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4-Norleucine, 7-D-phenylalanine-α-melanocyte-stimulating hormone: A highly potent α-melanotropin with ultralong biological activity

(amino acid racemization/peptide degradation/adenylate cyclase/tyrosinase/melanoma)

TOMI K. SAWYER*, PAULINE J. SANFILIPPO*, VICTOR J. HRUBY*†, MICHAEL H. ENGEL‡, CHRISTOPHER B. HEWARD§, JEAN B. BURNETT§¶, AND MAC E. HADLEY§

*Department of Chemistry and Biochemistry; ‡Laboratory of Organic Geochemistry, Department of Geosciences; and §Department of General Biology, University of Arizona, Tucson, Arizona 85721

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ABSTRACT **α-Melanocyte-stimulating hormone (α-MSH) reversibly darkens frog skins by stimulating melanosome movement (dispersion) within melanophores. Heat–alkali treatment of α-MSH results in prolonged biological activity of the hormone. Quantitative gas chromatographic analysis of the hydrolyzed heat–alkali-treated peptide revealed partial racemization particularly at the 4 (methionine) and 7 (phenylalanine) positions. [Nle⁴]-α-MSH, a synthetic analogue of α-MSH, reversibly darkens frog skins and also exhibits prolonged activity after heat–alkali treatment. Synthesis of [Nle⁴, D-Phe⁷]-α-MSH provided an analogue with prolonged biological activity, identical to that observed with heat–alkali-treated α-MSH or [Nle⁴]-α-MSH. [Nle⁴, D-Phe⁷]-α-MSH was resistant to enzymatic degradation by serum enzymes. In addition, this peptide exhibited dramatically increased biological activity as determined by frog skin bioassay, activation of mouse melanoma adenylate cyclase, and stimulation of mouse melanoma cell tyrosinase activity. This Nle⁴, D-Phe⁷ synthetic analogue of α-MSH is a very potent melanotropin, 26 times as potent as α-MSH in the adenylate cyclase assay. The resistance of the peptide to enzymatic degradation and its extraordinarily potent and prolonged biological activity should make this analogue of α-MSH an important molecular probe for studying the melanotropin receptors of both normal and abnormal (melanoma) melanocytes.**

α-Melanotropin (α-MSH, α-melanocyte-stimulating hormone) is a tridecapeptide (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) that is synthesized and secreted by the pars intermedia of the vertebrate pituitary (1). The amino acid residues that are important in the expression of melanotropic activity have been elucidated through systematic structure–function investigations of α-MSH and α-MSH fragments on amphibian melanophores (2, 3) and, to a lesser extent, on mammalian melanoma cells (4–6). Very little information is available, however, regarding the stereochemical and conformational correlates of biological activity in either of these two biological systems.

Earlier reports have shown that heat–alkali treatment of crude or purified preparations of naturally occurring α-MSH produces a partially racemized product with altered activity on amphibian melanophores both *in vivo* and *in vitro*. Such changes in biological effects have been discussed in terms of "potentiation," "prolongation," and "retardation" (7–12). Although the precise biochemical mechanism by which these unusual biological properties were produced is unknown, it appeared possible that synthetic stereostructural tailoring of α-MSH might produce an analogue that would also possess these properties. Utilizing a high-resolution gas chromatographic method to localize and quantitate specific sites of racemization within the primary sequences of peptides, we obtained additional evidence which suggested that stereochemical substitution at position 7 (replacement of L-phenylalanine by D-phenylalanine) of α-MSH or [Nle⁴]-α-MSH would provide an analogue with the desired biological properties. Previous investigations have shown that [Nle⁴]-α-MSH is more potent than α-MSH on both amphibian melanophores (2, 6) and on stimulating melanoma adenylate cyclase (6, 13), and it is also resistant to inactivation by chloramine-T (14, 15), an oxidant used in peptide iodination. Because heat–alkali treatment of this analogue also resulted in "potentiation," "prolongation," and "retardation," it was clear that alteration of the methionine residue was not a requirement for the expression of these properties. Thus, it was decided to retain the benefits of the norleucine substitution in position 4 in the synthesis of the "definitive" peptide.

We report here the synthesis of [Nle⁴, D-Phe⁷]-α-MSH and present data demonstrating its unique biological properties. These include prolonged biological activity, enhanced potency relative to α-MSH in a number of biological systems, and resistance to degradation by serum enzymes. The biological properties of this analogue provide insights into the structure–activity relationships of the melanotropins.

MATERIALS AND METHODS

The amino acids used were of the L configuration unless otherwise stated. N^{α}-*tert*-butyloxycarbonyl (N^{α}-Boc) amino acids and amino acid derivatives were purchased from Vega Biochemical (Tucson, AZ) or from Biosynthetica (Oberdorf, Switzerland), or were prepared by published procedures. The [Nle⁴]-α-MSH was purchased from Penninsula Laboratories (San Carlos, CA), or was prepared in our laboratories (15; unpublished).

Solid-Phase Peptide Synthesis. The α-MSH was prepared as described (16). [Nle⁴, D-Phe⁷]-α-MSH was synthesized by using a *p*-methylbenzhydrylamine resin (17). The method for the preparation of this resin from beaded polystyrene/1% divinylbenzene was identical to published procedures for the preparation of benzhydrylamine resin (18), except for the substitution of *p*-toluoyl chloride for benzoyl chloride in the intermediate step of ketone resin formation. Amino acids were coupled successively as their N^{α}-Boc derivatives. Reactive amino acid side-chains were protected as follows: serine, *O*-benzyl; tyrosine, *O*-2,6-dichlorobenzyl; glutamic acid, γ-benzyl

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Abbreviations: α-MSH, α-melanotropin, α-melanocyte-stimulating hormone; Nle, norleucine; N^{α}-Boc, N^{α}-*tert*-butyloxycarbonyl; 1-BuOH, 1-butanol; Pyr, pyridine.

† To whom reprint requests should be addressed.
¶ Present address: Department of Biomechanics, Michigan State University, East Lansing, MI 48824.

Table 1. Analytical properties of α-MSH analogues

Residue in position 4	Residue in position 7	Physicochemical properties: Thin-layer chromatography R_F in various systems A	B	C	D	$[\alpha]^{25}_{546}$ in 10% AcOH, degrees
Met	L-Phe	0.24	0.58	0.72	0.78	−58.0 (c = 0.50)
Nle	L-Phe	0.20	0.70	0.69	ND	−45.5 (c = 0.44)
Nle	D-Phe	0.24	0.79	0.74	0.72	−59.7 (c = 0.49)

		Amino acid analyses, mol/mol of analogue: Trp	Lys	His	Arg	Ser	Glu	Pro	Gly	Val	Met	Nle	Tyr	Phe
Met	L-Phe	0.90	1.04	0.90	0.98	1.72	1.00	1.08	1.08	1.07	0.97	—	0.92	1.06
Nle	L-Phe	0.85	1.04	0.99	1.04	1.58	0.95	0.97	0.92	0.87	—	0.93	1.05	1.10
Nle	D-Phe	0.93	1.07	1.00	1.01	1.75	1.00	1.06	1.07	1.00	—	0.91	0.87	0.96

c, Concentration, g/100 ml; ND, not determined.

ester; lysine, N^{ϵ}-2,4-dichlorobenzyloxycarbonyl; arginine, N^{g}-*p*-toluenesulfonyl; histidine, N^{im}-*p*-toluenesulfonyl; tryptophan, N^{i}-formyl. The coupling reactions in the solid phase syntheses were achieved with a 3-fold excess of N^{α}-Boc amino acid and a 2.4-fold excess of dicyclohexylcarbodiimide. Removal of the N^{α}-Boc protection at each step was effected by treatment (2 and 20 min) with 45% (vol/vol) trifluoroacetic acid in CH_2Cl_2 containing 2% (vol/vol) anisole. Other solid-phase procedures were similar to those used in the synthesis of α-MSH (16). After all of the amino acid residues had been coupled to the resin, the amino terminus of the peptide-resin was acetylated with *N*-acetylimidazole. The protected peptide was cleaved from the resin and all protecting groups were removed (except the N^{i}-formyl group of tryptophan) by treatment (30 min, 0°C) with anhydrous HF containing 16% anisole and 0.5% 1,2-ethanedithiol. The formylated (N^{i}-formyl-Trp) peptide was deformylated (19) by adding 4 M NaOH to pH 11.5 (3 min), and the reaction was terminated by addition of glacial acetic acid to a final pH of 4.5.

Purification and Homogeneity. The crude [Nle^4, D-Phe^7]-α-MSH was purified by ion-exchange chromatography on a carboxymethyl-cellulose column (2.0 × 18.0 cm) using a discontinuous gradient of 250 ml of 0.01 M ammonium acetate (pH 4.5) and then 250 ml of each of 0.1 M, 0.2 M, and 0.4 M ammonium acetate (pH 6.8). The major peak (280-nm absorbance detection) occurred during the 0.1 M ammonium acetate elution. On the basis of the starting N^{α}-Boc-Val-*p*-methylbenzhydrylamine resin, the overall yield of purified [Nle^4, D-Phe^7]-α-MSH was 25%.

The homogeneity of the peptides tested was demonstrated by thin-layer chromatography on silica gel plates using the following solvent systems (all vol/vol) (1-BuOH, 1-butanol; Pyr, pyridine): A, upper phase of 1-BuOH/HOAc/H_2O, 4:1:5; B, 1-BuOH/HOAc/Pyr/H_2O, 15:3:10:12, C, 1-BuOH/Pyr/HOAc/H_2O, 6:6:1.2:4.8; D, 2-propanol/25% aqueous NH_3/H_2O, 3:1:1. Optical rotation values were measured at the mercury green line (546 nm) in a Perkin–Elmer 241 MC polarimeter. Amino acid analyses were performed on a Beckman 120C amino acid analyzer after hydrolysis (100°C, 22 hr) in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole. No corrections were made for destruction of amino acids during hydrolysis. Analytical results for all peptides are shown in Table 1.

Table 2. Amino acid D/L ratios of α-MSH analogues

Residue in position 4	Heat–alkali treatment	D/L ratio*: Glu	Pro	Val	Met	Nle	Phe
Met	No†	0.022*	0.031	0.018	0.010	—	0.023
Met	Yes	0.078	0.027	0.033	0.230	—	0.219
Nle	No†	0.022	0.019	0.015	—	0.021	0.026
Nle	Yes	0.096	0.022	0.034	—	0.043	0.281

* D/L ratios are shown as a mean of at least three gas chromatographic determinations, and the SEM for each D/L ratio is less than 0.01.

† The low D/L ratios reported for the samples that were not heated in 0.1 M NaOH are primarily the result of (*i*) slight racemization during acid hydrolysis and (*ii*) (−)-2-butanol impurity present in the (+)-2-butanol used for esterification. D/L ratios reported for the heated samples have not been corrected for this contribution.

Quantitation of Heat–Alkali-Catalyzed Racemization. Gas chromatographic determination of the extent of racemization of heat–alkali-treated α-MSH and [Nle^4]-α-MSH was adopted from procedures previously described (20–22) with some modifications.

Heat–alkali treatment of α-MSH and [Nle^4]-α-MSH was accomplished by heating the peptides (100°C, 10 min) in 0.1 M NaOH in sealed tubes. The samples were then hydrolyzed in 6 M HCl (100°C, 24 hr) and desalted by cation-exchange chromatography (Bio-Rad AG 50-X8). The purified amino acid residues were esterified with 2–4 M (+)-2-butanol/HCl and acylated with pentafluoropropionic anhydride. The resultant amino acid diastereomeric derivatives were separated on a Carbowax-20 M nickel (wall coated open tubular) capillary column (60 m × 0.5 mm). The D/L ratios of the amino acids investigated were determined by an Infotronics CRS-208 digital integrator coupled to the Perkin–Elmer F-11 gas chromatograph and are given in Table 2. Standards consisted of duplicate samples of α-MSH and [Nle^4]-α-MSH that were not subjected to the heat-alkali treatment but were hydrolyzed, derivatized, and chromatographed by the above procedure.

Frog-Skin Bioassay. The α-MSH analogues were compared with respect to their ability to stimulate melanosome dispersion *in vitro* using the frog (*Rana pipiens*) skin bioassay as described (23–25).

Tyrosinase Activity. Cloudman S-91 NCTC 3960 (CCL 53.1) melanoma cells were obtained from the American Type Culture Collection Cell Repository and were grown and maintained as described (26). Melanoma cells (2×10^5) were seeded in 25-cm^2 flasks and allowed to attach overnight. At time $t = 0$, the medium in all the flasks was replaced with 4 ml of medium containing 0.1 μM melanotropin (i.e., α-MSH, [Nle^4]-α-MSH, or [Nle^4, D-Phe^7]-α-MSH) or medium containing no melanotropin (controls). At selected time intervals (t = 0.5, 4, and 8 hr) control medium and melanotropin-containing medium was removed. The cells were carefully rinsed three times with fresh melanotropin-free medium and then exposed to fresh melanotropin-free medium containing [^{3}H]-tyrosine (specific activity 48 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) at 1 μCi/ml. After 24 hr, this medium was removed and the tyrosinase activity of the melanoma cells was determined by assaying for the ^3H_2O released from the [^{3}H]tyrosine by the action of tyrosinase.

Adenylate Cyclase Activity. Adenylate cyclase activity of the particulate membrane fraction isolated from Cloudman S-91 mouse melanoma tumors was determined by assaying [α-^{32}P]ATP conversion to [^{32}P]cAMP as described (6, 13). [^{32}P]cAMP was isolated, purified, and detected according to the method of Salomon *et al.* (27).

RESULTS

Both synthetic α-MSH and [Nle^4]-α-MSH amino acid constituents were partially racemized in 0.1 M NaOH (100°C, 10 min). With respect to their melanophore-dispersing activity on frog skins, α-MSH, [Nle^4]-α-MSH, heat–alkali-treated α-MSH, and heat-alkali-treated [Nle^4]-α-MSH all showed similar biological activities (Fig. 1). The frog skin darkening effect of α-MSH and [Nle^4]-α-MSH could be rapidly reversed by simply rinsing the frog skins with fresh media and then placing them in fresh media without added hormone (Fig. 1 *A* and *B*). However, the effect of the heat–alkali-treated peptides *could not be reversed* by this method even after several washings over a period of many hours (Fig. 1 *A* and *B*). This "prolongation" effect was identical for the partially racemized samples of both α-MSH and [Nle^4]-MSH.

Using quantitative gas chromatographic methods, we examined the D/L ratios for amino acid residues in heat–alkali-treated α-MSH and [Nle^4]-α-MSH to determine the sites and extent of racemization in these analogues. The results for some of the amino acid residues are given in Table 2. The methionine-4 residue in α-MSH was racemized to a significant extent on heat–alkali treatment of the hormone, while the norleucine-4 residue in [Nle^4]-α-MSH was only slightly racemized by this treatment. This confirmed our expectation that racemization at position 4 in the native hormone, though substantial, was not responsible for the heat–alkali-induced changes in biological activity. Of particular interest was the substantial racemization of the phenylalanine-7 residue in both α-MSH and [Nle^4]-α-MSH (Table 2). The ratios of % D-phenylalanine to D-glutamic acid were 2.48 and 2.50 for heat–alkali-treated α-MSH and [Nle^4]-α-MSH, respectively. Previous racemization studies of peptides and proteins (22, 28–30) generally have revealed % D-phenylalanine to % D-glutamic acid ratios of approximately 1.

The preceding results and the earlier studies (10–12) led us to postulate that preparation of a diastereoisomeric analogue of α-MSH with a D-phenylalanine-7 residue would provide a peptide with biological properties similar to those of the heat–alkali-treated peptides. We, therefore, synthesized [Nle^4, D-Phe^7]-α-MSH, which was found to incorporate both the higher melanotropic potency found for [Nle^4]-α-MSH, and the prolonged *in vitro* biological activity observed for heat–alkali-treated α-MSH and [Nle^4]-α-MSH. The frog skin melanophore-dispersing activity of [Nle^4, D-Phe^7]-α-MSH is shown in Fig. 1*C*. Under these experimental conditions this analogue has about the same apparent potency as [Nle^4]-α-MSH. However, repeated rinsing of the frog skins treated with [Nle^4, D-Phe^7]-α-MSH did not lead to skin lightening as in the case of α-MSH (Fig. 1*A*) or [Nle^4]-α-MSH (Fig. 1 *B* and *C*). Instead, the frog skins remained darkened (Fig. 1*C*) for many hours (>24 hr) even after repeated rinsing of the skins. The remarkably prolonged (apparently irreversible) biological activity of [Nle^4, D-Phe^7]-α-MSH suggests that the prolonged biological effects observed for heat–alkali-treated α-MSH and [Nle^4]-α-MSH on amphibian melanophores may be due, in part, or solely, to racemization of phenylalanine at position 7 of the peptide. In this regard, it is interesting to note that in earlier studies Schnabel and Li reported (31) that the D-Phe analogue of α-MSH-(6–10), His-D-Phe-Arg-Trp-Gly, was more potent than the all-L α-MSH-(6–10), and Nakamura *et al.* (32) found that [β-Ala^1, D-Phe^7, Orn^{15}]ACTH-(1–18)-NH$_2$ possessed higher melanotropic activity than native α-MSH. However, neither compound was shown to possess prolonged melanotropic activity.

We were especially interested in utilizing [Nle^4, D-Phe^7]-α-MSH and related analogues to study mammalian melanocytes both *in vitro* and *in vivo*. Because the analogues would likely be exposed to proteolytic enzymes under various assay conditions, the stability of [Nle^4, D-Phe^7]-α-MSH in culture media used to maintain melanoma cells was investigated. The relative stabilities of the diastereoisomeric analogue [Nle^4, D-Phe^7]-α-MSH and of α-MSH and [Nle^4]-α-MSH over a 72-hr period are shown in Fig. 2. Over this time period essentially all of the biological activity of both α-MSH and [Nle^4]-α-MSH was lost, suggesting extensive proteolytic inactivation of these peptides. However, the diastereoisomeric analogue was completely resistant to this loss of activity, implying that it was not degradable under these conditions. This stability suggests that the compound might prove to be particularly useful in studying biological systems both *in vitro* and *in vivo*.

Next, the ability of [Nle^4, D-Phe^7]-α-MSH to stimulate melanoma adenylate cyclase activity was investigated. This seemed relevant because we (6) and others (4) have observed differences in structure–function relationships of melanotropin receptors in normal and transformed cell types. The results (Fig. 3) show that [Nle^4, D-Phe^7]-α-MSH is much more potent than α-MSH in this assay system and is by far the most potent

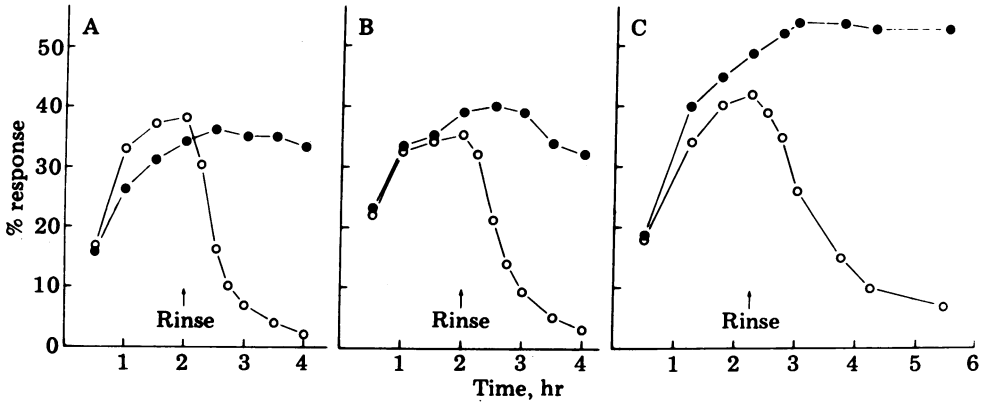

FIG. 1. *In vitro* demonstration of the effect of heat–alkali treatment on the biological activity of melanotropins. (*A*) α-MSH, before (○) and after (●) treatment; (*B*) [Nle^4]-α-MSH, before (○) and after (●) treatment. (*C*) Synthetic [Nle^4, D-Phe^7]-α-MSH (●) also exhibited prolonged biological action identical to that observed for heat–alkali-treated α-MSH (*A*) and [Nle^4]-α-MSH. (*B*); ○, [Nle^4]-α-MSH control. Values represent the mean darkening response of frog skins (n = 7 for each point) to each of the peptides (0.1 nM).

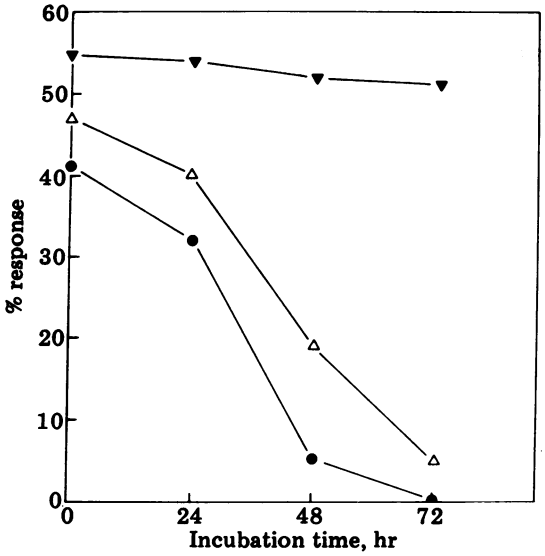

FIG. 2. Demonstration of the stability of [Nle^4, D-Phe^7]-α-MSH in tissue culture media under incubation conditions (37°C). Synthetic α-MSH (●), [Nle^4]-α-MSH (△), and [Nle^4, D-Phe^7]-α-MSH (▼) were incubated under sterile conditions in Corning flasks containing Ham's F10 medium containing 10% horse serum and 2% fetal calf serum. Samples of the medium containing the peptides (10 nM) were removed at time zero and at 24, 48, and 72 hr. The samples were immediately frozen and then assayed (frog skin darkening) for biological activity. Each value represents the mean darkening response of the skins (n = 6 per point) to the peptide-containing solutions.

α-MSH analogue we have studied, being about 26 times more potent than α-MSH. However, the diastereoisomer exhibited the same maximal adenylate cyclase stimulation as α-MSH.

Previous studies have demonstrated that α-MSH stimulates tyrosinase activity in melanoma cells in tissue culture (33). The results of exposure of melanoma cells to α-MSH, [Nle^4]-α-MSH, and [Nle^4, D-Phe^7]-α-MSH are shown in Fig. 4. α-MSH and [Nle^4]-α-MSH have similar activities in this assay system. However, [Nle^4, D-Phe^7]-α-MSH is much more active than the native hormone or its norleucine-4 analogue. The results of the frog skin, melanoma adenylate cyclase, and tyrosinase assays suggest that both amphibian and mammalian cell receptors for α-MSH recognize the specific stereostructural characteristics of the D-phenylalanine-7 analogue in a similar manner.

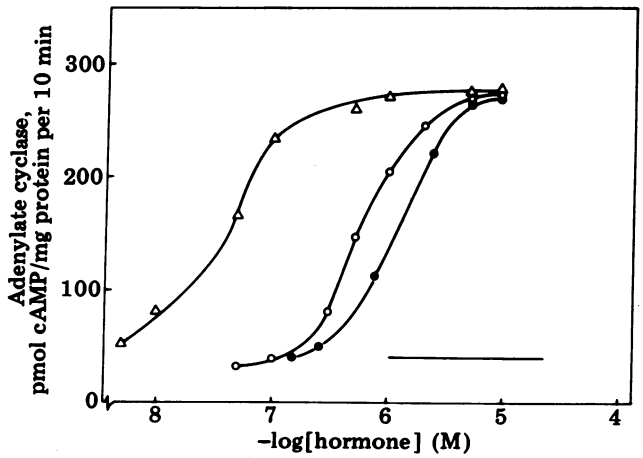

FIG. 3. Dose–response curves of α-MSH (●), [Nle^4]-α-MSH (○), and [Nle^4, D-Phe^7]-α-MSH (△) in the melanoma adenylate cyclase assay. —, Basal activity. All results were determined using 100 μg of protein per assay under standard conditions. Each point represents the mean value of triplicate determinations, and all SEMs were less than 5%.

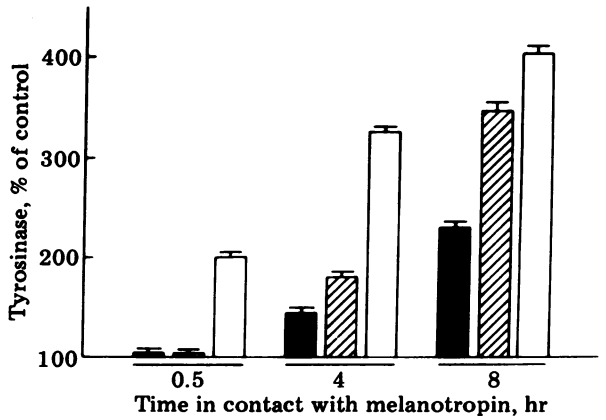

FIG. 4. Effects of α-MSH (filled bars), [Nle^4]-α-MSH (hatched bars), and [Nle^4, D-Phe^7]-α-MSH (empty bars) on the tyrosinase activity in cultured mammalian melanoma cells. Tyrosinase activities are expressed as percent activity of control cells (i.e., cells *not* exposed to melanotropin). Each bar represents the mean of four determinations ± SEM.

DISCUSSION

Potentiation of the biological effects of heat–alkali treatment of the melanotropic principle(s) of the pituitary gland were noted as early as 1924 (7), and subsequent investigations suggested that this treatment led to partial racemization of certain amino acid residues of the melanotropins. By use of quantitative gas chromatographic methods we were able to confirm the specific sites and quantitate the extent of racemization within the α-MSH structure. In α-MSH both methionine-4 and phenylalanine-7 were highly racemized. Interestingly, the norleucine-4 was not significantly racemized in [Nle^4]-α-MSH, but the extent of racemization at the other amino acid positions remained the same as in α-MSH.

Although the extent of racemization of phenylalanine-7 relative to other amino acids such as glutamic acid is unusual, it was not surprising considering the wide variety of parameters that appear to affect amino acid racemization rates in peptides. These rates are controlled by the position of an amino acid residue in a peptide chain, the nature of the adjacent amino acids, and structural features of the amino acid residues (which give rise to inductive, resonance, and steric effects), as well as intramolecular solvation and base action (29, 34–38). However, the extensive racemization of phenylalanine-7 led us to suspect that it might be related to the observed biological activities of the heat–alkali-treated α-MSH.

Indeed, synthetic [Nle^4, D-Phe^7]-α-MSH is a melanotropin analogue displaying the biological properties of heat–alkali-treated α-MSH (and [Nle^4]-α-MSH). This peptide exhibited prolonged biological activity in the frog assay without heat–alkali treatment. It was also discovered that the Nle^4, D-Phe^7 analogue, unlike the native α-MSH or its Nle^4 analogue, was resistant to degradative inactivation by serum enzymes. The principal route for enzymatic degradation of α-MSH that is "protected" at both its NH_2 and COOH terminals may be via an initial cleavage involving the phenylalanine residue, most likely between the phenylalanine-7 and arginine-8 residues of the peptide. Enzymatic stability may be responsible, at least in part, for the enhanced activity of the peptide in the melanoma tyrosinase assay. In regard to the ultralong action (24 hr or longer) of the peptide on frog skin darkening, it is possible that [Nle^4, D-Phe^7]-α-MSH is irreversibly bound to the receptor as a result of conformational properties of the diastereoisomer, which may be highly favorable to peptide–receptor interaction. The former explanation has been suggested to explain the potency of [D-Ala^2, Met^5]enkephalinamide on opiate receptors

(39), and the possibility of irreversible binding has been offered to explain some aspects of prolactin binding to liver membrane receptors (40). Yet a further possibility is that the analogue may have irreversibly effected a transduction signal between receptor and adenylate cyclase. Presently we have no direct evidence to support these or other possibilities. However, the cell-free melanoma adenylate cyclase response to the Nle^4, D-Phe^7 analogue clearly indicates that the increased potency is membrane related.

It is now recognized that α-MSH may function in a number of roles in mammals, including humans, in addition to its well-characterized role in color change mechanisms of poikilothermic vertebrates. α-MSH is found in the brain, and it has been suggested that it may function therein in neural mechanisms related to learning and memory; it also may have a role in fetal development (41–45). To further study the sites and mechanisms of MSH action, it will be necessary to identify and characterize the melanotropin receptor(s). Mammalian melanocytes, both normal and abnormal (melanoma) cells, respond to melanotropin. The hormone stimulates adenylate cyclase activity and, over longer periods of time, tyrosinase activity and melanin production. The high potency of [Nle^4, D-Phe^7]-α-MSH and its apparent resistance to enzymatic activity make it an especially attractive compound for studying these and other biological effects of melanotropins in both normal and abnormal (melanoma) melanocytes.

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