Topological isomers of human uroguanylin: interconversion between biologically active and inactive isomers

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Received 21 October 1997; revised version received 1 December 1997

Abstract The solution structures of the two compounds of human uroguanylin (I and II), which were generated during disulfide bond forming reaction, were found to be topological isomers by ^{1}H-nuclear magnetic resonance spectroscopy. These isomers are interconvertible in aqueous media at rates which vary with the pH and temperature of the solution. Because compound I is active in the cGMP producing assay, but compound II is not, this interconversion may be useful for evaluating the activity of human uroguanylin both in vivo and in vitro.

Key words: Uroguanylin; Guanylin; Topological isomer; Interconversion; HPLC analysis; Biological activity

1. Introduction

Uroguanylin and guanylin were discovered as endogenous peptide hormones in mammals based upon their structure similarity to heat-stable enterotoxins (STs) secreted by pathogenic bacteria. The primary structures of uroguanylin and guanylin from human, rat (mouse) and opossum have been reported as being comprised of 15 or 16 amino acid residues [1–5]; the human and rat peptide sequences are shown in Fig. 1. The sequence similarity among them is high and four Cys residues in all the peptides are conserved. These Cys residues participate in the formation of the two intramolecular disulfide linkages, one between Cys[4] and Cys[12] and the other between Cys[7] and Cys[15]. Uroguanylin and guanylin, as well as ST, are reported to be involved in the regulation of salt and water transport in the intestinal tract and kidney. In addition, these peptides are known to stimulate cGMP production by activating the guanylyl cyclase C in both enterocytes and T84 colon cancer cells. Therefore, endogenous uroguanylin and guanylin are suggested to play important roles in intestinal and renal dysfunction and salt-dependent hypertension [6].

In our previous paper on the chemical synthesis of human uroguanylin using a two-step selective disulfide forming method, two compounds (I and II) were found to be generated upon analyzing the second disulfide bond forming reaction by reversed-phase high performance liquid chromatography (RP-HPLC) at 40°C [7]. A typical chromatogram for the separation of a 1:1 mixture of these compounds is shown in Fig. 2. We have so far clarified the following characteristics for compounds I and II: (i) each compound can be isolated to a purity greater than 99% as determined by RP-HPLC; (ii) both have identical primary structures, molecular weights and disulfide connectivity patterns according to examination by suitable analytical methods; and (iii) significant differences exist between them in the optical rotation value and their biological activity. During the course of our previous study, Skelton et al. reported that two clearly separable signal connectivities were detected in the analysis of the amino-terminally extended or deleted human guanylin derivatives by nuclear magnetic resonance (NMR) spectroscopy. Based on these observations and structural refinements, they proposed that the heterogeneity of the NMR signals of human guanylin derivatives originated from the topological isomerism of the peptide, although such isomers were unseparable on RP-HPLC under the various analytical conditions used [8]. In contrast, we found in a previous study that human des-Leu[16]-uroguanylin and rat guanylin, both of which terminate the peptide chains at the fourth Cys residue like human guanylin, were detected as two base-line separable peaks on RP-HPLC when the analytical temperature was decreased to 8°C, although these peptides were eluted in a broad but single peak at 40°C [7]. From these observations, we assumed that the two well-separable human uroguanylin compounds on RP-HPLC were similar topological isomers with respect to the peptide backbone as reported for human guanylin derivatives. However, this assumption required definite confirmation by experimental evidence.

In the present study, we analyzed the solution structures of both compounds by NMR in an aqueous medium to gain further insight into the characteristics of the isolated compounds I and II of human uroguanylin. We report here con-

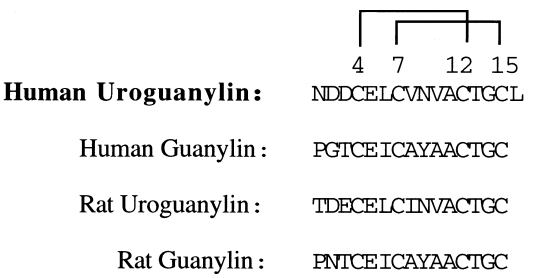

Fig. 1. Primary structures of human and rat uroguanylin and guanylin. Two intramolecular disulfide linkages are shown at the top of the sequences.

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Abbreviations: ST, heat-stable enterotoxin; cGMP, cyclic 3′,5′-guanosine monophosphate; RP-HPLC, reversed-phase high performance liquid chromatography; CD, circular dichroism; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; MD, molecular dynamics; RMSD, root mean square deviation; GdnHCl, guanidine hydrochloride; NEM, *N*-ethylmaleimide; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide

PII S0014-5793(97)01527-5

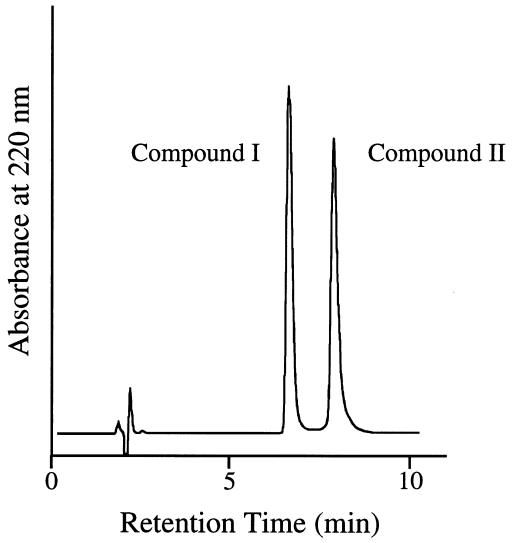

Fig. 2. Separation of the isolated compounds I and II of human uroguanylin (1:1 mixture) on RP-HPLC under isocratic conditions. See Section 2 for details of the analytical conditions.

firmation that the two compounds of human uroguanylin are indeed topological isomers. We then report on their stability in solution and in the solid state. These experiments demonstrate that the topological isomers of human uroguanylin are not stable in solution and are readily interconvertible, although they are stable during storage in a freezer as powders. This has important implications for evaluating the biological activity of human uroguanylin because compound I elicits the activity in the cGMP production assay, whereas compound II primarily aborts it [9].

2. Materials and methods

2.1. Peptide synthesis

Two compounds (I and II) of human uroguanylin were obtained following the reported procedure [7]. Briefly, the protected peptide was elongated on resin using an ABI 430A peptide synthesizer by applying Boc/Bzl chemistry. Pairs of the Cys residues which form intramolecular disulfide bonds were selectively protected by orthogonally cleavable groups, 4-methylbenzyl and acetamidomethyl. After treatment with anhydrous hydrogen fluoride, two intramolecular disulfide bonds were formed successively with $K_3[Fe(CN)_6]$ and then with iodine. Two compounds generated during the second disulfide bond formation in MeOH/50% AcOH (1:1, v/v) were isolated by RP-HPLC, lyophilized and stored in a freezer until use. Human des-Leu^{16}-uroguanylin, rat guanylin and rat uroguanylin-15 were synthesized by applying the same strategy as for human uroguanylin. The resulting major peaks from RP-HPLC were isolated at 40°C and stored in a freezer after the usual workup including lyophilization.

2.2. CD measurement

The CD spectrum was recorded on a JASCO J720 spectropolarimeter with a cell path length of 0.1 cm at 25°C. The sample was dissolved in 10 mM sodium phosphate buffer at pH 7.0 at a concentration of 100 μM.

2.3. NMR measurement and structure calculation

All the spectra were recorded on a Bruker DRX-500 spectrometer at 10°C. The peptide was dissolved in 10 mM sodium phosphate at pH 3.7 containing 10% deuterated water (D_2O) at a peptide concentration of 5 mM. Assignment of proton resonances was achieved according to the standard method developed by Wüthrich [10]. The nuclear Overhauser effect (NOE) distance constraints for compounds I and II were derived from two-dimensional NOESY spectra acquired for 24 h with mixing times of 120 and 250 ms, respectively. All the structure calculations were performed with the program X-PLOR.

2.4. RP-HPLC analysis

RP-HPLC was performed on a Shimadzu Model LC-6A with a YMC ODS column (4.6×150 mm). Standard conditions for analyses of the two compounds of human uroguanylin were isocratic elution at 25.5% acetonitrile in 0.1% TFA at 40°C. Analyses of 15-residue guanylin and uroguanylin derivatives were carried out both at 40°C and 8°C under linear gradient conditions; 1% to 60% acetonitrile (25 min) in 0.1% TFA. Absorbance was monitored at 220 nm.

2.5. Stability of compounds I and II in solution

Each isolated compound of human uroguanylin was dissolved in 50% AcOH, 0.05% TFA or 50 mM NH_4OAc at pH 7.7 containing 0.25 M guanidine hydrochloride (GdnHCl) at a concentration of 1 mg/200 μl. Half of each prepared solution was incubated at 37°C and the remaining half was kept at ambient temperature (15–20°C). In the case of the analysis in the presence of *N*-ethylmaleimide (NEM), each compound was dissolved in the same buffer as above at pH 7.7 which contains a slight molar excess amount of NEM. The change in purity of each peptide was analyzed at 24 h intervals by RP-HPLC. The amount of each compound in the individual solution was calculated by integration of the corresponding peak areas on the chromatogram.

2.6. Bioassay

Accumulation of cGMP in T84 cells was measured following a reported procedure [9].

3. Results

3.1. Solution structure of compounds I and II of human uroguanylin

In order to elucidate the secondary structure difference between compounds I and II of human uroguanylin, CD spectra of each compound were recorded at pH 7.0 (Fig. 3). A negative band around 200 nm was detected for both compounds, but the ellipticity was much greater in compound I, suggesting a difference in their structures.

Further structural analysis of the two compounds was carried out by NMR in 10 mM sodium phosphate at pH 3.7 and 10°C. From the distance constraints elucidated from the NOE data, the ensembles of 10 solution structures of compounds I and II were deduced with reasonably good convergence (RMSDs for compounds I and II backbone atoms are 0.67 Å and 0.48 Å, respectively) if the less well-defined amino-terminal 3-residues and the carboxyl-terminal Leu residue were excluded. The energy-minimized average structures of

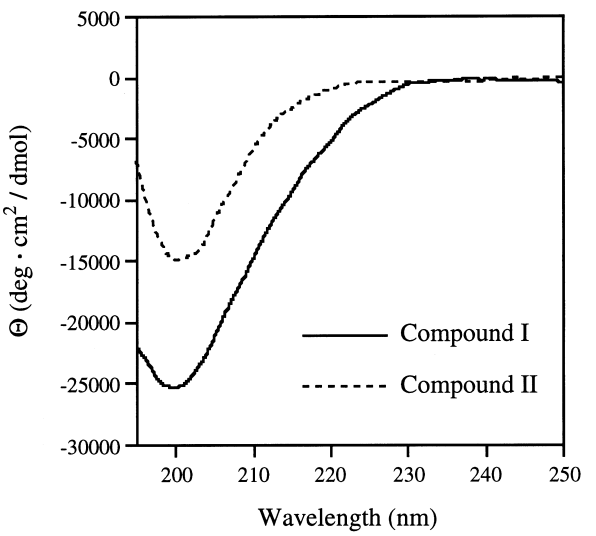

Fig. 3. CD spectra of compounds I and II of human uroguanylin in 10 mM sodium phosphate at pH 7.0.

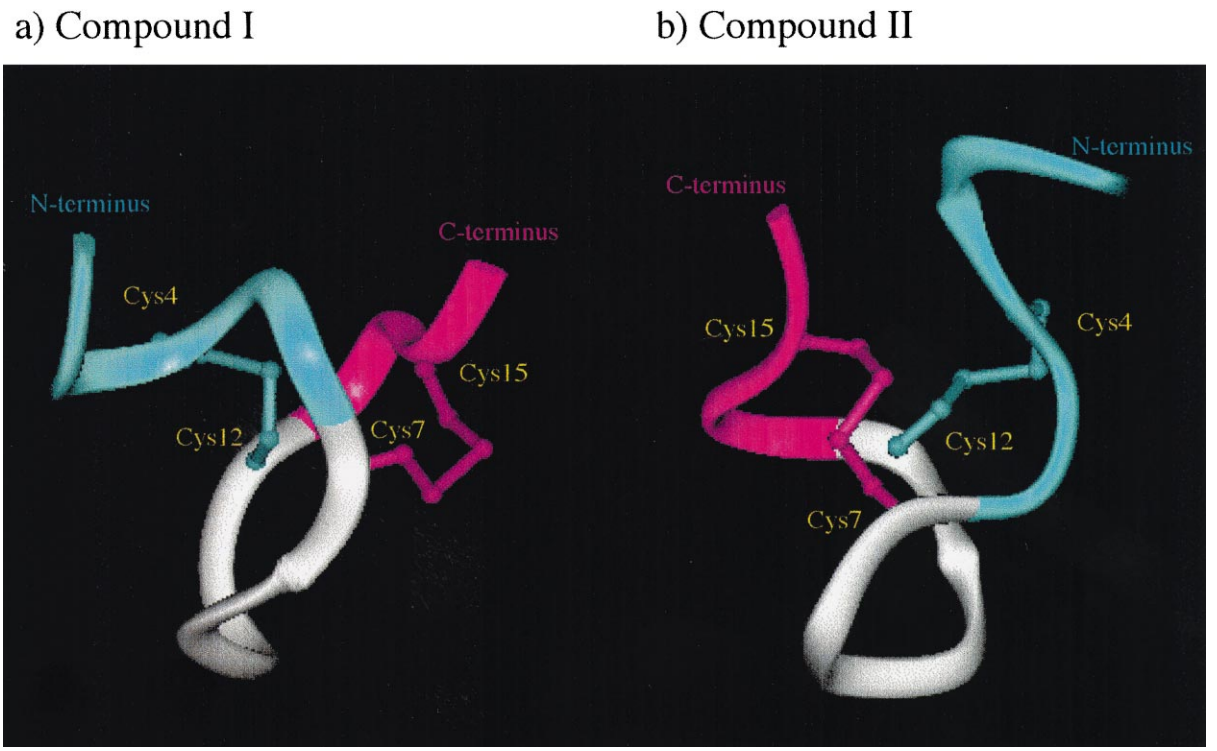

Fig. 4. Average favored solution structures of compound I (a) and compound II (b) of human uroguanylin.

compounds I and II are shown in Fig. 4. Both favored structures are depicted by fixing the loop structure composed of the residues 7–12 (central loop) to have the same spatial arrangement at their bottoms. As is obvious from the deduced structure of compound I shown in Fig. 4a, one segment comprising the amino-terminal residues 1–7 and the disulfide bond between Cys^4 and Cys^{12} is located at the left top side of the central loop and another segment encompassing the carboxyl-terminal residues 12–16 and the disulfide bond between Cys^7 and Cys^{15} is at the right top side. In contrast, the structure of compound II, shown in Fig. 4b, revealed that the above two segments extend outwards and directly opposite from the central loop. Therefore, the solution structure difference between the two compounds lies merely in the orientation of these two segments from the central loop, clearly demonstrating that the well-separable two compounds of human uroguanylin on RP-HPLC are topological isomers.

3.2. *Stability of compounds I and II of human uroguanylin*

Compounds I and II have distinctly different retention times on RP-HPLC at 40°C, and thus could be separately isolated at purities greater than 99%. The purities of the isolated compounds were confirmed to be maintained for more than one year when each compound was stored in a freezer as an amorphous powder. However, mutual contamination, comprising 0.8% of the total peak areas, was seen when the purities of the isolated compounds were evaluated by RP-HPLC after the compounds were lyophilized from 0.1%

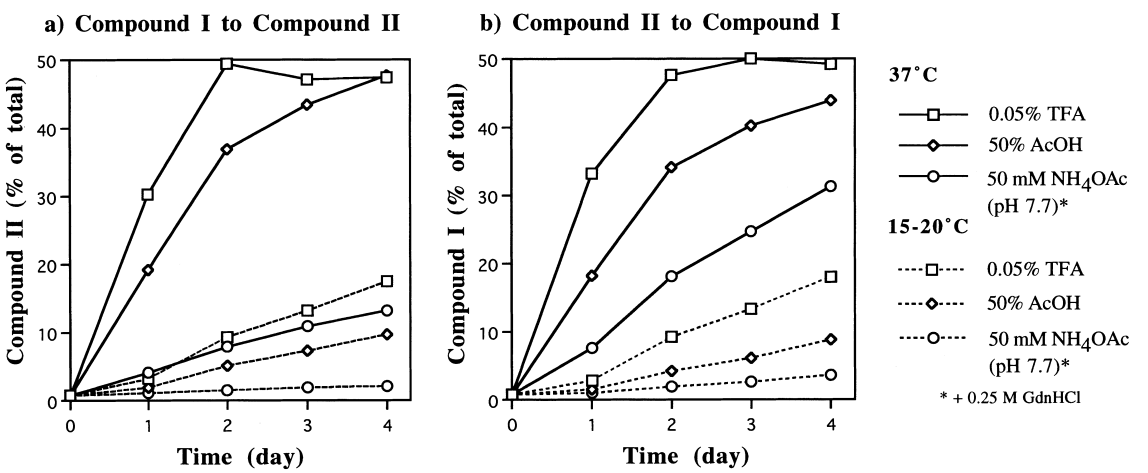

Fig. 5. Time course analyses of conversion of compound I (a) and compound II (b) of human uroguanylin in solution. The purity change of each compound was monitored by RP-HPLC under isocratic conditions. The peak area of each converted compound was shown as a % of the sum of those for compounds I and II on the individual chromatogram.

TFA solution. These results suggest that while the two compounds are stable once they are isolated and stored as powders at below −20°C, their stability may be less in solution, that is, compounds I and II are interconvertible in solution. To examine whether such interconversion occurred during the synthesis and analysis, the degree of purity of each compound in 50% AcOH, 0.05% TFA and 50 mM NH_4OAc (pH 7.7) containing 0.25 M GdnHCl was analyzed by RP-HPLC. These solvents were chosen as mimicking the media employed for disulfide bond formation, purification of the oxidized peptides by RP-HPLC and measurement of the biological activity, respectively. Each solution was kept at both ambient temperature (15–20°C) and 37°C.

RP-HPLC analyses of the constructed solutions of compounds I and II showed that the peak area of the starting compound decayed gradually and that of the converted material (compound I in the case of compound II and vice versa) increased comprehensively in all the media we examined. This clearly demonstrates that the interconversion of the two compounds indeed takes place in solution. The results of the analyses of the interconversion for each compound are summarized in Fig. 5 as a function of time. In the acidic milieu (0.05% TFA and 50% AcOH), the conversions proceeded in an almost comparable manner for both compounds. The slopes of the conversion rate in 0.05% TFA, however, were steeper than those in 50% AcOH, that is, the conversion from compound I to compound II and vice versa at 37°C in 0.05% TFA was complete and resulted in a steady state in 2 days, whereas more than 4 days were required for the same processes in 50% AcOH. The temperature dependence of the conversion was observed in both acidic media, in which the rate of the interconversion was always faster at 37°C than that at room temperature (15–20°C).

In 50 mM NH_4OAc buffer at pH 7.7, both compounds were generally more stable (slower conversion) than in acidic milieu. However, in contrast to the results in the two acidic solvents, their conversion rates were not identical in this neutral buffer, that is, compound I was converted more slowly than compound II. In addition, the slope of the conversion rate of compound I at pH 7.7 and 37°C was found to be lower than that in 0.05% TFA at ambient temperature. This rank order alteration in neutral buffer is distinct from all other experiments because regardless of the starting compounds, preferential conversion rates were always observed at 37°C except for this particular case.

The above results definitely demonstrate that the two compounds are interconvertible in solution, therefore, it is tempting to conjecture that disulfide linkage scission and reclosure is involved in the mechanism of the conversion. In order to address this, a stability test was done using the same buffer as above at pH 7.7, except NEM, a well-known SH trapping reagent, was added. Analysis by RP-HPLC showed that the slope of the conversion in the presence of NEM could be completely superimposed on that in its absence (data not shown), indicating that disulfide bond opening does not occur during the conversion.

4. Discussion

Structure analyses of two compounds (I and II) of human uroguanylin by NMR were carried out in both dimethyl sulfoxide (DMSO) and aqueous solutions. Distinct chemical shifts were observed for both compounds in either medium, but more Hα-signal overlapping was detected in DMSO. Therefore, the aqueous solution structures of the two compounds of human uroguanylin were refined with a simulated annealing protocol, by which they were definitely confirmed to be topological isomers. The elucidated backbone structure of the isolated compound I (a biologically active component) is similar to that of the reported human guanylin isomer 'A-form', which was determined in the mixture of the two topological isomers [8], as well as that of heat-stable enterotoxin (ST) with three disulfide bonds [11]. Although it has not been clarified as to whether the human guanylin 'A-form' is a biologically active component or not, the similar backbone topology among these family peptides may be a prerequisite for expressing the cGMP producing activity. Analyses of the side chain orientation of the two compounds of human uroguanylin is now underway in our laboratory, however, in a preliminary result, side chain location in the putative active site region around Ala^{11}, which is estimated from the active site of ST reported by Shimonishi et al. [12], seems to be different from each other (data not shown). Results of these analyses together with the experimental data for three-dimensional backbone structure resolution will be reported in the near future (T. Yoshida et al., manuscript in preparation).

In the RP-HPLC analyses of the two human uroguanylin isomers, we had already established that they are separable at 40°C. However, separations of the human des-Leu^{16}-uroguanylin isomers, as well as the rat guanylin isomers, were possible only at lower temperatures such as 8°C [7]. This separation characteristic has also been observed for a recently disclosed member of the uroguanylin and guanylin peptide family, rat uroguanylin-15 (unpublished result). Considering that the latter three peptides are composed of 15 residues with the sequence ending at the fourth Cys residue, we confirmed that the Leu residue at position 16 of human uroguanylin endows the topological isomers with a separation efficiency significantly higher than the other shorter uroguanylin and guanylin family peptides on RP-HPLC. In other words, the topological isomers of human uroguanylin may be stabilized significantly by the Leu residue lying outside the disulfide-linked loop structure.

As far as we know, including the case of human guanylin [8], few features have been characterized to date for the isolated individual topological isomers. In the present study, we have shown that these isomers of human uroguanylin are interconvertible without disulfide bond opening when left standing in solution over a period of time (days). Analyses of the stability of the two compounds at acidic and neutral pH values suggest that the conversion rates are affected by the ionization state of functional group(s) in the molecule. At acidic pH, both compounds are, in one sense, freely convertible (same conversion rates) and eventually come to a 1:1 equilibrium ratio. In contrast, conversions of both compounds at pH 7.7 seem to be hampered and thereby their rates are significantly decreased, especially for compound I. A more extensive analysis of the pH-dependent conversion rates of the two isomers is now underway in our laboratory.

In the NMR study of human guanylin derivatives reported by Skelton et al. [8], they commented on the interconversion of the topological isomers: (i) based on line broadening experiments at high temperature, the exchange must be very slow with a half-life of seconds or longer, and (ii) in restrained

molecular dynamics (MD) calculations, transition from one state to the other could not be induced with a realistic force field. In the present study, we observed by RP-HPLC analyses that the half-life of interconversion of the two human uroguanylin isomers in 0.05% TFA at 37°C was about 1 day; these are the conditions under which the interconversion proceeds fastest. In the case of the human des-Leu^{16}-uroguanylin topological isomers, they were more unstable than those of the parental peptide because they were mutually contaminated by approximately 20–30% after isolation by RP-HPLC at 8°C and subsequent concentration of the solvent at room temperature for 2 h (preliminary data). This phenomenon is also observed for rat uroguanylin-15, demonstrating that the topological isomers of the peptides with the fourth Cys residue at their carboxyl termini survive as a main fraction after 2 h even though the interconversion rate is faster than for human uroguanylin. These observations confirm that the interconversion rates of the topological isomers of uroguanylin and guanylin family peptides are much longer than that of NMR and MD time scales.

We have already reported that (i) in the chemical synthesis of human uroguanylin, the ratio of the topological isomers (compounds I and II) differs significantly with the order of the two intramolecular disulfide bond forming reactions, and that (ii) compound I shows significant activity in the cGMP producing assay, whereas compound II is practically inactive [7,9]. We have further clarified recently that compound I isolated after conversion from the inactive component, compound II, is fully active. Furthermore, compound II is a weak agonist without antagonistic activity because the cGMP production in T84 cells by the simultaneous stimulation with both compounds was equivalent to the sum of the individual stimulations (data not shown). In the former chemical synthesis, the second disulfide bond forming reaction proceeded very quickly (less than 20 min) and the reaction mixture was analyzed immediately after quenching of the reaction with ascorbic acid. In the latter case, measurement of the biological activity was performed using freshly prepared solution of each compound and the response in T84 cells was not retarded. Therefore, we are certain that all these findings are correct even though the two compounds are interconvertible in solution. We have also elucidated the endogenous molecular form of uroguanylin in humans by the combined analyses of RP-HPLC and radioimmunoassay using antibodies specific for each topological isomer [9]. As relatively long times and procedures were required for the isolation and quantification of the two isomers in the body, it is conceivable that the interconversion may occur during the course of the analysis. Actually, a low level of mutual cross-reactivity contaminations (3–3.5%) in each of the specific antibodies were observed during characterization of the specificity of the respective antibodies using two ^{125}I-Tyr^{0}-human uroguanylin isomers as tracers. This implies the possible conversion of the two isomers of the standard and/or radio-labeled peptides, although such cross-reactivity might be an intrinsic feature of the antibodies. Nonetheless, this does not invalidate the reported results concerning the endogenous form and amount of human uroguanylin because care was always taken during each experimental step to detect the occurrence of the conversion and to minimize experimental errors by keeping the peptide solutions at as a low temperature as possible. In the above NMR experiments, the structures of both compounds were determined without serious mutual contamination of signals probably because the measurements were carried out at a low temperature of 10°C.

In the present study, we found that human uroguanylin tends to isomerize topologically in solution, resulting in a mixture of the biologically active and inactive isomers. Interestingly, the 24-amino acid peptide with an 8-residue extension at the amino terminus of human uroguanylin could be isolated from hemofiltrate pools and was found to accumulate cGMP in T84 cells, that is, amino-terminally extended human uroguanylin is a biologically active form [13]. In the case of rat guanylin, its precursor with 94 amino acid residues as well as the amino-terminally Asp extended peptide with 16 amino acid residues are both biologically inactive [14]. Most of the biologically active 15-residue peptide of rat guanylin is reported to be generated by the artificial cleavage at the Asp-Pro bond in the precursor during the isolation process with hot AcOH [15]. Taken together, we speculate that exertion of the biological activity of the uroguanylin and guanylin family peptides is primarily determined by two factors: (1) peptide chain length and (2) topological isomerization.

Finally, we emphasize that the biological activity data of human uroguanylin and its derivatives, regardless of whether they are synthetic or natural products, may lead to confusing, variable potency results if the data are obtained after the peptide solutions have been left at room temperature for a few days, especially under acidic conditions.

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