Conformational Flexibility and Biological Activity of Salmon Calcitonin†

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Received July 22, 1985; Revised Manuscript Received December 6, 1985

ABSTRACT: We have assessed the biological activity of salmon calcitonin I (sCT) using an in vivo biological assay of hypocalcemic activity in rats. The changes in biological activity observed are explained on the basis of changes in the conformational properties of the hormone analogues. Helical content in the presence and absence of lipids and detergents was assessed by using circular dichroism, and the section of the molecule that folds into a helix was predicted on the basis of the helix–coil transition theory of Mattice and co-workers. In the amino acid sequence of sCT, residue 8 is valine and residue 16 is leucine. The synthetic calcitonin derivatives [Gly^8]sCT and [Ala^{16}]sCT have higher biological activity than the native hormone although they have a lower helical content. The increased biological activity of these derivatives is ascribed to an increase in their conformational flexibility resulting from the substitution of amino acid residues with less bulky side chains and less tendency to form helical structures. The derivative [Met^8]sCT has less substitution than sCT on the β-carbon at position 8, but it has increased helix-forming potential in the region of residues 8–12. These two factors affect conformational flexibility in opposite ways, resulting in the biological activity of [Met^8]sCT being slightly higher than that of sCT. However, increased conformational flexibility does not always increase biological activity. Substitution of the L-arginine at residue 24 for a D-arginine has little effect on the conformational properties or biological activity of sCT. However, [Gly^8,D-Arg^{24}]sCT is less active than sCT, [Gly^8]sCT, or [D-Arg^{24}]sCT. These results suggest that, in addition to conformational flexibility, long-range interactions affecting receptor binding and/or conformational properties of the receptor-bound hormone also affect biological activity.

Salmon calcitonin I (sCT)[1] is a 32 amino acid peptide hormone that has regularly spaced hydrophobic amino acids at every third or fourth residue in the central region of the chain (Potts & Aurbach, 1976; also, see Table III, below). This structural feature was fist suggested to be important for the binding of proteins to lipids in studies of serum apolipoproteins (Segrest et al., 1974). The regular spacing of hydrophobic amino acid residues would allow a peptide to fold into an amphipathic helix in which one face of the helix was hydrophobic and the other hydrophilic. The presence of such sequences in polypeptide hormones was first noted for glucagon (Epand et al., 1977), and it was demonstrated that this peptide hormone could act surprisingly like a serum apolipoprotein and solubilize phosphatidylcholine (Jones et al., 1978). This property is a common feature of several polypeptide hormones of diverse sequence (Epand, 1983) including sCT (Epand et al., 1983). Synthetic calcitonin analogues have been prepared based on this structural feature, which demonstrated that a number of amino acid replacements could be incorporated into the calcitonin molecule with retention of considerable biological potency (Moe et al., 1983; Moe & Kaiser, 1985). However, there is some question about the role of helix-forming ability in determining biological activity. All biologically active forms of calcitonin are able to solubilize dimyristoylphosphatidylglycerol (DMPG) (Epand et al., 1985a), but the solubilization of lipids by calcitonin is not always accompanied by a marked enhancement of helical content (Epand et al., 1983). Furthermore, [Gly^8]sCT is more active than sCT, yet it forms less helix under similar conditions (Epand et al., 1985a), while the synthetic calcitonin analogue MCT-I is more helical but has a lower biological activity (Moe et al., 1983). In this work we elucidate other factors, in addition to the formation of an amphipathic helix, that are important in determining the biological activity of sCT.

Experimental Procedures

Materials

Lipids. Dimyristoylphosphatidylglycerol (DMPG) from Avanti Polar Lipids, bovine lysophosphatidylcholine (LPC) from P-L Biochemicals, and sodium dodecyl sulfate (SDS) from Miles Laboratories were all high-purity commercial preparations.

Peptides. Synthetic sCT and analogues were synthesized by standard solid-phase methodology (Orlowski & Seyler, 1983a,b, 1984; Orlowski et al., 1985). After removal from the resin by treatment with anhydrous hydrofluoric acid and disulfide ring closure, the products were concentrated on SP-Sephadex, desalted in G-25, and purified on CM-52. [Met^8]sCT was further purified by partition chromatography (Orlowski et al., 1982) on G-25 Sephadex using 1-butanol, 95% ethanol, and 0.2 N ammonium acetate, pH 6.1, in the ratio 4:1:5. The R_f for [Met^8]sCT is 0.63 (Orlowski & Seyler, 1985), and the weight mean potency is 5500 IU/mg, P = 95% (range, 4952–6158). The amino acid composition of [Met^8]sCT is as follows: Asp, 2 (2); Thr, 5 (5); Ser, 3.8 (4); Glu, 3 (3); Pro, 2 (2); Gly, 3 (3); Cys, 0.91 (1); Leu, 5.1 (5);

† This work was supported by the Medical Research Council of Canada (Grant MA-7654).

[1] Abbreviations: sCT, salmon calcitonin I; DMPG, dimyristoylphosphatidylglycerol; LPC, bovine lysophosphatidylcholine; SDS, sodium dodecyl sulfate; CD, circular dichroism; Pipes, 1,4-piperazinediethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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Tyr, 0.91 (1); Met, 1 (1); Lys, 1.9 (2); His, 1 (1); Arg, 1 (1); NH_3, 5.2 (5). Comparable data on the other analogues can be found in the cited references. The biological activities are given under Results.

Methods

Buffer. The buffer used was generally 20 mM Pipes, 1 mM EDTA, and 150 mM NaCl containing 0.02 mg/mL NaN_3 and adjusted to pH 7.40 with NaOH. However, in order to penetrate further into the far ultraviolet region of the spectrum for the estimation of secondary structure, a 10 mM sodium phosphate buffer, pH 7.0, was used.

Addition of Protein to Lipids and Detergent. DMPG and LPC were first deposited as films from solution in chloroform/methanol by solvent evaporation under nitrogen, followed by removal of the last traces of solvent under vacuum for at least 1 h. Peptide solution was added to these films and the sample warmed to 40 °C and vortexed. The mixture was then cooled and heated between 10 and 40 °C to aid in the dissolution of the lipid. All of the samples containing a calcitonin analogue and DMPG, LPC, or SDS were visually transparent.

Circular Dichroism (CD). CD spectra were obtained with an Aviv Model 61DS solid-state CD instrument (Aviv Associates, Lakewood, NJ). This instrument is equipped with a 50-kHz photoelastic modulator and an end-on photomultiplier. The instrument was calibrated with *d*-10-camphorsulfonic acid (Chen & Yang, 1977). The concentration of peptide was determined by using a molar extinction coefficient of 1515 cm^{-1} M^{-1} at 275 nm (Epand et al., 1983). The CD was measured in a 1-mm sample cell that was maintained at 25 °C with a thermostated cell holder. Single scans were generally performed on duplicate samples and were corrected for the base line. The corrected data set were multiplied by a constant to obtain the mean residue ellipticity $[\theta]$. In the cases of the spectra used for secondary structure estimation, the accuracy of the data was improved by averaging 10 scans. The instrument is interfaced with a Columbia computer, which was used for all mathematical manipulations. The program for secondary structure analysis was kindly supplied to us by Prof. J. T. Yang, University of California, San Francisco, and was adapted for the Aviv instrument by James Laurino. The nonlinear, least-squares curve-fitting program for the estimation of helix, β-form, β-turn, and unordered form has been previously described (Chang et al., 1978).

Calculation of Helix Probability Profiles. The probability of folding into a helical conformation was calculated for each amino acid residue by the method of Mattice and co-workers (Mattice & Robinson, 1981; Hamed et al., 1983). The statistical weights for each amino acid at 30 °C (Mattice & Robinson, 1981; Hamed et al., 1983) were used to construct a series of 3 × 3 matrices to calculate the helical content in water and SDS and to construct a series of 13 × 13 matrices for the conformation in zwitterionic lipids. For the peptides in the presence of zwitterionic lipids, a statistical weight of $s \pm = 4$ was used for charged residues which were separated by two or three other residues from a charged residue of opposite sign.

Hypocalcemic Activity. Biological activity was assayed in vivo by measuring the decrease in blood calcium levels after peptide administration to rats as previously described (Schwartz et al., 1981).

RESULTS

The conformational properties of sCT and several analogues are summarized in Table I along with the measured hypocalcemic activity. The value of $[\theta]_{222}$ is indicative of helical content, reaching −28 400 deg cm^2 $dmol^{-1}$ for a fully helical peptide with the average length of a helical segment being 10 residues. None of the peptides exhibit a great deal of helical structure in the absence of lipids or detergent, but all show marked increases in helical content in the presence of DMPG, LPC, or SDS. Substitution of Met for Val at position 8 or of D-Arg for L-Arg at position 24 has little effect on the conformational properties of the peptides and only slightly increases the biological activity of the hormone. However, substitution of Ala for Leu at position 16 or of Gly for Val at position 8 markedly reduces helical content but enhances biological activity. The analogue [Gly^8,D-Arg^{24}]sCT has conformational properties very similar to [Gly^8]sCT, but the former analogue has a reduced biological activity.

Table I: Circular Dichroism of Calcitonin Peptides in the Presence and Absence of DMPG, LPC, or SDS[a]

derivative	$-[\theta]_{222}$ (deg cm^2 $dmol^{-1}$) no additions	DMPG	LPC	SDS	biological act. (IU/mg)
sCT	4160	11 500	10 800	11 400	4250
[Gly^8]sCT	2915	5 400	5 325	7 925	6500
[D-Arg^{24}]sCT	3050	10 000	11 000	10 300	5000
[Gly^8,D-Arg^{24}]sCT	3000	4 700	5 500	7 800	3500
[Met^8]sCT	4240	10 900	10 200	10 950	5500
[Ala^{16}]sCT	1750	7 940	5 300	12 300	6200

[a] Peptide (100 μM) was incubated in the presence or absence of 1 mM DMPG, 2.5 mM LPC, or 25 mM SDS at 25 °C in 20 mM Pipes, 1 mM EDTA, 0.15 M NaCl, and 0.02 mg/mL NaN_3, pH 7.40.

Table II: Secondary Structure Estimates Calculated from the CD Spectra of Figure 1 by the Method of Chang et al. (1978)[a]

sample	helix	β-form	β-turn	unordered form
sCT	0	0.48	0.04	0.47
sCT + DMPG	0.40	0.31	0	0.28
[Gly^8]sCT	0	0.49	0.05	0.46
[Gly^8]sCT + DMPG	0.31	0.38	0	0.31

[a] See legend of Figure 1 for details of conditions.

For a more detailed analysis of secondary structure, the CD spectrum of sCT and of [Gly^8]sCT was measured in the presence and absence of DMPG. The measured spectra were similar to but not identical with those used to obtain the data for Table I. In the present case, 2.4 mM instead of 1.0 mM DMPG was used to ensure the presence of saturating concentrations of lipid. The buffer was 10 mM sodium phosphate, pH 7.0, which had a much lower ionic strength than the Pipes buffer used to obtain the data for Table I. The phosphate buffer was used because it had lower absorbance below 210 nm. The conditions used to obtain the spectra for curve resolution tend to slightly increase the magnitude of $[\theta]_{222}$ compared with the values given in Table I, but they do not change the qualitative features of the results (Figure 1). The peptides in buffer have a low helical content which increases greatly in the presence of DMPG (Table II). [Gly^8]sCT has less helix than sCT under the same conditions. Both peptides have large amounts of β-structure, but it has been shown that estimates of β-structure are less reliable than those of α-helix (Chang et al., 1978). Nevertheless, one can conclude that it is highly probable that the peptides in buffer have little or no helical content but do contain some β-structure. The effect of DMPG is largely to increase the helical content of these peptides, and [Gly^8]sCT attains less helix than sCT.

The extension of statistical mechanical theories of the helix–coil transition to include effects of lipids (Mattice & Robinson, 1981; Hamed et al., 1983) correctly predicts the increased helical content of sCT in the presence of lipids

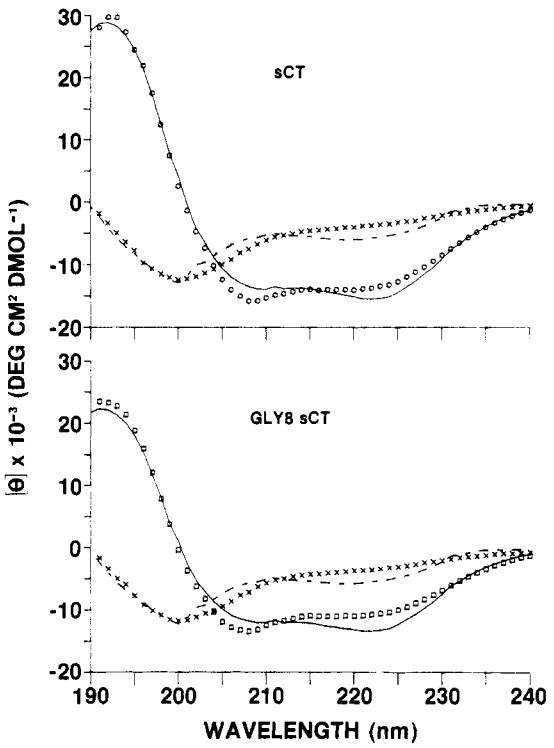

FIGURE 1: Circular dichroism spectra of [Gly^8]sCT and of sCT in the presence and absence of DMPG: 90 μM peptide in 10 mM sodium phosphate buffer, pH 7.0 (×, ---), and in the presence of 2.4 mM DMPG (□, —). Points are the experimentally determined values, and lines are the least-squares best-fit curves for the mixtrues of secondary structures given in Table II.

(Figure 2). In the case of zwitterionic lipids, the behavior of LPC is qualitatively predicted by the theory since it induces somewhat less helix formation than SDS. In contrast, the zwitterionic phospholipid dimyristoylphosphatidylcholine interacts with sCT only at high concentrations of the peptide (Epand et al., 1985b). The difference between [Gly^8]sCT and sCT is the decreased helical probability of residues 9 and 10 in the presence of anionic lipid. [Met^8]sCT by comparison has a higher helical probability for residues 9 and 10 in anionic lipid. This helix-promoting ability of Met extends from residue 8 to residue 18 in anionic lipids and from residue 8 to residue 22 in zwitterionic lipids. Substitution of Ala for Leu at position 16 causes a modest reduction in helix probability, but this reduction extends from residue 11 to residue 18 in anionic lipids and from residue 12 to residue 19 in zwitterionic lipids. Thus, the decreased helix-forming ability of [Ala^{16}]sCT (Table I) is correctly predicted by the Mattice treatment, but it would not have been predicted on the basis of the Chou–Fasman rules (Chou & Fasman, 1974).

Discussion

The role of an amphipathic helix in enhancing the potency of several peptide hormones has been recognized [see, for example, Epand (1983) and Kaiser and Kézdy (1984)]. However, in the cases of [Gly^8]sCT and [Ala^{16}]sCT the extent of helix formation in the presence of lipid, the hydrophobic moment of the helix that is formed, and the overall hydrophobicity of the peptide are all reduced. Thus other conformational features, in addition to the amphipathic helix, are likely to contribute to biological potency. Biological potency has been found to be proportional to receptor affinity for a number of calcitonin analogues, but the former is more sensitive to changes in peptide structure (Epand et al., 1985a; Findlay et al., 1985).

A conformational feature of [Gly^8]sCT and [Ala^{16}]sCT that can explain their increased biological activity is their increased conformational flexibility, i.e., their ability to attain a larger number of conformations. This increased flexibility could lead to higher activity either by allowing more energetically favorable access of the derivatives to conformers that better resemble the active, receptor-bound conformer or by allowing escape from a stable but less active conformer. We do not

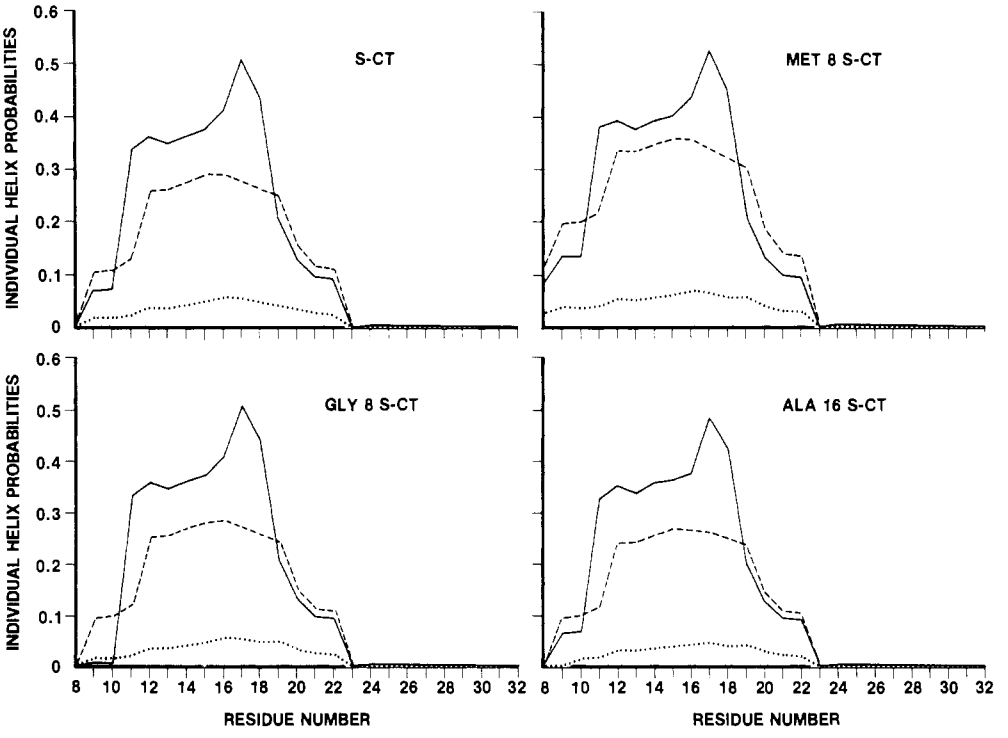

FIGURE 2: Helix probability profiles for sCT and analogues. The line of lowest helix probability represents peptide in water; that of intermediate probability, peptide in zwitterionic lipid; that of highest helical-forming probability, peptide in anionic lipid. Lines were calculated according to the method of Mattice & Robinson (1981) and Hamed et al. (1983).

use the term flexibility to specifically refer to molecular dynamics.

There are two different properties of the peptide that will affect the number of conformations available to the peptide. One property is the bulkiness of the side chain. This can be assessed with Ramachandran maps. The other property is the tendency to form specific secondary structures. When the peptide adopts a conformation with a large fraction of α-helix, it reduces the number of available conformational states. The extent of helix formation can be assessed by CD and the location of the helical residues predicted by helix–coil transition theory. In terms of Ramachandran maps of sterically allowed dihedral angles, ϕ and ψ, glycine allows the greatest conformational flexibility and valine one of the least (Ramachandran & Sasisekharan, 1968). Also an Ala at position 16 allows more combinations of ϕ and ψ angles than Leu. Both [Gly^8]sCT and [Ala^{16}]sCT also show lower helix-forming abilities in the presence of DMPG (Table I) as predicted by the statistical mechanical theory (Figure 2). Therefore, from the point of view of both Ramachandran maps and helix formation, [Gly^8]sCT and [Ala^{16}]sCT are more conformationally flexible than the native hormone. [Met^8]sCT has greater conformational flexibility from the point of view of Ramachandran maps but has less conformational flexibility as a result of a small local (residues 8 and 9) increase in helicity (Figure 2). Thus the rigid amphipathic helix extends toward the conformationally restricted N-terminal disulfide ring structure. These two factors of a less bulky side chain and increased helicity at position 8 would thus tend to cancel each other, resulting in little change in the activity of this analogue.

In emphasizing the role of conformational flexibility to explain the increased activity of [Gly^8]sCT and [Ala^{16}]sCT, we do not wish to imply that this is the only factor affecting biological potency or that maximizing flexibility will maximize potency. Clearly, peptides such as des-Leu^{16}-sCT or human calcitonin have less helix-forming potential and more conformational flexibility than sCT but are much less potent (Epand et al., 1985a). In fact, substituting Met at position 8 of human calcitonin for Gly increases the conformational flexibility to such an extent that Gly^8-substituted human calcitonin can no longer form structures of higher helical content in the presence of lipid or solubilize lipid (Epand et al., 1985a). This analogue shows no hypocalcemic activity (Findlay et al., 1985). Thus consideration only of conformational flexibility is not enough to predict the relative activity of calcitonin analogues, but neither is consideration of any other single factor such as hydrophobic moment, helix-forming potential, or overall peptide hydrophobicity. For certain analogues such as [Gly^8]sCT and [Ala^{16}]sCT the simplest way of explaining their increased potency is in terms of increased conformational flexibility. Conformational flexibility is also thought to be important for immunogenicity and possibly for protein–protein interactions in general (Tainer et al., 1984).

An additional factor that makes it more difficult to predict the potency of analogues is the conformational coupling among various regions of the molecule. For example, D-Arg at position 24 has almost no effect on the activity or conformational properties of sCT. However, substituting Gly at position 8 increases the activity of sCT but decreases the activity of [D-Arg^{24}]sCT. This result can be explained if there are "long-range" (with respect to the amino acid sequence) interactions between the amino- and carboxyl-terminal regions of the molecule, at least in the receptor-bound form. Another example is substituting Met at position 8 in sCT vs. making this substitution in salmon calcitonin II (sCT II). Salmon has

Table III: Amino Acid Sequences of Salmon Calcitonins[a]

Species	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32
Salmon I	Cys	Ser	Asn	Leu	Ser	Thr	Cys	Val	Leu	Gly	Lys	Leu	Ser	Gln	Glu	Leu	His	Lys	Leu	Gln	Thr	Tyr	Pro	Arg	Thr	Asn	Thr	Gly	Ser	Gly	Thr	Pro-NH_2
Salmon II	Cys	Ser	Asn	Leu	Ser	Thr	Cys	Val	Leu	Gly	Lys	Leu	Ser	Gln	Asp	Leu	His	Lys	Leu	Gln	Thr	Phe	Pro	Arg	Thr	Asn	Thr	Gly	Ala	Gly	Val	Pro-NH_2
Salmon III	Cys	Ser	Asn	Leu	Ser	Thr	Cys	Met	Leu	Gly	Lys	Leu	Ser	Gln	Asp	Leu	His	Lys	Leu	Gln	Thr	Phe	Pro	Arg	Thr	Asn	Thr	Gly	Ala	Gly	Val	Pro-NH_2

[a] Cys residues 1 and 7 are linked by a disulfide bond. The residues different from those in salmon calcitonin I are underlined.

three forms of calcitonin, the major component sCT I (generally referred to in this paper simply as sCT) and two minor components sCT II and sCT III (Table III). The sequence of sCT II differs from that of sCT I at residues 15, 22, 29, and 31, yet both forms have identical hypocalcemic activity (Pless et al., 1971). However, substitution of Met for Val at position 8 of sCT slightly increases the biological activity (Table I), but when Met is substituted at position 8 of sCT II to form sCT III (Table III), it results in a 3-fold decrease in activity (Pless et al., 1971). This result again suggests conformational coupling among various regions of the molecule. It is possible that hormone–receptor binding is coupled to cooperative conformational interactions among different regions of the peptide in an analogus manner to that which has been proposed for the binding of substrates to enzymes (Taniuchi & Bohnert, 1975; Taniuchi, 1984).

ACKNOWLEDGMENTS

We are grateful to Dr. Jen Tsi Yang of the Cardiovascular Research Institute, University of California, San Francisco, for providing us with the computer program and reference data for the secondary structure analysis. We thank Andy Epand and Victor Epand for their assistance in developing a computer program for the matrix analysis of helical structure. We thank Dr. E. T. Kaiser for making his manuscript available to us prior to publication.

Registry No. sCT, 47931-85-1; [Gly^8]sCT, 88433-78-7; [Ala^{16}]sCT, 98647-75-7; [Met^8]sCT, 100859-28-7; [Gly^8,D-Arg^{24}]sCT, 100937-77-7; [D-Arg^{24}]sCT, 93529-60-3; Ca, 7440-70-2.

REFERENCES

Chang, C. T., Wu, C.-S. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13–31.

Chen, G. C., & Yang, J. T. (1977) *Anal. Lett. 10*, 1195–1207.

Chou, P. Y., & Fasman, G. D. (1974) *Biochemistry 13*, 222–245.

Epand, R. M. (1983) *Mol. Cell. Biochem. 57*, 41–47.

Epand, R. M., Jones, A. J. S., & Schreier, S. (1977) *Biochim. Biophys. Acta 491*, 296–304.

Epand, R. M., Epand, R. F., Orlowski, R. C., Schlueter, R. J., Boni, L. T., & Hui, S. W. (1983) *Biochemistry 22*, 5074–5084.

Epand, R. M., Epand, R. F., & Orlowski, R. C. (1985a) *Int. J. Pept. Protein Res. 25*, 105–111.

Epand, R. M., Epand, R. F., Orlowski, R. C., Flanigan, E., & Stahl, G. L. (1985b) *Biophys. Chem. 23*, 39–48.

Findlay, D. M., Michaelangeli, V. P., Martin, T. J., Orlowski, R. C., & Seyler, J. K. (1985) *Endocrinology (Baltimore) 117*, 801–805.

Hamed, M. M., Robinson, R. M., & Mattice, W. L. (1983) *Biopolymers 22*, 1003–1021.

Jones, A. J. S., Epand, R. M., Lin, K. B., Walton, D., & Vail, W. J. (1978) *Biochemistry 17*, 2301–2307.

Kaiser, E. T., & Kézdy, F. J. (1984) *Science (Washington, D.C.) 223*, 249–255.

Mattice, W. L., & Robinson, R. M. (1981) *Biopolymers 20*, 1421–1434.

Moe, G. R., & Kaiser, E. T. (1985) *Biochemistry 24*, 1971–1976.

Moe, G. R., Miller, R. J., & Kaiser, E. T. (1983) *J. Am. Chem. Soc. 105*, 4100–4102.

Orlowski, R. C., & Seyler, J. K. (1983a) U.S. Patent 4 401 539.

Orlowski, R. C., & Seyler, J. K. (1983b) U.S. Patent 4 414 149.

Orlowski, R. C., & Seyler, J. K. (1984) U.S. Patent 4 469 632.

Orlowski, R. C., & Seyler, J. K. (1985) U.S. Patent Appl. 746 435.

Orlowski, R. C., Groginski, C. M., & Seyler, J. K. (1982) U.S. Patent 4 336 187.

Orlowski, R. C., Stahl, G. L., & Colescott, R. L. (1985) U.S. Patent 4 528 132.

Pless, J., Bauer, W., Bossert, H., Zehnder, K., & Guttmann, St. (1972) *Endocrinol., Proc. Int. Symp. 3rd, 1971 (1972)*, 67–70.

Potts, J. T., Jr., & Aurbach, G. D. (1976) *Handb. Physiol., Sect. 7: Endocrinol. 7*, 423–430.

Ramachandran, G. N., & Sasisekharan, V. (1968) *Adv. Protein Chem. 23*, 283–438.

Schwartz, K. E., Orlowski, R. C., & Marcus, R. (1981) *Endocrinology (Baltimore) 108*, 831–835.

Segrest, J. P., Jackson, R. L., Morrisett, J. D., & Gotto, A. M. (1974) *FEBS Lett. 38*, 247–253.

Tainer, J. A., Getzoff, E. D., Alexander, H., Houghten, R. A., Olson, A. J., Lerner, R. A., & Hendrickson, W. A. (1984) *Nature (London) 312*, 127–134.

Taniuchi, H. (1984) in *The Impact of Protein Chemistry on the Biomedical Sciences* (Schechter, A. N., Dean, A., & Goldberger, R. F., Eds.) pp 67–81, Academic Press, Orlando, FL.

Taniuchi, H., & Bohnert, J. L. (1975) *J. Biol. Chem. 250*, 2388–2394.