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Conformational and Biological Properties of the Ala[10] Analogue of Human Des-Trp[1],Nle[12]-minigastrin†

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ABSTRACT: Synthesis, conformation, and biological properties of the Ala[10] analogue of des-Trp[1],Nle[12]-minigastrin are reported. Replacement of the Gly residue in the original sequence with Ala remarkably changes the conformational preference of the hormone in trifluoroethanol. CD and NMR results indicate that the conformational change is mainly located in the C-terminal portion of the molecule, with probable extension of the N-terminal α-helix throughout the entire sequence. The structural modification causes a 10-fold decrease in the biological potency of the hormone, which is about as active as the C-terminal tetrapeptide amide. These findings support our previous hypothesis that the optimal bioactive conformation of the native hormone is U-shaped, with mutual interactions among the two end segments.

Previous investigations on the synthetic analogues of human gastrin, des-Trp[1],Nle[12]-minigastrin

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     2   3-7   8   9   10  11  13  13  14
H-Leu-[Glu]5-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH2
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and Nle[15]-little gastrin

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 1   2   3   4   5   6-10  11  12  13  14  15  16  17
pGlu-Gly-Pro-Trp-Leu-[Glu]5-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH2
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led to the hypothesis that the conformation assumed by these hormones in TFE[1] (Peggion et al., 1985; Peggion & Foffani, 1985) or in aqueous solutions containing detergent micelles (Mammi et al., 1987) is of biological relevance. A conformational model was proposed on the basis of CD results on both peptides and on their fragments, as well as high-resolution ^{1}H NMR studies on des-Trp[1],Nle[12]-minigastrin (Mammi et al., 1986, 1988). According to our hypothesis, the structure of this gastrin form is characterized by an α-helical segment at the N-terminus, comprising the $-(Glu)_5-$ sequence, and by a bend in the central part of the molecule. The hydrogen bonds involving amide protons in the C-terminal region, suggested by NMR results, are compatible with the presence of a segment of 3_{10}-helix starting from Ala[8]. Thus, the proposed model involves two helical segments at the chain ends, stabilized by mutual interactions in a U-shaped structure.

In the proposed conformation, the Gly residue in position 10 appears to play a key role in determining the conformation of minigastrin. According to Chou and Fasman analysis (Chou & Fasman, 1977), replacement of Gly with a helix-forming residue would change completely the conformational preference of the peptide backbone, extending the α-helix throughout the entire molecule. This in turn should have a substantial effect on the biological potency of the hormone.

From previous structure–function correlation studies on gastrins, we have learned that replacement of the methionine residue by nearly isosteric norleucine is without any effect on the biological activity (Wünsch et al., 1982). Similarly, the hormonal potency was practically fully retained when the human minigastrin sequence was shortened by one residue at its N-terminus (Göhring et al., 1984). Correspondingly, for the present study a minigastrin analogue was synthesized in which the Gly residue at position 10 of our reference compound (des-Trp[1],Nle[12]-minigastrin) has been replaced by the helix-forming residue Ala. This gastrin analogue is used to test our working, structural hypothesis by comparative conformational and biological studies.

Experimental Procedures

Synthesis. Melting points were determined on a capillary melting point apparatus (Büchi) and are uncorrected. Optical rotations were measured in a thermostated 1-dm cell on a

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[1] Abbreviations: DCC, dicyclohexylcarbodiimide; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; TFE, trifluoroethanol; DMF, dimethylformamide, NMP, *N*-methylpyrrolidone; NMR, nuclear magnetic resonance; CD, circular dichroism; UV, ultraviolet; TMS, tetramethylsilane; FID, free induction decay; TOCSY, total correlation spectroscopy; ROESY, rotating-frame nuclear Overhauser effect spectroscopy; CCK, cholecystokinin.

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Perkin-Elmer polarimeter (Model 141). Acid hydrolyses were conducted in 6 M HCl containing 2.5% thioglycolic acid.

Amino acid analyses were obtained on a Biotronic LC6001 amino acid analyzer. Gas chromatographic racemization tests were carried out with Chirasil-Val glass capillary columns according to the method of Frank et al. (1977). Analytical HPLC was performed on Waters instruments and preparative HPLC on Jobin-Yvon Prep 10. For TLC and HPTLC silica gel 60 plates (Merck AG, Darmstadt) were used with the following solvent systems: (1) 1-butanol/pyridine/acetic acid/water/AcOEt 60:20:6:24:110; (2) heptane/2-methyl-2-propanol/acetic acid 5:1:1; (3) 1-butanol/acetic acid/water/AcOEt 6:2:2:10; (4) cyclohexane/$CHCl_3$/acetic acid 45:45:10; (5) MeOH/AcOEt/CH_2Cl_2 1:2:3; (6) $CHCl_3$/TFE/MeOH 18:2:1; (7) $CHCl_3$/TFE/80% propionic acid 5:1:1; (8) $CHCl_3$/TFE 3:1; (9) 1-butanol/acetic acid/water/pyridine 60:6:24:20; and (10) 1-butanol/acetic acid/water/pyridine 45:6:24:20.

The amino acid derivatives used in the synthesis were prepared according to standard procedures (Wünsch, 1974).

Z-Tyr(tBu)-Ala-OH (**1**). Z-Tyr(tBu)-OSu (14.06 g; 30 mmol) was dissolved in dioxane (120 mL) and added under stirring to a chilled solution of H-Ala-OH (5.35 g; 60 mmol) in 1 M NaOH (60 mL). The reaction mixture was kept in an ice bath for 2 h and then at room temperature overnight. The bulk of dioxane was removed under reduced pressure, and the resulting aqueous solution was acidified with 5% $KHSO_4$ to pH 2 and extracted twice with EtOAc. The combined extracts were washed with 1% $KHSO_4$ and water, dried over Na_2SO_4, and evaporated to dryness. The product crystallized from EtOAc/ether: yield 11.3 g (85%); mp 126–127 °C; $[\alpha]^{20}_D = -5.5°$ and $[\alpha]^{20}_{546} = -6.4°$ (*c* 3, MeOH); TLC solvent systems 1, 2. Anal. Calcd for $C_{24}H_{30}N_2O_6$ (442.5): C, 65.14; H, 6.84; N, 6.33. Found: C, 65.26; H, 6.95; N, 6.34.

H-Tyr(tBu)-Ala-OH (**2**). Dipeptide derivative **1** (11.15 g; 25.2 mmol) was hydrogenated at room temperature and atmospheric pressure over palladized charcoal in MeOH (300 mL). The catalyst was removed by filtration, and the filtrate was evaporated. The product was reprecipitated from MeOH with ether: yield 6.8 g (82%); mp 285–287 °C; $[\alpha]^{20}_D = +17.9°$ and $[\alpha]^{20}_{546} = +21.7°$ (*c* 1, MeOH); TLC solvent systems 2, 3. Anal. Calcd for $C_{16}H_{24}N_2O_4 \cdot {}^1/_2H_2O$ (317.4): C, 60.55; H, 7.94; N, 8.83. Found: C, 60.71; H, 8.08; N, 8.84.

Z-Ala-Tyr(tBu)-Ala-OH (**3**). Dipeptide derivative **2** (6.5 g; 20.5 mmol) was reacted with Z-Ala-OSu (8.1 g; 25.3 mmol) at room temperature in DMF (150 mL). After 12 h, the solvent was removed under reduced pressure, and the residue was worked up as described for **1**; the product crystallized as DCHA salt from EtOAc/ether: yield 12.5 g (88%); mp 143–145 °C; $[\alpha]^{20}_D = -11.7°$ and $[\alpha]^{20}_{546} = -13.9°$ (*c* 1, MeOH); amino acid analysis Ala 2.00 (2), Tyr 1.00 (1); gas chromatographic racemization test D-Ala 0.7%, D-Tyr 0.7%; TLC solvent systems 1, 2. Anal. Calcd for $C_{27}H_{35}N_3O_7 \cdot C_{12}H_{23}N$ (694.9): C, 67.41; H, 8.41; N, 8.06. Found: C, 67.28; H, 8.46; N, 8.05.

Z-Ala-Tyr(tBu)-Ala-Trp-Nle-Asp(OtBu)-Phe-NH$_2$ (**4**). Tripeptide derivative **3** (2.07 g; 2.98 mmol) was desalted by distribution between EtOAc and 1% $KHSO_4$. The organic layer was washed with water, dried over Na_2SO_4, and evaporated. The residue was dissolved in DMF (40 mL), and H-Trp-Nle-Asp(OtBu)-NH_2·HCl·H_2O (Moroder et al., 1981) (2.05 g; 2.98 mmol) was added followed by *N*-methylmorpholine (0.33 mL; 2.98 mmol) and *N*-hydroxysuccinimide (0.69 g; 5.96 mmol). The resulting solution was cooled to −15 °C and then reacted with DCC (0.68 g; 3.28 mmol). The reaction mixture was allowed to reach room temperature in 2 h and was stirred for an additional 12 h. On addition of EtOH the precipitate was collected and washed extensively with MeOH and ether: yield 2.6 g (27%); mp 255–256 °C dec; $[\alpha]^{20}_D = -21.4°$ and $[\alpha]^{20}_{546} = -26.4°$ (*c* 1, DMF); amino acid analysis Asp 1.05 (1), Ala 2.00 (2), Tyr + Nle 1.92 (2), Phe 1.02 (1), Trp 0.94 (1); gas chromatographic racemization test D-Ala <0.5%, D-Asp 1.3%, D-Phe 0.8%, D-Tyr 0.5%, D-Trp <1.0%, D-Nle <0.5%; TLC solvent systems 4, 5, 6. Anal. Calcd for $C_{61}H_{79}N_9O_{12} \cdot H_2O$ (1148.5): C, 63.80; H, 7.11; N, 10.98. Found: C, 63.74; H, 7.09; N, 10.97.

H-Ala-Tyr(tBu)-Ala-Trp-Nle-Asp(OtBu)-Phe-NH$_2$ (**5**). Heptapeptide derivative **4** (2.4 g; 2.09 mmol) was hydrogenated in the usual manner at pH 5 in DMF (300 mL); the pH was kept constant by titrimetric addition of 0.1 M HCl. The catalyst was removed by filtration, and the filtrate was concentrated to half volume. Upon addition of Na_2CO_3 (0.18 g) in H_2O (10 mL) the product was precipitated with 5% $NaHCO_3$; it was collected by filtration, washed extensively with water and with ether, and then reprecipitated from DMF with CH_3CN: yield 1.7 g (80%); TLC solvent systems 6, 7. Anal. Calcd for $C_{53}H_{73}N_9O_{10} \cdot H_2O$ (1014.3): C, 62.76; H, 7.45; N, 12.43. Found: C, 62.51; H, 7.31; N, 12.36.

Boc-Leu-[Glu(OtBu)]$_5$-Ala-Tyr(tBu)-Ala-Trp-Nle-Asp(OtBu)-Phe-NH$_2$ (**6**). To a solution of Boc-Leu-[Glu(OtBu)]$_5$-OH (Moroder et al., 1978) (2.06 g; 1.78 mmol) and **5** (1.18 g; 1.16 mmol) in DMF (80 mL) and NMP (40 mL) was added *N*-hydroxysuccinimide (0.27 g; 2.36 mmol), followed by DCC (0.39 g; 1.87 mmol) at −15 °C. The reaction mixture was allowed to reach room temperature in 4 h and was then stirred for additional 48 h. On addition of MeOH the precipitate was collected and washed with warm MeOH and ether: yield 2.2 g (86%); mp 250–251 °C dec; $[\alpha]^{20}_D = +9.5°$ and $[\alpha]^{20}_{546} = +11.5°$ (*c* 1, TFE/$CHCl_3$ 1:1); amino acid analysis Asp 1.03 (1), Glu 4.87 (5), Ala 2.00 (2), Leu 1.04 (1), Tyr + Nle 2.06 (2), Phe 1.04 (1), Trp 0.89 (1); gas chromatographic racemization test D-Ala <1%, D-Leu <0.5%, D-Asp 2.3%, D-Phe 1.1%, D-Glu 1.2%, D-Tyr 0.3%, D-Trp <1%, D-Nle <0.5%; TLC solvent systems 6, 7, 8. Anal. Calcd for $C_{109}H_{167}N_{15}O_{28} \cdot CH_3OH$ (2167.7): C, 60.95; H, 7.95; N, 9.69. Found: C, 60.71; H, 7.97; N, 9.64.

H-Leu-[Glu]$_5$-Ala-Tyr-Ala-Trp-Nle-Asp-Phe-NH$_2$ (**7**). The fully protected tridecapeptide **6** (1.0 g; 0.46 mmol) was dissolved in ice-cold trifluoroacetic acid (25 mL) containing 10% anisole and 2.5% 1,2-ethanedithiol. The resulting solution was kept at room temperature for 90 min and then poured into precooled ether. The precipitate was collected by filtration, washed extensively with ether, and dried over KOH pellets. The crude product was dissolved in 1% ammonia (50 mL) and chromatographed by preparative HPLC on a μBondapak C18 (15–25 μm) column (4 × 40 cm) by isocratic elution with EtOH/0.1 M ammonium acetate (pH 6.8), 24:76 (v/v), at a flow rate of 20 mL/min; the elution pattern was monitored by absorbance measurements at 210 nm. The main peak was collected, diluted with water, and freeze-dried. The product was dissolved in 1% ammonia, and upon Millipore filtration (GY 22 μm) the filtrate was again lyophilized: yield 0.55 g (61%); homogeneous on HPLC [μBondapak C18 (30 × 0.4 cm), eluent, CH_3CN/1 M sodium phosphate buffer (pH 5.4); linear gradient from 18% to 36% CH_3CN in 30 min] and HPTLC (solvent systems 9, 10); amino acid analysis of the HCl hydrolysate Asp 1.00 (1), Glu 4.79 (5), Ala 1.90 (2), Leu 0.99 (1), Tyr + Nle 1.95 (2), Phe 1.03 (1), Trp 1.03 (1); peptide content 83% calcd for M_r = 1642.8; gas chromatographic racemization test D-Ala 0.7%, D-Leu 0.4%, D-Asp

2.1%, D-Phe 1.3%, D-Glu 1.1%, D-Tyr 0.5%, D-Trp <1%, D-Nle 0.6%.

Spectroscopic Methods. UV absorption measurements were performed by using a Perkin-Elmer Model Lambda 5 spectrophotometer.

CD measurements were performed by using a Jasco Model J-500A automatic recording spectropolarimeter, equipped with a Jasco DP-500 N data processor. The spectra were recorded at 25 °C by using a thermostatable cell assembly. The signal-to-noise ratio was improved by accumulating 8 and 64 scans, in the far-UV and near-UV absorption regions, respectively. The dichrograph was equipped with a sample alternator, which allowed us to record and subtract the base line immediately after each scan (Peggion et al., 1985). Addition or subtraction of the spectra was performed directly by the data processor. All spectra reported in this paper are original, computer-drawn CD spectra, reported in terms of ellipticity units per mole of peptide residue ($[\vartheta]_R$) and per mole of hormone ($[\vartheta]_M$) in the peptide absorption region and in the aromatic absorption region, respectively. As previously reported (Peggion et al., 1985), the poor solubility of the gastrin peptides in 98% TFE required special care in the preparation of the solutions. A weighed amount of the Ala[10] analogue of minigastrin was treated for several hours with a minimum volume of a 1:1 (v/v) TFE–H_2O mixture. The solution was then diluted with TFE to a final TFE content of 98%. To avoid intermolecular aggregation, the peptide concentration never exceeded 5×10^{-5} M in 98% TFE. The concentration was determined by weight and peptide content and by absorption measurements in the near UV (Peggion et al., 1985). The agreement between concentration values determined by the two methods was always within 3%.

The NMR spectra were recorded on a Bruker AM 400 spectrometer and processed on a Bruker X-32 computer. Peak positions were measured relative to TMS as internal standard. The NMR samples were prepared at 4.5 mM peptide concentration in 90% TFE (Merck Uvasol) and 10% D_2O containing 15 mM NH_4OH and at 4.9 mM peptide concentration in 90% TFE-d_3 (Stohler Isotopes, Inc.) and 10% H_2O. The CD spectra of the NMR samples were measured and found to be identical with those recorded in 98% TFE at sample concentration $<5 \times 10^{-5}$ M, indicating that aggregation did not occur. The presence of 10% water in a solution of minigastrin in TFE increases its solubility without appreciable effects on the peptide conformation (Mammi et al., 1986, 1988).

The 2D experiments were performed at 30 °C on the 90% TFE-d_3/10% H_2O solution. The water resonance was eliminated by a DANTE sequence. The pure absorption mode spectra were obtained by using the TPPI method (Marion & Wüthrich, 1983). Prior to transformation, Gaussian functions were applied in the t_2 dimension and shifted squared sine bell functions were applied in the t_1 dimension. Zero-filling was carried out in the t_1 dimension only, to give final real matrices of 1K by 1K data points. Base-plane correction was performed after transformation.

The 2D homonuclear Hartmann–Hahn (TOCSY) spectrum was obtained by using the sequence described by Bax and Davis (1985b). The MLEV-17 spin-locking sequence was cycled 40 times, and the "trim" pulses were 3 ms each, for a total mixing time of 72 ms. The spin-locking power was $\gamma B_2/2\pi \approx 10.0$ kHz. The spectral width was 4505 Hz in both dimensions, and 512 FIDs of 128 scans each were collected.

The ROESY spectrum (Bax & Davis, 1985a) was obtained by using a 120-ms continuous spin-locking field of $\gamma B_2/2\pi \approx$ 8.47 kHz; 512 FIDs of 272 scans each were collected.

The temperature study was carried out on the 90% TFE/10% D_2O solution between 22 and 54 °C. The quartet arising from the TFE methylene protons was eliminated by selectively presaturating each resonance line for 50 ms in a cycle that lasted a total of 4.2 s.

Biological Assays. Stimulation of gastric acid secretion was determined in anaesthetized male rats according to the experimental procedure of Pham-Thanh-Chi et al. (1978). Parietal and nonparietal cells were isolated from rabbit gastric fundus as previously described (Magous et al., 1987). Both cell preparations are known to bind gastrins and CCK-octapeptide with comparable affinities. Correspondingly, the binding affinities of the gastrin peptides for both cell preparations were determined by using radiolabeled CCK-octapeptide (Amersham, U.K.) as tracer ligand.

Results and Discussion

Synthesis. The minigastrin analogue was synthesized by classical solution methods using the fragment condensation strategy. The synthetic route relies on our previous work in the field (Moroder et al., 1978) to allow for maximal utilization of common suitably protected fragments, i.e., of the C-terminal tetrapeptide amide derivative H-Trp-Nle-Asp(OtBu)-Phe-NH_2 (Moroder et al., 1981) and of the N-terminal hexapeptide derivative Boc-Leu-[Glu(OtBu)]$_5$-OH (Moroder et al., 1978). The central tripeptide derivative Z-Ala-Tyr(tBu)-Ala-OH was obtained in good overall yield by stepwise synthesis via the *N*-hydroxysuccinimide esters. Subsequent fragment condensation in sequence order by the DCC/*N*-hydroxysuccinimide procedure led to the fully protected human minigastrin analogue, which was then deprotected by exposure to trifluoroacetic acid in the presence of scavengers at ratios optimally suited for this synthetic step in the case of gastrin-related peptides (Moroder et al., 1983; Moroder & Wünsch, 1984). Preparative HPLC served for the final purification step that produced the desired des-Trp[1],Ala[10],Nle[12]-minigastrin at a high degree of purity in satisfactory yields.

CD Results. The CD spectrum in the far-UV absorption region of the minigastrin analogue in 98% TFE is reported in Figure 1. The CD pattern of unmodified minigastrin is also reported for comparison. With respect to the normal minigastrin we observe an appreciable enhancement of the negative band at 207 nm to 14 000 molar ellipticity units and also of the positive band at 190 nm to 28 000 molar ellipticity units. The CD pattern of the modified analogue becomes almost identical with that of the 17 amino acid residue hormone little gastrin, in which the N-terminal α-helix should be longer than in the case of des-Trp[1],Nle[12]-minigastrin. In little gastrin in fact, the additional residues should adopt the α-helical conformation starting with Pro[3] (Peggion et al., 1981). Even if a precise estimation of the helix content according to the analysis of Greenfield and Fasman (1969) cannot be performed because of the difficulty of quantifying the contribution of the aromatic chromophores, our findings suggest that the helix content of the Ala[10] analogue is increased with respect to that of des-Trp[1],Nle[12]-minigastrin.

The CD spectra in the near UV of the two analogues are also slightly different (Figure 2). The intensity of the positive band envelope around 265 nm is reduced in the modified analogue, and the fine structure in the region of L_b Trp transitions at 282 and 290 nm is less evident. These results suggest that the environments of the aromatic chromophores in the two cases are not identical.

^1H NMR Results. The chemical shifts of the assigned resonances of the two analogues in 90% TFE are reported in

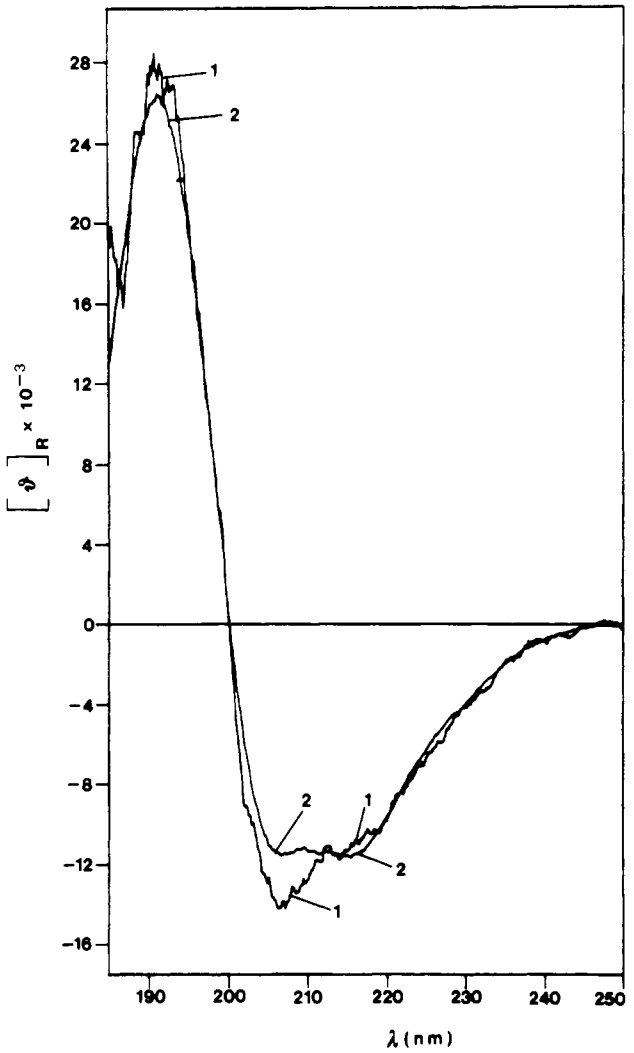

FIGURE 1: Far-UV CD spectra of des-Trp[1],Ala[10],Nle[12]-minigastrin (curve 1) and des-Trp[1],Nle[12]-minigastrin (curve 2) in 98% TFE. The hormone concentrations were 1.79×10^{-5} and 1.98×10^{-5} M, respectively, in a 0.1 cm optical path length cell.

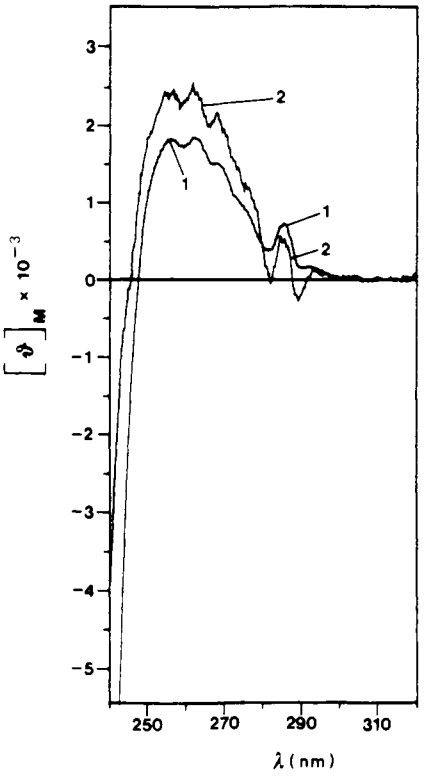

FIGURE 2: Near-UV CD spectra of des-Trp[1],Ala[10],Nle[12]-minigastrin (curve 1) and des-Trp[1],Nle[12]-minigastrin (curve 2) in 98% TFE. The hormone concentrations were 3.19×10^{-5} and 1.86×10^{-5} M, respectively, in a 5.0 cm optical path length cell.

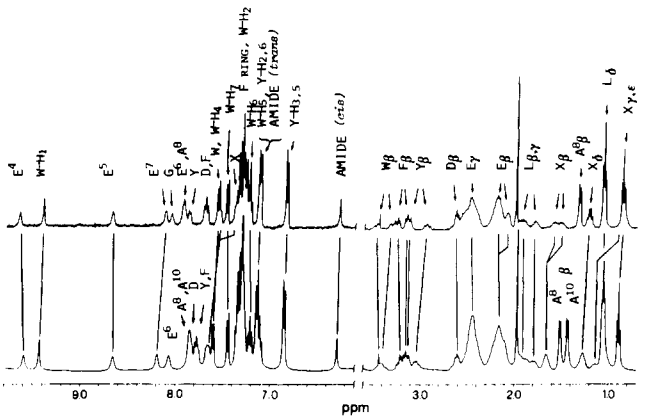

FIGURE 3: Assigned 400-MHz spectra of des-Trp[1],Nle[12]-minigastrin (top) and des-Trp[1],Ala[10],Nle[12]-minigastrin (bottom) in 90% TFE/10% D_2O. Residues are labeled by using the one-letter code, and X stands for Nle.

Figure 3. The resonance assignment was accomplished with a 2-dimensional homonuclear Hartmann–Hahn experiment and by using the assignment of the Gly[10] analogue as a reference. The main features of the TOCSY spectrum are reported in Figure 4. The resonance positions of the amide protons and the corresponding temperature coefficients are compared in Table I. In the assignment of the spectrum of des-Trp[1],Nle[12]-minigastrin (Mammi et al., 1988), ambiguities remained for the amide resonances of the Glu residues. The resonances at 8.68 and 7.93 ppm were tentatively assigned to Glu[6] and Glu[7] amide protons, respectively, while Glu[4] and Glu[5] could not be distinguished. Subsequent work on selectively α-deuterated analogues dissipated this doubt (unpublished results), and the correct assignment of the Glu amide resonances for the two analogues is given in Table I and Figure 3.[2] The ROESY experiment confirmed the assignment and allowed us to distinguish the two Ala methyl groups.

The resonance position of the amide protons of des-Trp[1],Ala[10],Nle[12]-minigastrin are all very different from those of the Gly[10] analogue with the exception of Trp NH, the C-terminal *cis*-amide, and all but one of the Glu NH's. The Nle NH is the most different one, with a downfield shift of 0.29 ppm. This residue shows also considerable differences in the chemical shift of its aliphatic protons, notably for the $C^{\gamma}H_2$ protons and for the appearance of the $C^{\beta}H_2$ protons, which are separated in normal minigastrin and overlap in the Ala[10] analogue. One of the Tyr $C^{\beta}H_2$ protons is downfield shifted by 0.11 pm, and both Ala methyl sets resonate further downfield than the Ala methyl group of the Gly[10] analogue by 0.11 and 0.19 ppm. All the other aliphatic and aromatic protons resonate within ±0.08 and ±0.05 ppm, respectively, in the two analogues. These findings indicate that the structural differences between Ala[10]-minigastrin and Gly[10]-minigastrin, visible in the CD spectrum, involve mostly the C-terminal portion of the molecule. This is confirmed by the values of the temperature coefficients of the amide protons (Table I). The temperature coefficient of Ala[10] NH is lower

[2] Throughout this paper, the nomenclature is according to that of Walsh et al. (1974), and the numbering is different from our previous paper (Mammi et al., 1988).

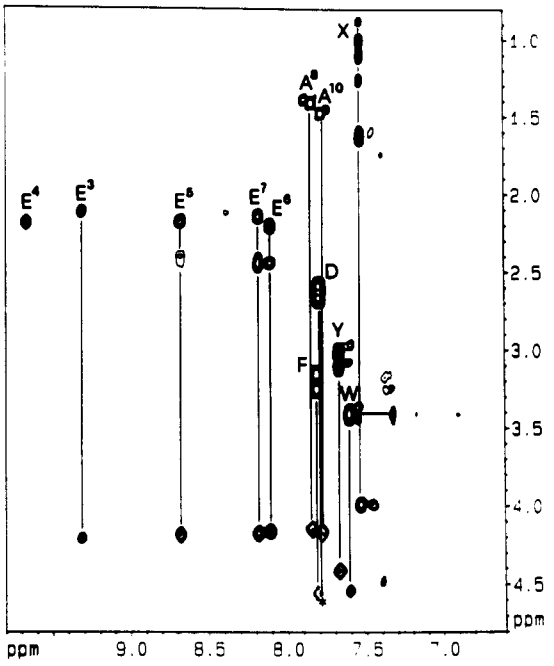

FIGURE 4: Aliphatic (F 1 axis)-NH and aromatic (F 2 axis) region of the two-dimensional homonuclear Hartmann–Hahn spectrum of des-Trp[1],Ala[10],Nle[12]-minigastrin in 90% TFE-d_3/10% H_2O. The experimental conditions are described in the text. Only positive peaks are plotted. Residues are labeled by using the one-letter code, and X stands for Nle.

Table I: Position at T = 296 K (ppm) and Temperature Coefficients (Δppb/K) of Amide Resonances in 90% TFE[a,b]

	des-Trp[1],Nle[12]-minigastrin		des-Trp[1],Ala[10],Nle[12]-minigastrin	
Glu[4]	9.660	−8.9 ± 1.4	9.660	−6.7 ± 0.2
Glu[5]	8.681	+0.9 ± 1.3	8.659	+0.8 ± 0.4
Glu[6]	7.934	−0.7 ± 0.8	8.071	−1.6 ± 0.4
Glu[7]	8.126	−4.5 ± 0.9	8.196	−3.7 ± 0.2
Ala[8]	7.934	−4.8 ± 0.9	7.858	−4.3 ± 0.6
Tyr	7.879	−7.1 ± 0.7	7.767	−6.3 ± 0.6
Gly	8.063	−5.3 ± 0.7		
Ala[10]			7.858	−4.3 ± 0.6
Trp	7.651	−2.3 ± 0.6	7.668	−4.2 ± 0.2
Nle	7.373	−1.6 ± 0.8	7.659	−5.2 ± 0.7
Asp	7.710	−1.8 ± 0.9	7.844	−4.0 ± 0.6
Phe	7.689	−3.0 ± 0.7	7.787	−3.1 ± 0.4
amide (cis)	6.269	−4.4 ± 1.4	6.286	−4.3 ± 0.2

[a] 95% confidence intervals are reported for the temperature coefficients. [b] The chemical shifts of some amide protons are slightly different in the 90% TFE solution with respect to the shifts in the 90% TFE-d_3 solution used for the two-dimensional experiments. These differences are very likely due to a small difference in the pH of the two solutions. The apparent pH of the 90% TFE-d_3 solution was 6.7 and allowed the identification of the Glu[3] NH, very broad in 90% TFE solution. However, the comparison with previous results is made on the basis of the chemical shift values in the 90% TFE solution because these conditions are more closely related to our previous work.

than that of Gly[10] NH, and those of Ala[8] NH and Tyr NH are smaller than in the Gly[10] analogue, while those of Nle NH and Asp NH are substantially higher.

With the exception of Nle NH and Tyr NH, all the temperature coefficients of the amide protons range from −1.6 to −4.3 ppb/K and are of the same order of magnitude as those reported by Ribeiro et al. (1985) for oligoglutamates in TFE with an estimated α-helical content of about 30%. These results and the close resemblance of the CD pattern with that of little gastrin suggest that in the Ala[10] analogue there is an extension of the N-terminal helical segment toward the C-terminus. In this connection, we should point out that the

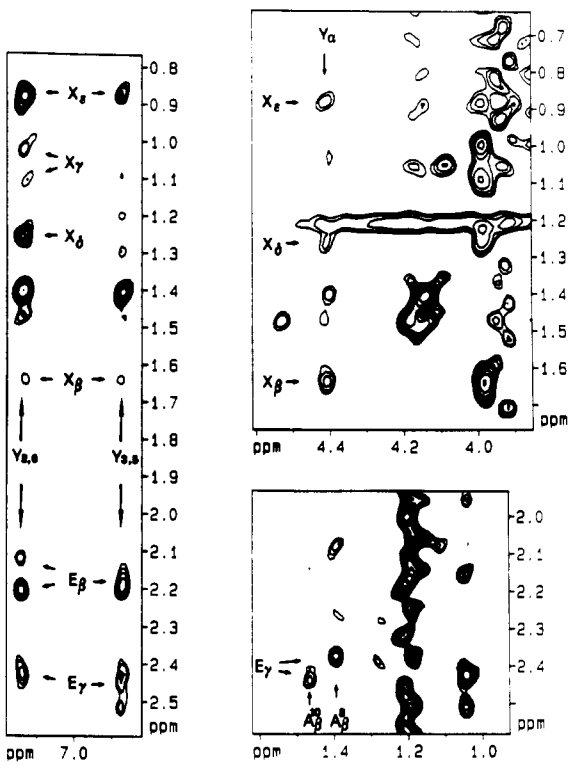

FIGURE 5: Portions of the two-dimensional ROESY spectrum of des-Trp[1],Ala[10],Nle[12]-minigastrin in 90% TFE-d_3/10% H_2O. The experimental conditions and the peaks indicated in the figure are described in the text. Only negative peaks are plotted. Residues are labeled by using the one-letter code, and X stands for Nle.

removal of the central bend and of the stabilizing interactions between the two chain ends makes the structure more flexible than in normal minigastrin. In the new situation, the conformer population fluctuates with a certain preference for the α-helical structure. Under these circumstances, we should not expect a highly tight hydrogen-bonded structure, which is reflected in the moderately low temperature coefficients of the amide protons.

The conclusion that the N-terminal α-helix is extended toward the C-terminus is supported by several cross-peaks in the ROESY experiment (Figure 5). The Tyr $C^{\alpha}H$ shows a cross-peak to Nle C^{β} protons, and such interaction is diagnostic for α-helical structure (Wüthrich, 1986). Cross-peaks are also found between the Tyr ring protons and Nle side chain, notably between Tyr $C^{2,6}H$ and Nle $C^{\epsilon}H_3$ and $C^{\delta}H_2$. The Tyr ring protons are also close in space to some Glu $C^{\beta}H_2$ and $C^{\gamma}H_2$, though specific assignment is not possible at present. Both Ala methyl groups display connectivities to Glu $C^{\gamma}H_2$. In the case of Ala[10], this is an interaction of the type $i - (i + 3)$ or even further removed. These results demonstrate that both Tyr and Ala[10] are part of the α-helical region, which therefore extends beyond Glu[7] at least through Nle.

Biological Studies. Des-Trp[1],Ala[10],Nle[12]-minigastrin was found to exhibit agonistic activity on gastric acid secretion but was devoid of antagonistic properties against gastrin-stimulated acid secretion. However, the potency of this gastrin analogue was remarkably lower than that of des-Trp[1],Nle[12]-minigastrin, i.e., only about 10% (Figure 6). This residual activity corresponds, within the limit of error of the assay system, to that detected for C-terminal gastrin fragments, e.g., acetyltetrapeptide amide (Previero et al., 1982). Similarly, replacement of Gly[10] by Ala produced a significant reduction of binding affinity (Figure 7). In fact, the affinity for parietal and nonparietal cell receptors is reduced by a factor of 10 and 5, respectively (Table II).

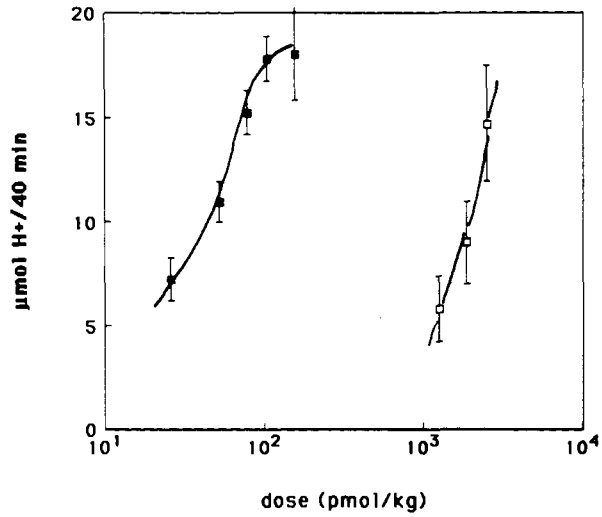

FIGURE 6: Dose–response curve to des-Trp[1],Nle[12]-minigastrin (■) and to des-Trp[1],Ala[10],Nle[12]-minigastrin (□) on in vivo gastric acid secretion in rats. Each point represents the average of 2–17 separate experiments. Results are expressed as micromoles of H^+ secreted in 40-min experiments.

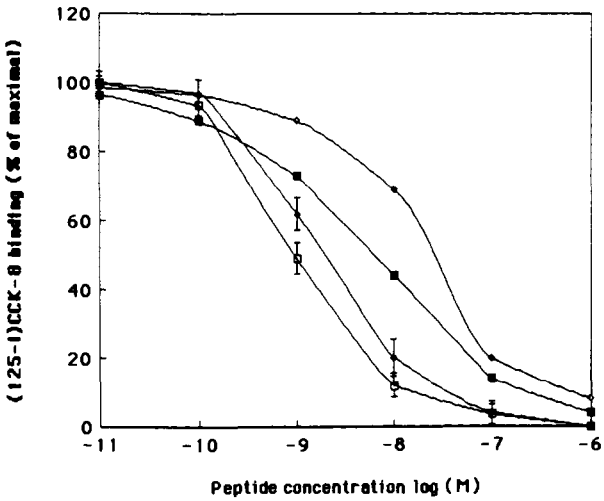

FIGURE 7: Displacement curves of ^{125}I-labeled CCK-8 by des-Trp[1],Ala[10],Nle[12]-minigastrin and CCK-8 on parietal (F 3) and nonparietal (F 1) cells from rabbit gastric funds. Results are expressed as percent of radioactivity specifically bound to cells in the presence of various gastrin analogue concentrations (average ± SD of 3–10 separate experiments run in duplicate). (□) ^{125}I-labeled CCK-8/CCK-8/F 1; (◆) ^{125}I-labeled CCK-8/CCK-8/F 3; (■) ^{125}I-labeled CCK-8/des-Trp[1],Nle[12],Ala[10]-minigastrin/F 1; (◇) ^{125}I-labeled CCK-8/des-Trp[1],Nle[12],A[10]-minigastrin/F 3.

CONCLUSIONS

Substitution of Gly in position 10 of the minigastrin sequence with the helix-forming residue Ala remarkably affects the conformation of the hormone and its biological potency. CD and NMR data provided evidence that the structural modification is mainly produced in the C-terminal portion of the peptide chain, probably with an extension of the N-terminal α-helix throughout the entire sequence. This conformational change has a significant impact on the hormonal potency and supports the hypothesis of a U-shaped structure as the optimal bioactive state of the hormone. When the preference for this conformation is reduced, only the intrinsic potency of the C-terminal portion of the molecule is present. On the other hand, in our previous work, we have shown that replacement of the $-(Glu)_5-$ sequence with the $-(Asp)_5-$ sequence prevents the formation of the α-helix at the N-terminus and reduces substantially the biological potency of the hormone (Wünsch et al., 1986). Our findings suggest that refolding of the molecule in a hairpin mode stabilizes the bioactive structure of the C-terminus with a concomitant 10-fold increase of the potency of the gastrin tetrapeptide to the full biological activity of the circulating forms of gastrins.

Table II: IC-50 Values As Determined from Displacement Curves of Binding of Radiolabeled CCK-Octapeptide on Parietal (F 3) and Nonparietal (F 1) Cells from Rabbit Gastric Fundus by Minigastrin Related Peptides[a]

ligand	F 1	F 3
CCK-8	1.0×10^{-9} M	2.0×10^{-9} M
des-Trp[1],Ala[10],Nle[12]-minigastrin	5.0×10^{-9} M	2.2×10^{-8} M

[a] The reported values are the average of 3–10 separate experiments, run in duplicate.

Registry No. **1**, 121886-97-3; **2**, 121886-98-4; **3**, 121886-99-5; **3**·DCHA, 121918-55-6; **4**, 121887-00-1; **5**, 121887-02-3; **6**, 121887-03-4; des-Trp[1],Nle[12]-minigastrin, 75679-13-9; Z-Tyr-(tBu)-OSu, 10068-67-4; H-Ala-OH, 56-41-7; Z-Ala-OSu, 3401-36-3; H-Trp-Nle-Asp(OtBu)-NH_2·HCl, 121887-01-2; BOC-Leu-[Glu-(OtBu)]$_5$-OH, 65734-32-9.

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Extrinsic 33-Kilodalton Protein of Spinach Oxygen-Evolving Complexes: Kinetic Studies of Folding and Disulfide Reduction†

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ABSTRACT: The 33-kDa protein is one of the three extrinsic proteins in the oxygen-evolving photosystem II complexes. The protein has one intrachain disulfide bond. On reduction of this disulfide bond, the protein was unfolded and lost its activity. On the basis of the unfolding equilibrium curve obtained by using guanidine hydrochloride, the free energy change of unfolding in the absence of guanidine hydrochloride was estimated to be 4.4 kcal/mol using the Tanford method [Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95] and 2.8 kcal/mol using the linear extrapolation method. The unfolding of the 33-kDa protein caused by reduction was explained in terms of the entropy change associated with reduction of the intrachain disulfide bond. The kinetics of the reduction of the disulfide bond using dithiothreitol were studied at various concentrations of guanidine hydrochloride at pH 7.5 and 25 °C. The disulfide bond was reduced even in the absence of guanidine hydrochloride. The unfolding and refolding kinetics of the 33-kDa protein using guanidine hydrochloride were also studied under the same conditions, and the results were compared with those for the reduction kinetics. It was shown that the reduction of the disulfide bond proceeds through a species in which the disulfide bond is exposed by local fluctuations.

The 33-kDa protein is an extrinsic component of the oxygen-evolving photosystem II (PS II)[1] complexes located on the thylakoid membrane of chloroplasts (Inoue et al., 1983, 1984; Miyao & Murata, 1984; Abramowicz & Dismukes, 1984). The 33-kDa protein consists of 247 amino acid residues and has only 1 intrachain disulfide bond between Cys-28 and Cys-51 and 1 Trp at position 241 (Oh-oka et al., 1986). Recently, Tanaka and Wada (1988) reported that the intrachain disulfide bond is essential for maintaining the functional conformation of the molecule. In this study, we investigated the role of the disulfide bond in the stability of the 33-kDa protein. We also examined the kinetics of the reduction of the disulfide bond with DTT at various concentrations of Gdn-HCl, and the results were compared with those of the unfolding and refolding kinetics obtained with Gdn-HCl. We found that the disulfide bond is located in the interior of the molecule and that the reduction proceeds through a species in which the disulfide bond is exposed by local fluctuations.

Materials and Methods

Materials. The 33-kDa protein was extracted from NaCl-treated PS II complex by 1.0 M $CaCl_2$ treatment and purified by column chromatography on DEAE-Sepharose CL-6B as described by Kuwabara and Murata (1982). The purified protein was dialyzed against 10 mM NH_4HCO_3 and lyophilized. All the procedures were done at 0–7 °C. The reduced 33-kDa protein was prepared by reduction of the intrachain disulfide bond with a 1000 molar excess of 2-mercaptoethanol in the presence of 6 M Gdn-HCl for 2 h at 25 °C and then separated from the residual reagents on a column of Sephadex G-25 equilibrated with 10 mM acetate buffer at pH 4.0 containing 30 mM NaCl and 5 μM EDTA.

DTT and Gdn-HCl (specially purified grade) were obtained from Nakarai Chemicals, and DEAE-Sepharose CL-6B was obtained from Pharmacia Fine Chemicals. Other reagents were obtained from Wako Pure Chemicals and Nakarai Chemicals and were used without further purification.

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[1] Abbreviations: CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Gdn-HCl, guanidine hydrochloride; reduced 33-kDa protein, 33-kDa protein in which the intrachain disulfide bond is reduced; PS, photosystem.

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