the fragments of amyloid \(\beta\) peptides showed that the charge to charge interactions stabilize the \(\beta\)-sheet conformation and promote the assembly of protofibrils into longer amyloid fibers (30). In contrast to the \(\beta\)-amyloid peptides, hCT has a limited number of ionic charged amino acids, such as Asp^{15}, Lys^{18}, and His^{20}, and the present study of the hCT fibril formation could be served as a more straightforward means to elucidate the effect of the electrostatic interaction on the formation of amyloid fibril.

The local charges in the hCT and D15N-hCT molecules are as follows: hCT at pH 7.5: Asp^{15}(-), Lys^{18}(+), NH_{3}(+); hCT at pH 3.3: Lys^{18}(+), His^{20}(+), NH_{3}(+); D15N-hCT at pH 7.5: Lys^{18}(+), NH_{3}(+); D15N-hCT at pH 3.3: Lys^{18}(+), His^{20}(+), NH_{3}(+). As for the molecular association, we suggested that hCT fibril at pH 7.5 forms the antiparallel \(\beta\)-sheet by a favorable electrostatic interaction between Asp^{15} and Lys^{18}, in addition to the hydrophobic interaction among the amphiphilic helices of the core region of hCT during the nucleation process (13). This kind of electrostatic interaction is also important for the stabilization of the coiled-coil structure (11). The hCT fibril formed the mixtures of antiparallel and parallel \(\beta\)-sheets up to the most C-terminal region at pH 3.3 because of the absence of an attractive ionic interaction to determine the direction for molecular association (13). On the other hand, the molecular structure of D15N-hCT fibril at pH 7.5 was similar to that of D15N-hCT fibril at pH 3.3. They are also similar to that of the hCT fibril at pH 3.3 and are quite different from that of the hCT fibril at pH 7.5. This result clearly shows that the presence of
a negative charge at Asp$^{15}$ determines the direction to lead the antiparallel $\beta$-sheet during molecular association (Fig. 7).

**Effect of Local Charges on Kinetics of hCT Fibrillation**—Fibril formation of D15N-hCT also showed the same two-step autocatalytic reaction mechanism as hCT. In the reaction, we can compare the rates for samples of one concentration with those of different concentrations, because the rate constant $k_1$ (s$^{-1}$) is independent of sample concentration and $ak_2$ (s$^{-1}$) is the comparative value where $a$ is the initial sample concentration (13). The rate constant, $k_1$, obtained for D15N-hCT in the acidic condition showed a smaller value by 5-fold as compared with that of hCT at pH 3.3, although the $k_2$ values are comparable ($k_1 = 3.28 \times 10^{-6}$ s$^{-1}$; $ak_2 = 4.10 \times 10^{-5}$ s$^{-1}$ for hCT) (13). Interestingly, both $k_1$ and $k_2$ values of D15N-hCT at neutral condition are larger than those of hCT at pH 7.5 ($k_1 = 2.79 \times 10^{-6}$ s$^{-1}$, $ak_2 = 1.34 \times 10^{-4}$ s$^{-1}$) (13).

Fig. 8A summarizes the local and the net charges in the central core region of hCT and D15N-hCT at various pH values as compared with their rate constants (Table II) (13). The sign of inequalities indicates the order of rate constants summarized in Table II. B, $\beta$-strands in the central core region of hCT and D15N-hCT (Leu$^9$-Phe$^{22}$).

![Fig. 7. Schematic representation of a proposed model for the fibril formation of D15N-hCT.](image)

![Fig. 8. A, local charges in the central core region of hCT and D15N-hCT at various pH values with the respective and net charges (Net). Net charges for Lys and His (Net*) are also shown. The sign of inequalities are also shown. The sign of inequalities indicates the order of rate constants summarized in Table II. B, $\beta$-strands in the central core region of hCT and D15N-hCT (Leu$^9$-Phe$^{22}$).](image)

**Table II.** Net charges for Lys and His in hCT and D15N-hCT.

| pH 3.3 | pH 7.5 |
|--------|--------|
| hCT    | D15N-hCT |
| Lys$^{18}$ | 0.90 | Lys$^{18}$ + 1.0+ |
| His$^{20}$ | 1.0+ | His$^{20}$ + 1.0+ |
| Net | 0.90 + | Net* 2.0 + |
| $k_1$ | $k_1$ | $k_1$ |
| $k_2$ | $k_2$ | $k_2$ |

**CONCLUSION**

We demonstrate by using D15N-hCT that absence of the negatively charged group in hCT leads to the fibril structure as a mixture of parallel and antiparallel $\beta$-sheets. This result demonstrates that the favorable electrostatic interaction among the charged side chains of Asp$^{15}$, Lys$^{18}$, and His$^{20}$ residues or amide nitrogen in hCT plays an important role to determine the direction of the molecular association to form the antiparallel $\beta$-sheet structure. It was found that the absence of a negative charge at Asp$^{15}$ does not reduce the ability of fibril formation, as D15N-hCTs form the amyloid fibrils even faster than hCT does. On the contrary, positively charged side chains of Lys$^{18}$ and His$^{20}$ residues standing near the $\beta$-strand structure delay the maturation of the fibril formation.

**Acknowledgments**—We thank Drs. S. Sonobe, T. Shimmen, S. Kimura, and T. Iyanagi of Himeji Institute of Technology for help and advice in the electron microscopy and the CD measurements.

**REFERENCES**

1. Copp, D. H., Cameron, E. C., Cheney, B. A., Davidson, A. G. F., and Henze, K. G. (1962) Endocrinology 70, 638–649
2. Kumar, M. A., Foster, G. V., and MacIntyre, I. (1963) Lancet 2, 480–482
3. Austin, L. A., and Heath, H. D. (1981) N. Engl. J. Med. 304, 269–278
4. Sieber, P., Rinkler, B., Brugger, M., Kamber, B., and Rittel, W. (1970) Helv. Chim. Acta 53, 2135–2150
5. Sipe, J. D. (1982) Annu. Rev. Biochem. 61, 947–975
Electrostatic Interaction of hCT during Fibrillation

6. Sipe, J. (1994) Crit. Rev. Clin. Lab. Sci. 31, 325–354
7. Kelly, J. (1998) Curr. Opin. Struct. Biol. 8, 101–106
8. Lansbury, P. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3342–3344
9. Berger, G., Berger, N., Guillaud, M., Truvillas, J., and Vanzelle, J. (1998) Verh. Dtsch. Ges. Pathol. 82, 543–551
10. Byard, R., Thorner, P., Chan, H., Griffiths, A., and Cutz, E. (1990) Pediatr. Pathol. 10, 581–592
11. Branden, C., and Tooze, J. (1999) Introduction to Protein Structure, 2nd Ed., pp. 35–37, Garland Publishing, Inc., New York
12. Jeon, Y. H., Kanaori, K., Takashima, H., Koshiba, T., and Nosaka, Y. A. (1998) Abstracts of XVIIIth International Conference on Magnetic Resonance in Biological Systems, p. 61, Tokyo
13. Kamihira, M., Naito, A., Tuzi, S., Nosaka, Y. A., and Saitò, H. (2000) Protein Sci. 9, 867–877
14. Kanaori, K., and Nosaka, A. Y. (1995) Biochemistry 34, 12138–12143
15. Saitô, H. (1986) Magn. Reson. Chem. 24, 835–852
16. Saitô, H., and Ando, I. (1989) Annu. Rep. NMR Spectrosc. 21, 209–290
17. Wishart, D. S., Sykes, B. D., and Richards, F. M. (1991) J. Mol. Biol. 222, 311–333
18. Saitô, H., Tuzi, S., and Naito, A. (1998) Annu. Rep. NMR Spectrosc. 36, 79–121
19. Moe, G. B., and Kaiser, E. T. (1985) Biochemistry 24, 1971–1976
20. Orpiszewski, J., and Benson, M. D. (1999) J. Mol. Biol. 289, 413–428
21. Torchia, D. A., and Lyerla, J. J. (1974) Biopolymers 13, 97–114
22. Wishart, D. S., Bigam, C. G., Holm, A., Hodges, R. S., and Sykes, B. D. (1995) J. Biomol. NMR 5, 67–81
23. Epand, R. M., Epand, R. F., and Orlowski, R. C. (1990) Eur. J. Biochem. 188, 633–635
24. Arvinte, T., Cudd, A., and Drake, A. F. (1993) J. Biol. Chem. 268, 6415–6422
25. Epand, R. M., Epand, R. F., Orlowski, R. C., Schlueter, R. J., Beni, L. T., and Hui, S. W. (1983) Biochemistry 22, 5074–5084
26. Kazantzis, A., Waldner, M., Taylor, J. W., and Kapurniotu, A. (2002) Eur. J. Biochem. 269, 780–791
27. Saitô, H., Tuzeta, R., Asakura, T., Iwanaga, Y., Shoji, A., Ozaki, T., and Ando, I. (1984) Macromolecules 17, 1405–1412
28. Ishida, M., Asakura, T., Yokoi, M., and Saitô, H. (1990) Macromolecules 23, 88–94
29. Hijiida, D. H., Gian Do, H., Michal, C., Wong, S., Zax, D., and Jelinski, L. W. (1996) Biophys. J. 71, 3442–3447
30. Fraser, P. E., Nguyen, J. T., Surewicz, W. K., and Kirschner, D. A. (1991) Biophys. J. 60, 1190–1201
31. Fraser, P. E., McLachlan, D. R., Surewicz, W. K., Mizen, C. A., Snow, A. D., Nguyen, J. T., and Kirschner, D. A. (1994) J. Mol. Biol. 244, 84–73
32. Wood, S. J., Maleeff, B., Hart, T., and Wetzel, R. (1996) J. Mol. Biol. 256, 870–877