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Biological and physical properties of a model calcitonin containing a glutamate residue interrupting the hydrophobic face of the idealized amphiphilic α-helical region

(hormone model/hypocalcemic activity/peptide design)

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ABSTRACT **A new calcitonin analogue, model calcitonin III (MCt-III), has been synthesized, and its biological and physical characteristics have been studied. This analogue has an idealized α-helix from residue 8–22 with glutamate at position 15 interrupting an otherwise continuous surface of aliphatic side chains (those of leucine residues) on the hydrophobic face of the helix. MCt-III differs from a previous model, MCt-II, only by the substitution Leu^{15}→Glu and is here compared with salmon calcitonin I (sCt-I) and MCt-II to elucidate further the role of the putative amphiphilic α-helix in determining biological and physical properties of the hormone. MCt-III shows physical properties intermediate between those of sCt-I and MCt-II, demonstrating the influence of appropriately positioned single residues on properties of amphiphilic structures. In our two biological assays, a brain-binding assay and an *in vivo* hypocalcemic assay, MCt-III reproduces the sigmoidal dose–response curves of sCt-I; this contrasts with the behavior of MCt-II, which demonstrated unusual dose–response curves in these two assays. MCt-III is almost three times more potent than sCt-I in our hypocalcemic assay; this activity groups MCt-III among the most potent known analogues of sCt-I.**

The importance of amphiphilic secondary structural units has been demonstrated for many peptide hormones in recent years (1, 2). In previous reports we described our understanding of the structural features of calcitonin important to its modulation of calcium ion homeostasis—in particular, the presence in the hormone of an amphiphilic α-helix (3, 4).

Calcitonin is a 32-residue calcium-regulating peptide hormone containing a seven-residue cyclic loop at the N terminus, formed by a disulfide bond between cysteines at positions 1 and 7 and also containing a carboxyl-terminal amide. This peptide hormone is produced by the parafollicular cells, which are located in the thyroid in mammals but which are associated with a distinct organ, the ultimobranchial body, in lower animals. Calcitonins from many species have been sequenced and compared in potency; those from salmon (salmon calcitonin I, or sCt-I) and from eel (eel calcitonin) are the most potent (5). In discussing the structure and function of calcitonin we focus on the sequence of sCt-I, except where indicated.

From examination of the relationships between primary sequences versus potencies of native hormones, as well as from observations of previous workers on the effects of modifying these natural hormones, we propose a model for the structural regions in calcitonin. Consistent with our design of two previous analogues, models calcitonin I and II (MCt-I and MCt-II), we identified three important regions in the hormone: an N-terminal seven-residue disulfide-bridged loop, an amphiphilic α-helical region comprising residues 8 through 22, and an extended linker region of the remaining 10 residues and leading to the C-terminal proline amide (which appears to be a crucial group for biological activity).

In both models MCt-I and MCt-II we idealized the hydrophilic face of the amphiphilic helical region and minimized its sequence homology to the corresponding part of sCt-I. For MCt-I, the less-active model (hypocalcemic potency approximately that of porcine calcitonin), the hydrophobic face of the helix was altered appreciably from that of sCt-I. However, in MCt-II, a model with biological activity similar to sCt-I, the only major change from sCt-I sequence on the hydrophobic face was a leucine residue at position 15 of the model peptide rather than the glutamic acid residue of the natural hormone. In other words, in MCt-II (Fig. 1) the amphiphilic helix has a continuous hydrophobic face, whereas in sCt-I this face is interrupted by a glutamic acid residue. In many naturally occurring surface-active peptides and proteins, the amphiphilic helical regions contain one or more residues that are "out of phase"; for instance, in apolipoprotein A-I, hydrophilic arginine residues seem to interrupt the otherwise hydrophobic faces of amphiphilic helical regions (6). To probe systematically the roles of the various proposed structural regions of calcitonin we have introduced a glutamic acid residue at position 15 in another model, MCt-III (Fig. 1), where this residue interrupts the hydrophobic face of the helical region. In model MCt-III the idealized hydrophilic face in the helical regions of MCt-I and MCt-II is maintained. Only the Glu-15 residue in MCt-III differentiates its sequence from that of MCt-II. We report the properties of MCt-III and compare them to those of sCt-I and MCt-II.

EXPERIMENTAL METHODS

Peptide Synthesis and Purification. MCt-III was prepared by standard Merrifield solid-phase techniques with a benzhydrylamine-substituted polystyrene resin cross-linked with 1% (vol/vol) divinylbenzene (7). The peptide was synthesized in a Beckman (model 990) peptide synthesizer, using symmetric anhydrides of Boc-Arg(N^G-Tos), Boc-Cys(*S*-4-MeOBzl), Boc-Glu(O^γ-Bzl), Boc-Gly, Boc-Leu, Boc-Lys(N^ϵ-2-ClZ), Boc-Pro, Boc-Ser(*O*-Bzl), Boc-Thr(*O*-Bzl), and Boc-Tyr(*O*-2,6-Cl_2Bzl) (protected amino acids from Peninsula Laboratories, San Carlos, CA; *N*-*tert*-butyloxycarbonyl, Boc; benzyl, Bzl; tosyl, Tos; Z, benzyloxycarbonyl). For Boc-Asn and Boc-Gln the HOBt/DCC (*N*-hydroxybenzotriazole/*N*,*N'*-dicyclohexylcarbodiimide) method was used (8). The coupling and deprotection program was similar

Abbreviations: Boc, *N*-*tert*-butyloxycarbonyl; Bzl, benzyl; MCt-I, -II, and III, model calcitonin I, II, and III; sCt-I salmon calcitonin I; Tos, tosyl; Z, benzyloxycarbonyl.

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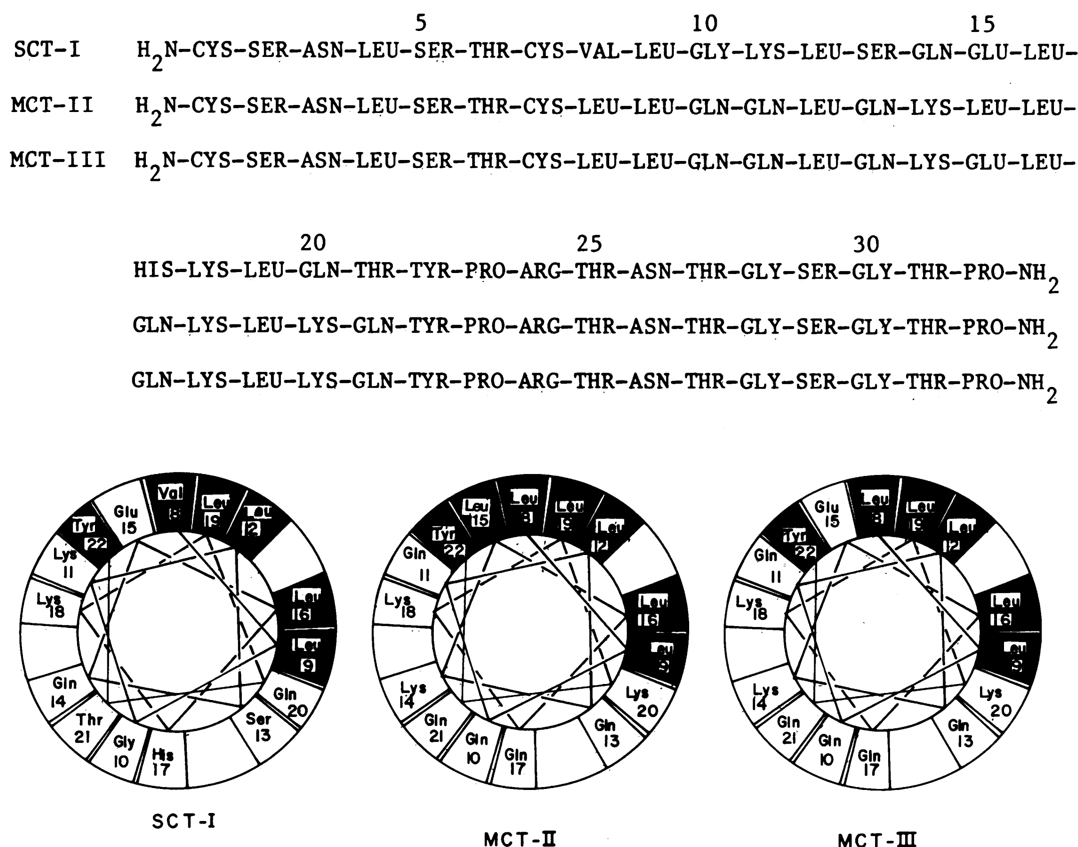

FIG. 1. (*A*) Amino acid sequences of sCt-I, MCt-II, and MCt-III. (*B*) Axial projection of the α-helical region (residues 8–22) of sCt-I, MCt-II, and MCt-III.

to that employed by Yamashiro and Li (9), except that Boc-Gln was deprotected using 6 M HCl in dioxane for 30 min (10) instead of the usual 50% trifluoroacetic acid in methylene chloride for 20 min. Cleavage from the resin and removal of protecting groups from the peptide followed by washing the peptide off the resin and cyclization of the disulfide-bridged loop were done as described (4). Purification of MCt-III was accomplished by ion-exchange chromatography on carboxymethyl-Sephadex C-25 followed by reverse-phase HPLC on a Vydac (Hesperia, CA) C_4 preparative column using an eluting gradient from 30% to 40% aqueous acetonitrile containing 0.1% trifluoroacetic acid. Purity was established by HPLC. Amino acid analysis (hydrolysis in 6 M HCl for 24 hr) yielded the following numbers of residues found versus expected: Asx, 2.12 (2); Arg, 0.96 (1); Cys, — (2); Glx, 6.03 (6); Gly, 2.00 (2); Leu, 5.50 (6); Lys, 2.89 (3); Pro, 1.90 (2); Ser, 3.08 (3); Thr, 3.93 (4); Tyr, 0.96 (1); NH_4^+, 7.42 (8). Mass spectral analysis using the ^{252}Cf fission-fragment ionization method (11) gave an $(M+H)^+$ peak at 3576.88 (calculated value, 3577.06; $\Delta = -0.18$).

Circular Dichroism. Circular dichroism spectra of MCt-III were taken on an Aviv (Philadelphia) modification of a Cary model 60ds spectropolarimeter. All spectra were recorded in filtered 0.16 M KCl/0.02 M phosphate buffer, pH 7.4, or alternatively this same buffer system plus 50% trifluoroethanol (a structure-inducing solvent) (12). High-concentration scans were done in a 0.5-mm cell, whereas the lower-concentration scans were done in a 2-cm cell. The spectrometer cells were washed with concentrated nitric acid, rinsed with deionized water, soaked in 2% aqueous poly(ethylene glycol) (M_r 15–20 kDa) solution for 1 hr and finally rinsed with water and dried before using. Concentrations of the stock solutions were determined by amino acid analysis.

Film Balance Experiments. Film balance experiments were done on a Lauda film balance with an Apple IIE controller interface. The subphase used was 0.1 M KCl/0.01 M Tris, pH 7.4, in glass-distilled water. Surface-active contaminants were removed by bubbling air through the solution for 10 min and then aspirating the surface of the buffer. The force versus area isotherms obtained were analyzed between 5 and 9.5 dynes/cm (1 dyne = 1×10^{-5} newtons) using the equation $\pi[A - A_\infty(1 - \kappa\pi)] = nRT$, where π is the force (in dynes/cm), A is the area in cm^2, A_∞ is the limiting molecular area extrapolated to 0 surface pressure, and κ is a constant reflecting the monolayer compressibility (4, 13). Concentrations of the stock solutions were determined by amino acid analysis.

Molecular Weight Determination by Ultracentrifugation. Peptide or protein was dissolved in a buffer of 0.16 M KCl/0.02 M sodium phosphate, pH 7.4, with Dextran T-40 (7 mg/ml; Pharmacia) added. Concentrations of peptides and of egg-white lysozyme from chicken (peptidoglycan *N*-acetylmuramoylhydrolase; EC 3.2.1.17) were as follows: lysozyme, 1×10^{-5} M, sCt-I, 1 mM, and MCt-III, 3 mM. For each experiment, 100 μl of the respective solution was spun in a Beckman Spinco Airfuge at 90,000 rpm for 24 hr. Successive 10-μl aliquots were removed, diluted to 2 ml in 0.04 M borate buffer, pH 8.0, and assayed for peptide concentration using the fluorescamine method (14). Analysis of the concentration gradient generated gave the apparent molecular weight (14, 15).

Brain-Binding Assays. MCt-III was compared with sCt-I in a competitive brain-binding assay as described by Nakamuta *et al.* (16). sCt-I was iodinated with ^{125}I (New England Nuclear) using the chloramine-T method (17). The iodinated peptide was purified by ion-exchange chromatography on SP-Sephadex C-25 and stored until needed in 100-μl aliquots

at −20°C. Crude brain-membrane homogenates were prepared using 250–300 g male Sprague–Dawley rats and frozen as pellets at −80°C for up to a month. The assay was done as follows. Brain membranes were thawed and suspended in ≈40 ml of 0.02 M Tris buffer/0.1% bovine serum albumin, pH 7.4 [protein concentration as determined by the bicinchoninic acid method (ref. 18; reagents from Pierce) was, on average, 2.4 mg/ml]. In 1.5-ml polypropylene Microfuge tubes 20 μl of ^{125}I-sCt-I (≈20,000 cpm for a final concentration of ≈0.05 nM) was combined with 0.9 ml of brain homogenate, 0–100 μl of cold peptide in assay buffer, and 0–100 μl of buffer to a total volume of 1.0 ml. The tubes were mixed in a Vortex mixer and then shaken at 4°C for 1 hr, after which tubes were spun in a Beckman Microfuge at 11,000 *g* for 4 min; the resulting pellets were counted.

***In Vivo* Hypocalcemic Potency Assay.** Male Sprague–Dawley rats at 3–4 weeks of age were injected with sCt-I or MCt-III in 0.9% NaCl with 0.1% bovine serum albumin s.c. to the dorsal neck; 1 hr after injection the rats were sacrificed, and blood was collected by cardiac puncture. Blood was centrifuged, and aliquots of serum were assayed by atomic absorption for Ca^{2+} concentration. Results are calculated as compared with saline controls and plotted as $-\Delta[Ca^{2+}]$ (meq/liter) versus logarithm[peptide dose].

RESULTS

Circular Dichroism. The near-UV CD spectra of MCt-III between 250 and 195 nm are shown in Fig. 2, which illustrates curves for the peptide in aqueous buffer at both high and low concentrations and for the peptide in 50% trifluoroethanol at high concentration. From the shape of the curves significant α-helical structure exists under both high-concentration conditions (as demonstrated by minima at 208 and 222 nm). The lower-concentration curve shows reduced helical content, as evidenced by both a lower CD absorbance and a shift of the mimima around 208 nm to lower wavelengths. The trifluoroethanol and aqueous curves at high concentration were analyzed by three different secondary structure-prediction algorithms: the methods of Chang *et al.* (19), Hennessey and Johnson (20), and Provencher and Glockner (21). All three methods gave similar results: we report here the predictions using the method of Provencher and Glockner, which has been found the most accurate method (22). Table 1 shows the percentages of secondary structure calculated from the CD

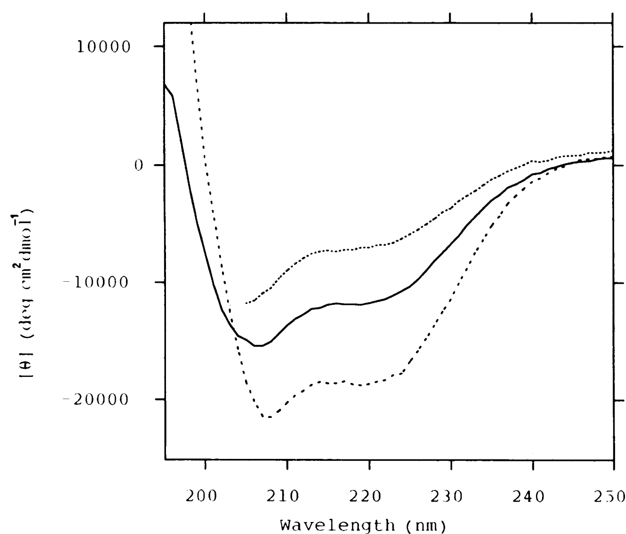

FIG. 2. CD spectra of MCt-III. (*A*) 9.8 × 10^{-5} M in 0% trifluoroethanol (—). (*B*) 9.8 × 10^{-5} M in 50% trifluoroethanol (---). (*C*) 2.4 × 10^{-6} M in 0% trifluoroethanol (···).

Table 1. Percentages of secondary structure calculated from CD spectra (21)

	α-helix	β-sheet	β-turn	Remainder
MCt-III aqueous	34	24	6	36
50% trifluoroethanol	59	13	8	20

data. There is a change from 34% to 59% α-helix going from aqueous buffer at MCt-III concentration of 9.8 × 10^{-5} M to 50% trichloroethanol, with decreases in the amounts of β-sheet structure and "remainder" structure. The amount of β-turn is predicted to be low but stable. The low-concentration curve was not analyzed by this method because the curve is cut off at 205 nm (due to increased noise at lower wavelengths in the longer-path-length cells), and the region between 195 and 205 nm is crucial for this analysis. sCt-I is reported to have 20% α-helix in aqueous buffer (3), whereas the amount of α-helix present in MCt-II is calculated to vary from 20% to 35% in aqueous buffer as it aggregates. Both estimates of α-helix structure of MCt-III, in trifluoroethanol and in aqueous buffer, are consistent with a helix in the region of the sequence between residues 8 and 22 (47% of the peptide sequence). At peptide concentrations used to obtain the CD spectra there is evidence for aggregation. A plot of θ_{222} versus concentration shows clear concentration dependence (data not shown). Analysis of these data to see whether they could be fit to a specific order of aggregation (23) was inconclusive. Within the accuracy of the CD measurements data for MCt-III fit equally well with a monomer–tetramer, or a monomer–trimer equilibrium, and we found it impossible to choose between the two alternate models with this experiment (however, see the ultracentrifuge experiment).

Film Balance. At the air–water interface, MCt-III forms an insoluble monolayer with a collapse pressure of 11.5 dynes/cm. This compares with 12 dynes/cm for sCt-I and 22 dynes/cm for MCt-II. Analysis of the force/area curve of MCt-III gave parameters of A_∞ = 530 Å^2 and κ = 0.02 cm/dyne. These values are closer to those of sCt-I (A_∞ = 560 Å^2 and κ = 0.03 cm/dyne) than those of MCt-II (A_∞ = 362 Å^2 and κ = 0.01 cm/dyne) (3, 4). Our results suggest that sCt-I and MCt-III have larger, more compressible structures at the air–water interface than does the more perfectly amphiphilic MCt-II. Because the only change between the peptides MCt-II and MCt-III is the presence of the glutamate residue at position 15 in the latter peptide, this observation demonstrates the sensitivity of amphiphilic structures to imperfections in the segregation of hydrophobic and hydrophilic residues.

Ultracentrifuge. The M_r values of MCt-III and sCt-I were determined by sedimentation-equilibrium ultracentrifugation and compared with that of lysozyme. Lysozyme was chosen as a control because its monomeric M_r is similar to that expected for aggregated species of the lower-M_r peptides. Table 2 shows the results of this experiment. The M_r for sCt-I is 0.86 times the expected value, consistent with sCt-I existing as a monomer at this concentration. The slightly low value indicates the inherent problems in using this method to analyze peptides in a M_r range below ≈6 kDa (14). At a concentration of 3 mM, MCt-III has an apparent M_r 3.7 times the actual M_r of monomeric peptide (or 3.9 times when all

Table 2. M_r determined by Airfuge

	Monomeric M_r	M_r*	M_r ratio†
Lysozyme	14,100	13,400	0.95
sCt-I	3,435	2,960	0.86
MCt-III	3,576	13,200	3.69

*By Airfuge (14, 16).

†Airfuge M_r/monomeric M_r.

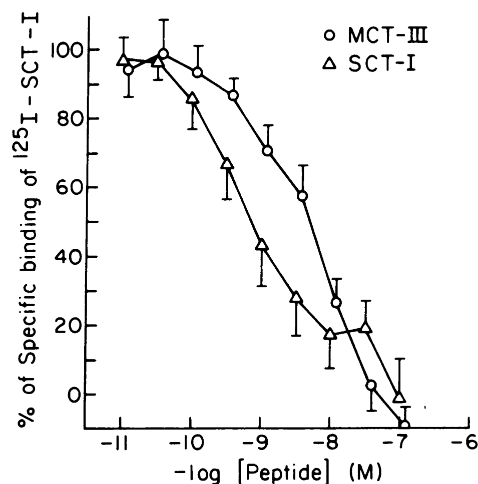

FIG. 3. Competitive inhibition of ^{125}I-sCt-I binding to brain particulate membranes by sCt-I (△) and MCt-III (○). Each point is the mean of four triplicate experiments. Error bars represent the SE.

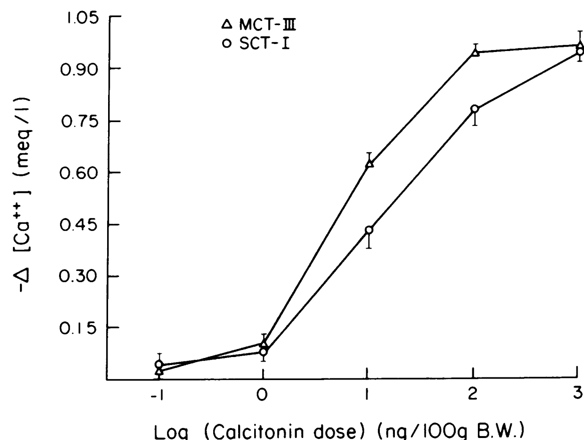

FIG. 4. Hypocalcemic potency of sCt-I and MCt-III. Each point represents the difference between the mean serum [Ca^{2+}] for rats given only saline and the mean for those given a particular dose of either sCt-I or MCt-III. Each point represents a total of 10 rats. Error bars indicate the SE.

values are corrected so that lysozyme Airfuge M_r/actual M_r = 1.0). The relatively high concentration of MCt-III used was required to observe complete aggregation. Below this concentration the apparent M_r decreases. These data are consistent with the concentration dependency of the CD data, which imply aggregation over the ranges studied (2×10^{-6} M to 3×10^{-3} M). Thus, at sufficiently high concentration, MCt-III appears to aggregate into tetramers. MCt-II was reported to aggregate to the trimeric form, whereas sCt-I does not aggregate in this concentration range (3, 4).

Brain Binding. A comparison of the relative abilities of MCt-III and sCt-I to displace ^{125}I-sCt-I from crude rat brain homogenate shows that MCt-III displaces ^{125}I-sCt-I specifically and completely, although with a lower potency than is seen using sCt-I (Fig. 3). The IC_{50} of sCt-I is 0.7 nM, whereas that of MCt-III is 5.0 nM. Therefore, MCt-III is some seven times less potent than sCt-I in this assay. In assays for MCt-II reported previously the tubes had to be pretreated with peptide because of nonspecific binding problems (4); no such problems were encountered with MCt-III.

***In Vivo* Hypocalcemic Potency Assay.** Results of this assay are shown in Fig. 4. Comparing the two peptides at points on the curves equivalent to 50% of the total biological effect (−Δ Ca^{2+} of 0.46 meq/liter) gives a relative potency of MCt-III that is 2.7 times the potency of sCt-I in this assay. MCt-II in this assay, as reported previously, gave a very flat, nonsigmoidal dose–response curve that is difficult to use in the potency comparison discussed above (4). In contrast, MCt-III reproduces the sigmoidal shape of the sCt-I dose–response curve.

DISCUSSION

The importance of an amphiphilic α-helix in the region 8–22 of calcitonin has been demonstrated in previous analogues we have described. The most potent of these previous analogues is MCt-II, which has no hydrophilic residues interrupting the hydrophobic face of the amphiphilic α-helix. This peptide is approximately as potent as sCt-I in the *in vivo* hypocalcemic assay, but the shape of its dose–response curve is nonsigmoidal, demonstrating that peptides with idealized amphiphilic α-helices can behave unusually. Potential problems include increased nonspecific binding and possible effects of secondary structure on adjacent regions of the peptide. For example, if our structural hypothesis is correct, the putative α-helix in calcitonin must terminate with residue 22 (such a termination may involve a reverse turn). Too strong an induction of helix in the region 8–22 may reduce the propensity for the helix to terminate at residue 22.

Such considerations have led to the synthesis and analysis of our third calcitonin model, MCt-III. The physical characteristics of this model are intermediate between those of sCt-I and MCt-II. Our film-balance experiments show similar behaviors for MCt-III and sCt-I. The solution properties of MCt-III are more like MCt-II, showing a concentration-dependent aggregation and a higher percentage of α-helical structure in solution than for sCt-I.

Biological results show MCt-III to reproduce the *in vivo* hypocalcemic effects of sCt-I and, indeed, to be more potent than the native hormone. In the brain-binding assay, however, MCt-III is less potent than sCt-I. This lower relative potency in the brain-binding assay as compared with the hypocalcemic assay was seen previously with model MCt-II and has several explanations. (*i*) mRNA coding for calcitonin has never been found in brain. This observation raises the question of whether calcitonin is actually present in the brain, despite the presence of high-affinity binding sites there (24). (*ii*) Receptors studied in the brain may be different from those responsible for hypocalcemic effects elsewhere in the body. They could, for instance, actually be receptors for another peptide (such as calcitonin gene-related peptide, CGRP), or they might represent a different subclass of receptor (similar to the situation in the opioid receptor family). (*iii*) Or possibly MCt-III exhibits higher potency than sCt-I by acting at the receptor in a slightly different manner—for instance, binding in the initial stages with somewhat less affinity, but activating the receptor more effectively, or for a longer time. Further experiments will be required to differentiate between these alternate possibilities.

Recently Epand *et al.* (25, 26) have synthesized a series of deletion and substitution analogues of sCt-I in the region of the putative amphiphilic α-helix and have used the relative potencies of these compounds to argue for the primacy of "conformational flexibility" in this region over amphiphilicity. An examination of these analogues by helical wheel and helical net diagrams shows that a correlation remains between amphiphilicity and potency. An amphiphilic α-helix is required; however, this helical region can be distorted or somewhat shortened, and the peptide retains activity. In this series of analogues the C-terminal region of the putative α-helix seems to be more amenable to distortion and elimination than the N-terminal region. For example, the most potent analogue is des-Leu^{19}-sCt, which clearly preserves unchanged most of the region from 8–22 in the native

hormone, consistent with our results in studies of MCt-III. Such interpretation is missed by relying on a too-rigid definition of an amphiphilic α-helix [specifically, using the hydrophobic-moment method of Eisenberg *et al.* (27)]. The region being modeled does not have to be postulated as a "perfect" amphiphilic α-helix, and, indeed, the variability of the calcitonin sequences from different species also argues against such a hypothesis.

In conclusion, the potency of MCt-III seems to make it a good model for calcitonin structure. Our results with MCt-III demonstrate that the α-helical region from residues 8–22, while exhibiting amphiphilicity, is amenable to sequence variation and that at least some properties of the hormone "benefit" from imperfections in the segregation of hydrophobic and hydrophilic residues.

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1. Kaiser, E. T. & Kézdy, F. J. (1984) *Science* **223**, 249–255.
2. Kaiser, E. T. & Kézdy, F. J. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1137–1143.
3. Moe, G. R., Miller, R. J. & Kaiser, E. T. (1983) *J. Am. Chem. Soc.* **105**, 4100–4102.
4. Moe, G. R. & Kaiser, E. T. (1985) *Biochemistry* **24**, 1971–1976.
5. Guttman, S. (1981) in *Calcitonin 1980: Chemistry, Physiology, Pharmacology and Clinical Aspects*, ed. Pecile, A. (Excerpta Medica, Amsterdam), pp. 11–23.
6. Fukushima, D., Yokoyama, S., Kézdy, F. J. & Kaiser, E. T. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2732–2736.
7. Hruby, V. J., Upson, D. A. & Agarwal, N. S. (1977) *J. Org. Chem.* **42**, 3552–3556.
8. Mojsov, S., Mitchell, A. R. & Merrifield, R. B. (1980) *J. Org. Chem.* **45**, 555–559.
9. Yamashiro, D. & Li, C. H. (1978) *J. Am. Chem. Soc.* **100**, 5174–5177.
10. Beyerman, H. C., Lie, T. S. & van Veldhuizen, C. H. (1973) in *Peptides 1971*, ed. Nesvadba, H. (North-Holland, Amsterdam), pp. 162–164.
11. Chait, B. T., Agosta, W. C. & Field, F. H. (1981) *Int. J. Mass Spectrom. Ion Phys.* **39**, 339–360.
12. Greff, D., Toma, F., Fermandjan, S., Low, M. & Kisfaludy, L. (1976) *Biochim. Biophys. Acta* **439**, 219–231.
13. Taylor, J. W. (1983) Dissertation (University of Chicago, Chicago).
14. Pollet, R. J., Haase, B. A. & Standaert, M. L. (1979) *J. Biol. Chem.* **254**, 30–33.
15. Bothwell, M. A., Howlett, G. L. & Schachman, H. K. (1978) *J. Biol. Chem.* **253**, 2073–2077.
16. Nakamuta, H., Forukawa, S., Koida, M., Yajima, H., Orlowski, R. C. & Schlueter, R. (1981) *Jpn. J. Pharmacol.* **31**, 53–60.
17. Hunter, W. M. & Greenwood, F. G. (1962) *Nature (London)* **194**, 495–496.
18. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimato, E. K., Goeka, N. M., Olsen, B. J. & Klenk, P. C. (1985) *Anal. Biochem.* **150**, 76–85.
19. Chang, C. T., Wu, C. S. C. & Yang, J. T. (1978) *Anal. Biochem.* **91**, 13–21.
20. Hennessey, J. P. & Johnson, W. C., Jr. (1981) *Biochemistry* **20**, 1085–1092.
21. Provencher, S. W. & Glockner, J. (1981) *Biochemistry* **20**, 33–37.
22. Wollmer, A., Strussburger, W. & Glatter, U. (1983) in *Modern Methods in Protein Chemistry: Review Articles*, ed. Tschesche, H. (de Gruyter, Berlin), pp. 361–384.
23. DeGrado, W. F. & Lear, J. D. (1985) *J. Am. Chem. Soc.* **107**, 7684–7689.
24. Rosenfeld, M. G., Mermod, J.-J., Amara, S. G., Swenson, L. W., Sawchenko, P. E., Rivier, J., Vale, W. W. & Evans, R. M. (1983) *Nature (London)* **304**, 129–135.
25. Epand, R. M., Seyler, J. K. & Orlowski, R. C. (1986) *Eur. J. Biochem.* **159**, 125–127.
26. Epand, R. M., Epand, R. F., Orlowski, R. C., Seyler, J. K. & Colescott, R. C. (1986) *Biochemistry* **25**, 1964–1968.
27. Eisenberg, D., Weiss, R. M. & Terwilliger, T. C. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 140–144.