Design of a New Class of Superpotent Cyclic α-Melanotropins Based on Quenched Dynamic Simulations

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Abstract: A new highly potent, receptor-selective and prolonged-acting cyclic lactam analogue of α-melanotropin (α-MSH) has been designed and synthesized. Molecular dynamics simulations and molecular mechanics calculations were used in conjunction with results from previous conformational structure–biological activity studies to design a new class of linear and cyclic α-melanotropin (α-MSH) analogues. Examination of these properties of α-MSH and [Nle^4,D-Phe^7]α-MSH led to the design of the potent linear fragment analogue Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$, in which the Gly^{10} residue of α-MSH$_{4-10}$ was replaced by Lys^{10} as the major novel change from previous investigations. This in turn led to the synthesis of the cyclic lactam analogue Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$, which was exceptionally potent in the lizard skin (90 times that of α-MSH) and mammalian melanoma tyrosinase (100 times that of α-MSH) assays and in addition exhibited prolonged biological activity.

α-Melanotropin (α-MSH, α-melanocyte stimulating hormone, **1**) is a linear tridecapeptide, Ac-Ser^1-Tyr^2-Ser^3-Met^4-Glu^5-His^6-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH$_2$,[2] with peripheral biological effects related to pigmentation[3] and a number of putative central nervous system (CNS) effects[4] as well. Extensive structure–biological activity studies have been made especially with regard to its pigmentary (melanotropic) activities[5] by use of the classical frog (*Rana pipiens*) skin bioassay. These studies have led to the design of superpotent (up to 60 times that of α-MSH), ultraprolonged-acting (hours in vitro, days in vivo) analogues such as [Nle^4,D-Phe^7]α-MSH (**2**).[5,6] Further conformational, structural, and model building studies led to the design of the cyclic pseudoisosteric analogue of α-MSH, [Cys^4,Cys^{10}]-α-MSH (**3**), which was found to be superpotent but which lacked the prolonged biological effects of **2**.[7] Interestingly, the structure–activity relationships for analogues of **3**[5-7d,e,f] were consistent with those predicted in the frog skin bioassay system but not with the lizard (*Anolis carolinensis*) skin assay, which generally gives structure–biological activity relationships which parallel the mammalian melanoma tyrosinase assay.[5] In view of the clinical importance of using melanotropic peptides for the possible treatment of human pigmentary disorders and the detection and eradication of melanoma cancer,[5,7,8] we have sought to develop potent and prolonged-acting cyclic, conformationally constrained[9] analogues of α-melanotropin which are particularly potent and prolonged acting in the mammalian tyrosinase and lizard skin bioassays. Here we report on the successful use of molecular mechanics calculations and molecular dynamics simulations in the development of a new class of superpotent, prolonged-acting cyclic melanotropins.

Experimental Section

General Synthetic and Analytical Methods. The peptide syntheses reported here were performed by the solid-phase method[10] using procedures very similar to those previously used in the synthesis of α-melanotropin fragment analogues.[11] Protected amino acids were either purchased from Bachem (Torrance, CA) or were prepared by literature methods.[12] The syntheses were performed on a *p*-methylbenzhydrylamine (pMBHA) resin which was prepared by literature methods.[6b,12] The coupling of amino acids to the growing peptide chain was accomplished with a 3.0 M excess of the amino acid and a 2.4 M excess of dicyclohexylcarbodiimide and of *N*-hydroxybenzotriazole (HOBt). The coupling was monitored by the quantitative ninhydrin test,[13] and an additional coupling was done with a 1.5 M excess of amino acid and coupling reagents if the first coupling was not greater than 99%. The peptides were purified by reverse-phase high-pressure liquid chromatography (RP-HPLC) on a Vydac C-18 reverse-phase column with a Spectra Physics 8100 instrument. Purity of the final products was assessed by TLC in three different solvent systems, by RP-HPLC, by fast atom bombardment mass spectrometry (FAB-MS) for the cyclic analogue, and by amino acid analysis. TLC solvent systems were (A) 1-butanol/acetic acid/pyridine/water (5:5:1:4 v/v), (B) 2-propanol/25%

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(2) All amino acids are of the L configuration unless otherwise indicated. Abbreviations and symbols for amino acids and other abbrevations are in accord with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). Other abbreviations include the following: DCM, dichloromethane; α-MSH, α-melanotropin, α-melanocyte stimulating hormone; N^{α}-Boc, N^{α}-*tert*-butyloxycarbonyl; HOBt, *N*-hydroxybenzotriazole; Tos, tosyl; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; CMC, carboxymethylcellulose; DPPA, diphenylphosphoryl azide.

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aqueous ammonia/water (3:1:1 v/v), (C) ethyl acetate/pyridine/acetic acid/water (5:5:1:3 v/v), and (D) 1-butanol/acetic acid/water (4:1:5 v/v), upper phase). TLC was run on Baker 250-mm analytical silica gel-glass plates. Detection was by ninhydrin, fluorescamine, and iodine vapor.

Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$. N^{α}-Boc-Lys(N^{ϵ}-2-ClZ) (2.7 mM) was coupled to 1.35 g (0.94 nM) of pMBHA resin containing 0.7 mM/g NH_2 by the dicyclohexylcarbodiimide/*N*-hydroxylbenzotriazole method followed by N^{α}-Boc deprotection by trifluoroacetic acid (TFA) in dichloromethane (DCM) (2% anisole added) for 2 and 20 min. Following neutralization,[11] stepwise coupling and deprotection of N^{α}-Boc-Trp(N^{in}-For), N^{α}-Boc-Arg(N^{g}-Tos), N^{α}-Boc-D-Phe, N^{α}-Boc-His(N^{im}-Tos), N^{α}-Boc-Asp(*O*-Bzl), and N^{α}-Boc-Nle were accomplished by methods previously reported.[11] Then the peptide–resin was treated with TFA to remove the N^{α}-Boc protecting group on Nle, and the N-terminal amino group was acetylated with a 2-fold excess of a 1:1 mixture of acetic anhydride/pyridine in dichloromethane for 1 h. The finished protected peptide–resin, Ac-Nle-Asp(*O*-Bzl)-His(N^{im}-Tos)-D-Phe-Arg(N^{g}-Tos)-Trp(N^{in}-For)-Lys(N^{ϵ}-2-ClZ)-pMBHA resin (2.1 g), was obtained. A 1.5-g portion of the protected peptide–resin was treated with anhydrous liquid HF–anisole–1,2-dithioethane (17 mL–2 mL–1 mL) for 1 h at 0 °C. The volatile materials were evaporated off in vacuo, and the dried peptide–resin mixture was washed with 3 × 30 mL of anhydrous diethyl ether and extracted with 3 × 30 mL of 30% aqueous acetic acid. The aqueous extract was lyophilized to give 370 mg of a white powder, half of which was dissolved in 1.5 mL of NH_4OAc acetate buffer (pH 4.5), filtered through a cartridge filter to the top of the CMC column (2.0 × 30.0 cm), and eluted with 250 mL each of 0.01 (pH 4.5), 0.10 (pH 6.8) and 0.2 M (pH 6.8) NH_4OAc. The major peak (280 nm) was eluted between the end of the 0.1 M and the first half of the 0.2 M NH_4OAc fractions and was lyophilized to give 112 mg of the title peptide. This compound was purified by preparative RP-HPLC on a Vydac 218TP15-16 C_{18} RP column (25 mm × 25 cm) using a gradient of CH_3CN/0.1% aqueous TFA (20:80 to 50:50 over 20 min) at a flow rate of 3 mL/min. There was obtained 64 mg of the title compound: $[\alpha]^{23}_{540}$ −28.0° (*c* = 0.05, 10% HOAc); TLC (A) R_f 0.36, (B) 0.40, (D) R_f 0.07; amino acid analysis Nle 1.08 (1.0), Asp 1.06 (1.0), His 0.90 (1.0), Phe 0.97 (1.0), Arg 1.00 (1.0), Trp 0.90 (1.0), Lys 0.93 (1.0); HPLC *K'* 2.25 (15% CH_3CN–85% 0.1% aqueous TFA for 5 min; then a gradient to 45% CH_3CN–55% 0.1% aqueous TFA for 15 min).

Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$. A 40-mg sample of pure Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH_2 was dissolved in 3 mL of dry, purified (vacuum distillation from ninhydrin) *N*,*N*-dimethylformamide. To the peptide solution was added 120 mg of anhydrous K_2HPO_4. The mixture was cooled to 0 °C in an ice–salt water bath, and 17 μL of diphenylphosphoryl azide (DPPA) was added.[14] The mixture was stirred at 0 °C for 1 h and then at 12 °C in a cold room overnight, and the cyclization reaction was followed by RP-HPLC. After completion of the cyclization (about 24 h), the reaction was stopped with 10% aqueous acetic acid and desalted on a P_4 polyacrylamide column (80 × 1.0 cm) with 30% aqueous acetic acid as the eluent solvent. Final purification was by RP-HPLC on the 25 mm × 25 cm column used above. With the same procedure as before, there was obtained 12 mg of the cyclic title compound: $[\alpha]^{23}_{546}$ −13.3° (*c* = 0.015, 10% HOAc); TLC (A) R_f 0.89, (C) R_f 0.65, (D) R_f 0.18; HPLC *K'* 5.87 (see above for conditions); FAB-MS, found 1040 (calcd 1040.1).

Energy Calculations and Dynamic Simulation. The molecular dynamics trajectory calculations as well as the subsequent energy minimization were carried out with the CHARMM program.[15] The empirical energy functions used to obtain the energy-minimized structures included harmonic potential energy terms for bond angles, bond lengths, bond dihedral angles, and improper dihedral angles, van der Waal and electrostatic terms for nonbonded interactions, and a hydrogen-bond potential. The computation methodology and the general form of the potential functions correspond to those described previously.[15]

Biological Assay Methods. The frog and lizard skin bioassays were determined by the ability of the melanotropin peptides to stimulate melanosome dispersion in the frog and lizard in vitro assay systems as previously described[16-18] with photometric reflectance measurements. Prolongation was measured by continued monitoring of the melanosome dispersion for 4 h *following removal of the peptide in the bathing solution and thorough washing of the skin.* The frogs (*R. pipiens*) used in these studies were obtained from Kons Scientific (Germantown, WI), and the lizards (*A. carolinensis*) were from the Snake Farm (La Place, LA).

In the mouse melanoma tyrosinase assay, the activity of tyrosinase from Cloudman S-91 mouse melanoma cells following stimulation by the melanotropin peptide was determined by the generation of tritiated water from ^{3}H-labeled L-tyrosine. The details of the methods used for this assay and for measurement of prolongation in this assay have been previously given.[7e,19]

Results and Discussion

A few computational studies of α-MSH, β-MSH, and some of its linear and cyclic analogues have been carried out previously by rigid geometry minimization techniques.[20-23] In the studies of α-MSH the full calculations were limited to fragments only, which limits the conformational possibilities for the full structures. To the best of our knowledge, no computational studies have been made on the dynamic properties of α-melanotropin, nor has the effect of D-Phe^7 substitution on the conformational and dynamic properties been examined before the studies reported here. However, a number of other reports have appeared (reviewed by Hagler[24]) in which molecular mechanics computations, and in some cases associated molecular dynamics calculations, have been made in an effort to relate calculated low-energy conformations to biological activities. These include extensive calculations on peptides which release growth hormone,[25] on enkephalins and related opiate peptides,[26] and on gonadotropin-releasing hormone (GnRH) and related peptides[27] and other peptides.[25,27a] These previous studies have indicated that conformational calculations when used in conjunction with known structure–biological activity relationships can provide useful insights for further structure–activity studies.

Simulation Modeling. Molecular dynamics simulations are capable of sampling conformations and configurations for a wide variety of complicated molecular systems.[28] In particular, the use of simulated annealing or the so-called "quenched dynamics" methods have been shown to be very useful in exploring the types of inherent structures that a given system may possess.[29] To apply this technique to the problem of peptide conformation, one runs a trajectory on either a solvent-modified or vacuum potential surface for a given period of time. Subsequently, one takes coordinate samples from the trajectory at various intervals and subjects each of them to an exhaustive energy minimization. The

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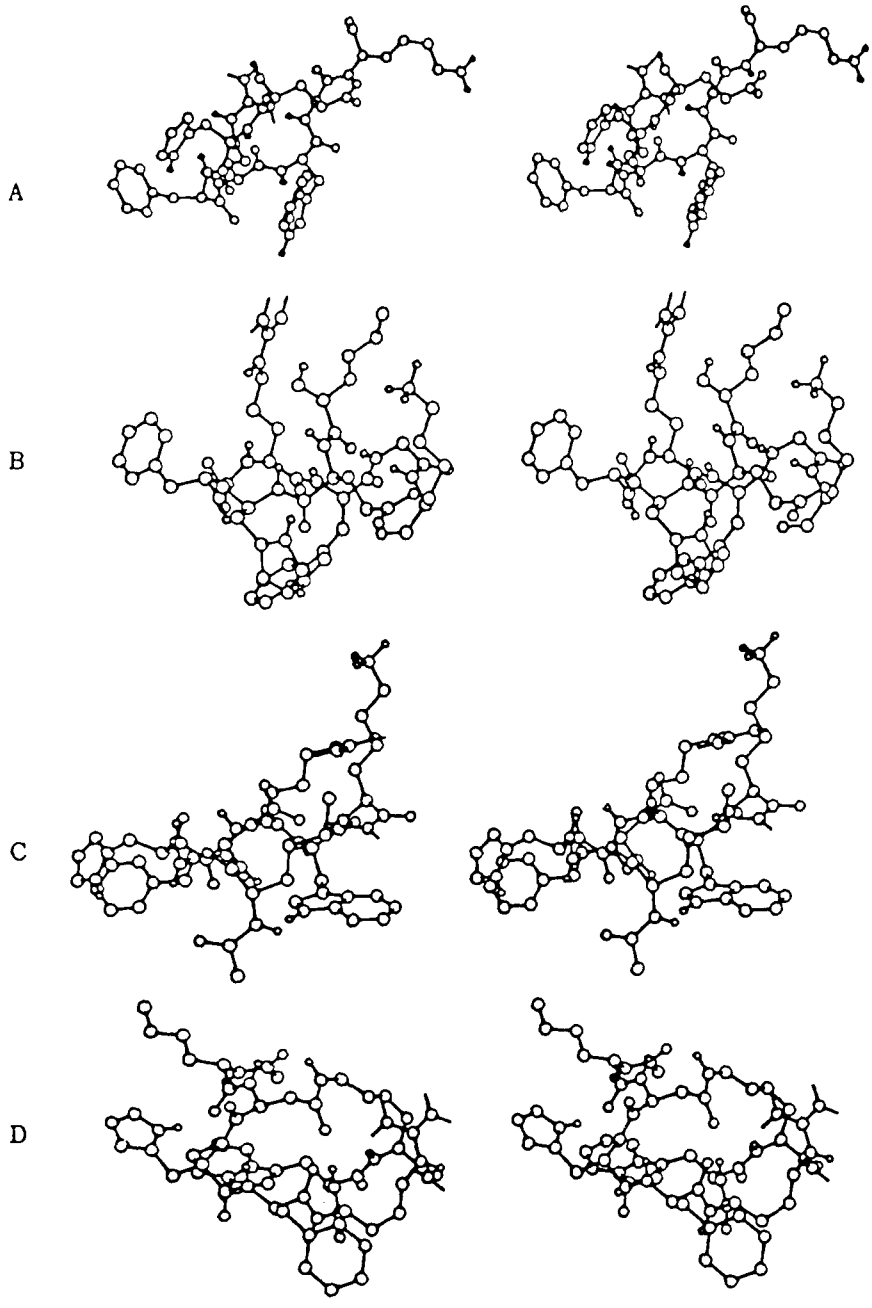

Figure 1. Stereoviews of prototypical energy-minimized conformations resulting from structures obtained during molecular dynamics simulations (see text for explanation) of (A) α-MSH (**1**) (only residues 5–11 are shown for clarity) and (B) [Nle^4,D-Phe^7]α-MSH (**2**) (only residues 4–11 are shown for clarity), (C) Ac-[Nle$_4$,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$ (**5**), and (D) Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$ (**6**). The figure is in cross-eyed stereo.

resulting structures can then be assigned to categories based on energy and shape.

For this study, molecular dynamics simulations were performed on both α-MSH and [Nle^4,D-Phe^7]α-MSH. The temperature used was 300 K, and to include some partial solvent effects, a dielectric constant of 80 was incorporated. No explicit use of model solvent molecules was deemed necessary. The program used for the trajectory calculations as well as the subsequent minimizations was CHARMM.[15] The potential functions were essentially those found in the CHARMM library for models containing polar hydrogens, with only small modifications added to reproduce unnatural but simple amino acid residues such as Nle (straight chain alkane) and D-Phe (a simple stereoisomer of an enantiomeric pair).

In the absence of any preconceived bias, the simulations were started with an energy-minimized "all-trans" conformation (ϕ, Ψ, ω, λ = 180°) in both cases.[30] Structures along the dynamics trajectory of 20 ps in length were energy minimized at 1-ps intervals, according to the adopted basis Newton–Raphson minimization algorithm.[15] The set of structures obtained were examined in detail for their conformational features, energies, and hydrogen-bonding patterns before and after the energy minimizations. Not all of the structures so obtained were relevant as several were caught in very high energy positions far from the lowest minimum obtained and therefore are less likely as representative structures.[29] Interestingly, it was noticed that α-MSH and its Nle^4, D-Phe^7 analogue both assumed folded conformations within a few picoseconds after the dynamic simulation began. A typical quenched low-energy conformation for **1** (only residues 5–11 are shown for clarity) and **2** (only residues 4–11 are shown

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Table I. Relative Biological Potencies of α-Melanotropin (α-MSH) and Related Analogues[a]

melanotropin	bioassay systems: frog skin	lizard skin	melanoma tyrosinase
(1) α-MSH	1.0	1.0	1.0
(2) [Nle^4,D-Phe^7]α-MSH[b]	60.0[d]	5.0[d]	100.0[d]
(3) [Cys^4,Cys^{10}]α-MSH[c]	20.0[e]	4.0	1.0
(5) Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$	1.0	5.0	
(6) Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$	0.5[d]	90.0[d]	100.0[d]

[a] All potencies are reported relative to α-MSH = 1.0 in the assay systems indicated. [b] The bioactivities reported here are from ref 6a, 7e, and 19. [c] The bioactivities reported here are from ref 7a and 19. [d] Prolonged biological activity for this analogue in this assay. All other analogues were not prolonged acting. [e] The minimum effective potency was 100–1000 times that of α-MSH.

for clarity) is shown in Figure 1 (panels A and B, respectively) in stereoview.

Use of Simulations for Design of New Analogues. Several features of the conformational properties of the α-melanotropin structures were particularly evident upon examination of the structures along the dynamic trajectory and in the corresponding energy-minimized structures. First, both **1** and **2** rapidly formed folded conformations, and the ϕ,Ψ backbone angles for residues -His-L(or D)-Phe-Arg-Trp- often were in C_7 or β-turn conformational space. Second, it was noticed that lipophilic or aromatic side-chain groups, especially for L- or D-Phe^7, Trp^9, and His^6, tended to segregate on one face of the molecule, while the side chains of the hydrophilic residues, Glu^5, Arg^8, and Lys^{11}, segregated on the other face of the structure. A somewhat different amphiphilic-like structure was recognized in a series of enkephalin analogues recently.[31] Third, it was noted further that despite the location of the Glu^5 and Lys^{11} side-chain groups on the same side of the molecule their charged groups generally were not close enough for a strong Coulomb interaction. However, it could be seen that if the Lys^{11} side chain was moved to the Gly^{10} position, strong interactions would be much more probable.

To test this hypothesis, we generated the [Nle^4,D-Phe^7]α-MSH$_{4-10}$ analogue in which position 5 contained a Glu^5 (native hormone) or Asp^5 residue, and position 10 a Lys^{10} residue (instead of Gly^{10}) to give the fragment analogues Ac-[Nle^4,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$ (**4**) and Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$ (**5**). Quenched molecular dynamics simulations were again performed on these structures, and indeed, as seen in Figure 1C for **5** (and also for the Glu^5 analogue—not shown), folded conformations were obtained in which the Asp^5 (or Glu^5) side-chain carboxylate group was in close proximity to the Lys^{10} side-chain ε-amino group, and a reverse turn type conformation was obtained. We then synthesized[11] compound **5**, and as seen in Table I we found that a potent fragment analogue of α-MSH was obtained which showed higher relative potency in the lizard skin assay than in the frog skin assay.

It occurred to us that if, indeed, a favored conformation for **5** would place the Asp^5 carboxylate and Lys^{10} ε-amino group in close proximity, they should easily form the cyclic lactam-bridged structure Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$ (**6**). Indeed, this analogue was readily prepared by treatment of the linear peptide **5** with DPPA[14] in DMF followed by purification by preparative reverse-phase high-pressure liquid chromatography (see Experimental Section). Quenched molecular dynamics simulation gave the structure shown in Figure 1D. One must be very cautious at this stage in examining the conformational properties of the cyclic analogue **6** and the related linear peptide **5** and conformations observed along the dynamic trajectory such as those of α-MSH (**1**) and [Nle^4,D-Phe^7]α-MSH (**2**) (panels A and B, respectively, in Figure 1). It is interesting to note that in all of these cases the presence of reverse turn structures and the segregation of lipophilic and hydrophilic side-chain residues on different faces of the peptides are retained. Most interestingly, the cyclic analogue **6** obtained was found to be superpotent in both the lizard skin and tyrosinase assays (Table I) but had low potency at the frog skin assay. Furthermore, analogue **6** had prolonged biological activity in all of the assays (not shown). Thus we have derived a new class of cyclic α-melanotropin analogues with very high potency and prolonged biological activity in several melanotropin systems. It is interesting to note that **6** possesses only 7 amino acid residues whereas α-MSH has 13.

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