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SYNTHESIS AND BIOACTIVITY STUDIES OF TWO ISOSTERIC ACYCLIC ANALOGUES OF MELANIN CONCENTRATING HORMONE

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Summary

Salmon melanin concentrating hormone (MCH) is a cyclic heptadecapeptide. MCH stimulates perinuclear aggregation of melanosomes within integumental melanocytes of teleost fishes resulting in skin blanching. MCH contains a disulfide bridge forming a 10-residue ring (H-Asp-Thr-Met-Arg-Cys-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val-OH). It has been proposed that the ring is necessary for maintenance of potency. In order to test this proposal, we have synthesized two pseudo-isosteric analogues of MCH that cannot cyclize. They differed only in the polarity of the side chain group of positions 5 and 14. Serine was substituted for Cys^5 and Cys^{14} in one peptide and L α-aminobutyrate (Abu) was the substitution at the two positions in the other peptide. Using a fish skin bioassay we determined that these analogues exhibit less than $1/10{,}000^{th}$ the potency of the native hormone. These results suggest that the disulfide bridge is necessary to maintain the correct conformational and topographical features of the hormone for receptor binding and transmembrane signal transduction.

Salmon melanin concentrating hormone (MCH) is a cyclic heptadecapeptide (Fig. 1). The hormone was originally isolated from chum salmon pituitaries by Kawauchi and coworkers (1), and the total synthesis was first achieved by Wilkes et al. (2,3). An acyclic analogue of MCH was previously synthesized by Eberle and coworkers and showed $1/300^{th}$ the potency of the native hormone (4). A second acyclic analogue synthesized by Kawazoe and coworkers employed reduction of the disulfide bond followed by subsequent carboxamidomethylation to achieve the desired product. Contrary to Eberle's work, Kawazoe et al. found a complete loss of biological activity (5). This apparent discrepancy in results led us to question whether or not the acyclic analogues employed were adequate models since the cysteine residues were

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derivatized with groups of considerable steric bulk. It could be argued that, because of the bulky substitutions, they may not allow the peptide to fold properly into a structure similar to the native hormone.

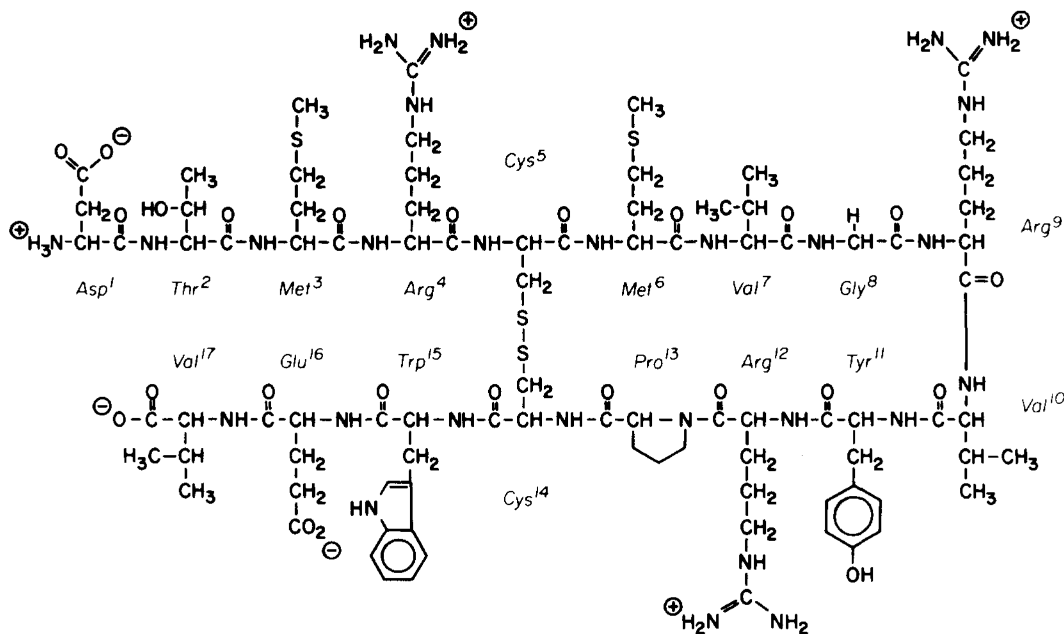

Fig. 1
Primary structure of salmonid melanin concentrating hormone (MCH).

We have therefore addressed this question by synthesizing acyclic analogues with pseudoisosteric residues to replace the Cys^5-Cys^{14}-bridge. To assess the effects of these side chain modifications we used both a polar (L-serine) and a non-polar (L-α-aminobutyrate) moiety to determine whether such substitutions would influence the structural integrity of the peptide and hence its bioactivity. The substituted structures in the two MCH analogues are shown below (Fig. 2).

Materials and Methods

All Boc-L-amino acids were either commercially obtained from Bachem (Torrance, CA), or synthesized in our laboratory. Peptide synthesis was performed on a DuPont 2200 automated synthesizer. The chloromethylated resin (Merrifield) was used as the support to introduce the first amino acid. Resin substitution was 0.48 mmole/g. The amino acid resin was prepared according to the method of Gisin (6). Resin couplings were accomplished using the appropriate Boc-amino-acid, 3 equivalents of hydroxybenzotriazole, and 3 equivalents of diisopropylcarbodiimide. All couplings were performed using N-methylpyrrolidinone as solvent.

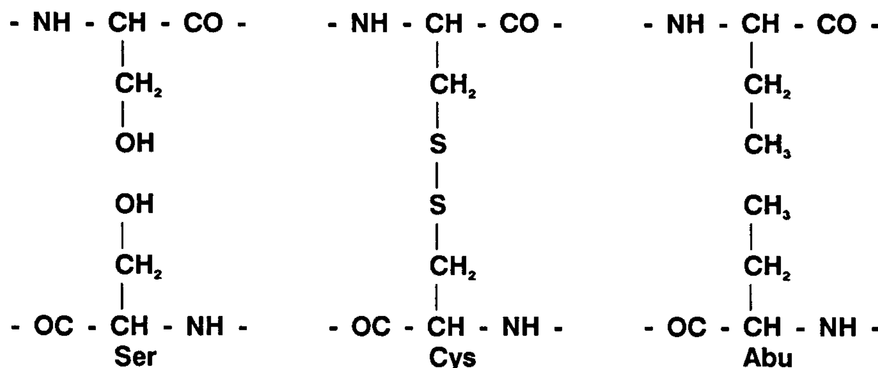

Fig. 2

Structure of the Cys^5,Cys^{14} disulfide bridge of MCH and the related structures in the two acyclic analogues of MCH demonstrating the pseudoisosteric substitution of Cys in position-5 and -14 by Ser or Abu.

The intact peptides were dried overnight in vacuo and cleaved with HF (10 ml/g), anisole (1.0 ml/g) and ethanedithiol (0.3 ml/g). The cleaved peptides were then washed with ethyl acetate and extracted via filtration with aqueous acetic acid. The products were isolated by lyophilization and dissolved in 1.6 liter of degassed, argon-purged, deionized water. The pH was adjusted to 8.4 with aqueous ammonia. Cyclization was achieved by the dropwise addition of $K_3Fe(CN)_6$.

The peptides were further purified by passing through a 2.5 cm x 90 cm carboxymethyl cellulose cation exchange column (Bio-Rad) and eluting with a linear gradient of 200 ml of 0.1 M $NH_4(OAc)$ in the mixing chamber and 200 ml of 0.7 M $(NH_4)OAc$ in the reservoir chamber. Fractions were then collected and monitored at 280 nm. Appropriate fractions were collected and lyophilized. Final purification was by HPLC using a Vydac TP-1010 semi-prep reverse phase column (1.0 x 25 cm) and a Perkin Elmer Series 410 BIO HPLC system equipped with a Perkin-Elmer LC-235 diode-array detector.

Amino acid analysis was performed at the Biotechnology Center, Department of Biochemistry, University of Arizona. All samples were hydrolyzed with 300 μl of 3N mercaptoethanesulfonic acid or 4N methanesulfonic acid (Pierce Chemical Co.) and heated for 22 hrs at 110°C. Samples were then diluted to a final concentration of 10^{-5}M with citrate buffer and analyzed quantitatively for amino acid composition on a Beckman 7300 amino acid analyzer. Table I lists the observed amino acid compositions of the synthesized peptides.

TABLE I

Amino Acid Analysis of $Ser^{5,14}$ MCH and α-$Aminobutyrate^{5,14}$ MCH

Peptide	Gly	Glu	Thr	Val	Met	Tyr	Arg	Asp	Pro	Trp	Ser	Abu
$Ser^{5,14}$ MCH	1.00	1.03	0.98	3.00	1.71	0.86	2.83	0.91	1.04	N.A.	1.96	-
$Abu^{5,14}$ MCH	1.00	1.05	0.92	2.87	1.69	0.89	2.76	0.86	1.19	N.A.	-	1.90

N.A. = Not assayed
Abu = α-aminobutyrate

Mass spectrometry was performed at the Midwest Center for Mass Spectrometry in Omaha, Nebraska. The mode was fast atom bombardment. Samples were suspended in either dithiothreitol, dithioerythreitol or ethylene glycol. Table II lists the calculated and observed values.

TABLE II

Mass Spectrometric Data - Fast Atom Bombardment

	Molecular Weight Calculated	Molecular Weight Observed
$Ser^{5,14}$ MCH	2067	2067 (M/z)
$Abu^{5,14}$ MCH	2063	2063 (M/z)

Thin-layer chromatography was performed on a silica gel matrix (Analtech) 10 cm x 2.5 cm plates with glass backings and are listed in Table 3.

TABLE III

Rf Values of $Ser^{5,14}$ and $Abu^{5,14}$ MCH in Different Solvents*

	A	B	C	D
$Ser^{5,14}$ MCH	0.03	0.63	0.81	0.73
$Abu^{5,14}$ MCH	0.05	0.66	0.81	0.79

*The solvents were comprised of the following:

A) N-butanol: acetic acid: water: pyridine (15:3:8:10 v/v)
B) N-butanol: acetic acid: water (4:1:1 v/v)
C) pyridine: acetic acid: water (50:30:15 v/v)
D) ethyl acetate: pyridine: acetic acid: water (5:5:1:3 v/v)

The in vitro fish skin assay developed by Castrucci and coworkers (7) was used to determine the relative MCH-like potency of the synthesized analogues. The teleost fish, Synbranchus marmoratus, an eel, was obtained from the Pantanal ("Big Swamp") of Brazil. Skins were removed from decapitated animals and immediately bathed in a physiological saline solution (teleost Ringer). Many smaller pieces of skin could be obtained from a single fish. Solutions of the acyclic analogues and MCH were prepared in teleost Ringer, immediately before use. MCH stimulates perinuclear aggregation of melanosomes (melanin granules) within integumental pigment cells (melanocytes). This results in lightening of the skins which can be measured objectively as a change in reflectance of light from the skins. In response to the peptides, the change in reflectance from the initial (time zero) base values were recorded by a Photovolt reflectometer (Model 670). A graph of -log analogue concentration vs. percent of skin lightening was used to determine the bioactivity (potency) of the peptides.

Results

Both acyclic analogues exhibited less than 1/10,000th the potency of the native hormone. Because our current purification procedures cannot unequivocally purify to this degree of accuracy (1 part in 10,000), we cannot definitively state that the acyclic analogues were active at all (e.g., not contaminated by MCH from previous syntheses). In order to confirm our results, one of the acyclic analogues, $Ser^{5,14}$ MCH was synthesized independently in another laboratory (ML). Although data is not shown, both $Ser^{5,14}$ MCH samples exhibited similar bioassay profiles, thus confirming that the acyclic analogue, at best, is very weakly potent.

Discussion

The almost total lack of activity of these pseudoisosteric, acyclic analogues of MCH supports the contention that the disulfide ring is a very important requirement for maintenance of the proper topographical features necessary for receptor binding and signal transduction. It is conceivable that the disulfide bridge of MCH obligates the ring to form a reverse turn at some point in the ring sequence. This has been supported by NMR observations (11), which show a modified Type I ß turn between the Val^7 through Val^{10} residues. Because previous results have pointed to the importance of either residues 5-14 or residues 5-15 for maintenance of equipotency in the fish skin bioassay (5,12), it becomes clear that the 10-residue ring is very important for optimal activation of integumental melanocytes. In addition, previous work by Lebl and coworkers demonstrated that ring-contracted analogues of MCH led to drastically reduced potency in the fish skin assay (13). Although ring expanded analogues have not yet been synthesized, present evidence suggests that ring size cannot be altered without loss of MCH-like activity. Our present work is in agreement with that of Kawazoe et al. who found that the reduced carboxamidomethyl acyclic analogue was totally inactive (5).

Elimination of the disulfide bond could conceivably increase the entropic behavior of the molecule, allowing a much different molecular conformation or subpopulation of different conformations to be assumed. Chou-Fasman analysis of the serine MCH analogue reveals a preference for a pleat over a bend in the 7-10 region of this acyclic analogue. Thus, the acyclic analogue may assume a more extended structure in this region.

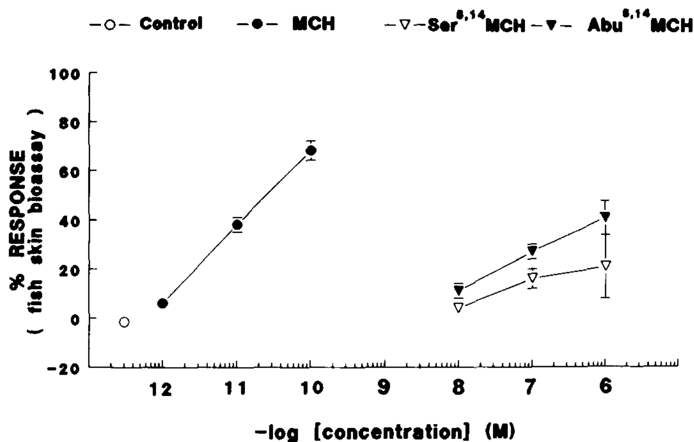

Fig. 3
In vitro demonstration that the acyclic MCH analogues of MCH possess little or no biological activity, α-Abu$^{5-14}$MCH, was very inactive compared to MCH (less than 1/10,000th the potency of the native hormone). The Ser$^{5-14}$MCH analogues were equally very weakly potent. Each value (n = 11) is the mean, ± S.E., response (lightening) of the fish skins to the melanotropic peptides at the concentrations noted.

An acyclic analogue [S-acetamidomethyl^{5,14}]MCH, was synthesized by Eberle and coworkers and found to possess about 1/300th the potency of native hormone (4). Similar results were obtained most recently by Baker and coworkers who synthesized a variety of analogues and found progressively increasing potencies as they synthesized the linearized analogues from MCH(15-17) to Cys(Acm)^{5,14}MCH(1-17) respectively. Molecular dynamics simulations on MCH, MCH(5-14), and linear MCH(5-14) showed that the overall conformations adopted by linear and cyclic sequences were similar (14). Thus, despite the presence of rather bulky Acm groups, the proper secondary structure for receptor binding and transduction could be achieved with the linear acetamidomethylated analogues.

Presently, we cannot explain the wide disparity of potency between our acyclic analogues ($\leq$ 1/10,000) and the acetamidomethyl Cys^{5,14} MCH ($\approx$ 1/300) prepared elsewhere (4). It is possible that the acetamidomethyl group could fold into a more favorable conformation that is recognized by the MCH receptor. Alternatively, it is also possible that one acetamidomethyl group may have been cleaved under conditions of isolation followed by attack of the subsequent sulfide anion on the sulfur of the other acetamidomethyl cysteine group. Thus, in this way, the native MCH hormone may have been regenerated, leading to a higher than expected potency. In closer agreement with our results, Kawazoe et al. (5) reported that the carboxamidomethyl analogue was totally devoid of biological activity. Ser^{5,14}MCH and α-Abu^{5,14}MCH, on the other hand, are unable to cyclize and thus, reflect very weakly potent acyclic analogues.

In our view, it is not surprising that the acyclic analogues we have synthesized are very weak or completely inactive in our assay system. Recent evidence of the gene for MCH in the rat has revealed that the sequence of the peptide is very well conserved in the 5-14 region; the only exception being a substitution of an Ile for Val in the seventh position (15). This evidence not only points to the importance of the disulfide bridge, but, even more importantly, the surprising conservation of this sequence may imply a functionality for MCH in mammals as well. NMR studies of MCH in our laboratory have found a surprising amount of apparently stable secondary structure as well as some evidence for tertiary structure (12).

Acknowledgements

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