HORMONE–RECEPTOR INTERACTIONS: MELANOTROPIC ACTIVITIES OF COVALENT SERUM ALBUMIN COMPLEXES WITH α-MELANOTROPIN, α-MELANOTROPIN FRAGMENTS, AND ENKEPHALIN

Alex EBERLE, Verena Marly KRIWACZEK and Robert SCHWYZER
Institute of Molecular Biology and Biophysics, Swiss Federal Institute of Technology (ETH), CH-8093 Zürich, Switzerland

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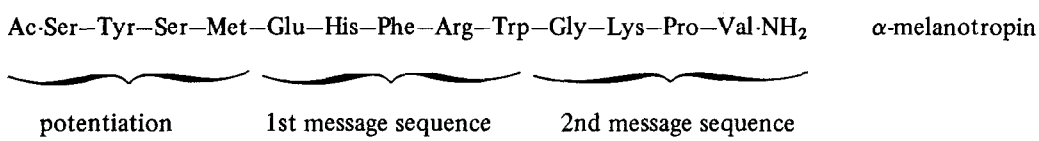
1. Introduction

The investigation of the biological properties of about 60 α-melanotropin analogues and fragments has led to the conclusion that this tridecapeptide consists of two message sequences which are capable of triggering the hormonal stimulus independently of one another [1–3]. The first message sequence is centered around –His–Phe–Arg–Trp– [4] and the second within the C-terminal tetrapeptide, –Gly–Lys–Pro–Val·NH_2 [1]. The N-terminal tetrapeptide, Ac·Ser–Tyr–Ser–Met·OH, is devoid of melanotropic activity; it potentiates, however, the activity of the C-terminal nonapeptide about 100-fold [2]:



A recent claim by Medzihradszky and Medzihradszky-Schweiger [5] that the N-terminal tetrapeptide, as well as a number of peptides related to the enkephalins, exhibit melanosome-dispersing properties necessitated a re-investigation of our results. At the same time the question should be answered whether the fragments of α-MSH elicit their stimulus by the same mechanism as the parent hormone, namely by acting on a cell surface membrane receptor [6]. To this end, α-MSH derivatives and fragments have been attached covalently to human serum albumin, and the complexes have been purified, analyzed and tested for melanotropic activity in vitro.

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Abbreviations: Amino acids and peptides: according to the tentative rules and recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature, Eur. J. Biochem. (1972) 27, 201–207. α-MSH = α-melanotropin (α-melanocyte-stimulating hormone); $\alpha\text{-MSH}_{5-13}$ = α-melanotropin-(5–13)-nonapeptide; $\alpha\text{-MSH}_{9-13}$ = α-melanotropin-(9–13)-pentapeptide; Pfp = *ar*-pentafluoro phenylalanine

2. Materials and methods

α-MSH, its fragments, and the enkephalin derivatives were synthesized by a classical approach and were analytically pure. They were attached to human serum albumin either by reaction of the corresponding N^{α}-bromoacetyl derivatives with thiol-containing albumin (modification of the albumin with *S*-acetyl thiosuccinic acid anhydride [^{14}C]: 4 to 6 –SH groups

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per protein molecule) or by formation of a defined isopeptide bond between hormone and protein [7]. The complexes were purified by dialysis, electrodialysis, and column chromatography, and were analyzed by chromatography and electrophoresis. The coupling rate ranged between 4 and 6 peptide molecules per protein molecule as determined with tritium-labelled α-MSH derivatives. Details of these syntheses and protein modifications will be described elsewhere (Eberle et al., in prep. for Helv. Chim. Acta).

The melanotropic activity of the complexes and of the corresponding free, N^{α}-acetylated peptides was determined in vitro using the reflectometric assay of Shizume et al. [8] with some modifications [1]. All assays were performed with specimens of *Rana pipiens* (northern), 7.5–9 cm long, which had been kept under constant illumination for at least 48 h preceding the experiment. The values of melanotropic activities are the means of the measurements of twelve different skins, four skins each, at three different concentrations. The melanotropic activity of the tetrapeptide, H·Ser–Tyr–Ser–Met·OMe, HCl, was tested furthermore by the Anolis skin assay [9] by Drs Coert, Greven, and Meijer (Organon, Oss, The Netherlands).

The time-response curves of the peptide–protein complexes and of the free peptides were measured in the following way: the change of reflectance of twenty frog skins for each substance was recorded every 5 min during stimulation with hormone and during the washing phase. The values were computed as the percentage of the total change for each skin and their means represented by the curves of fig.1.

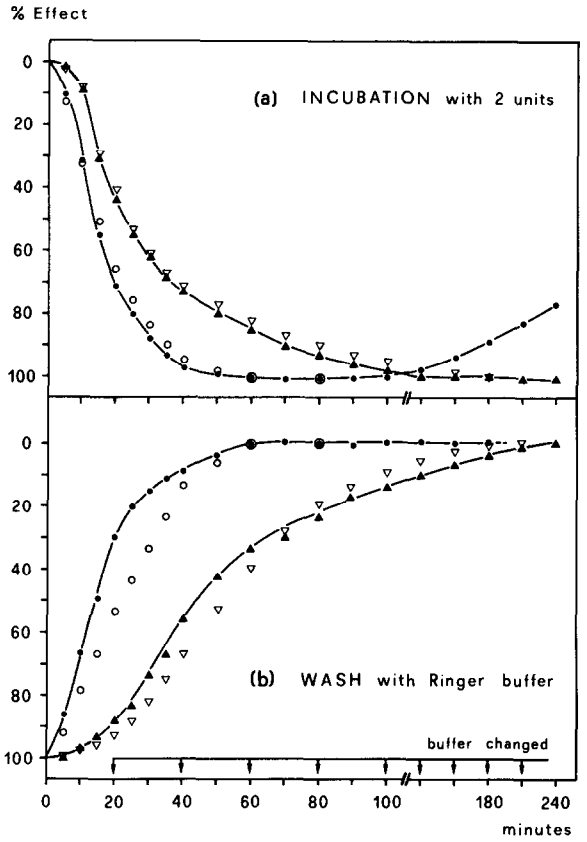

Fig.1. Time dependence of the melanotropic response of 20 Rana pipiens skins each for α-MSH (●—●—●), Ac·Lys–Pro–Val·NH_2 (○), albumin-[D-Ala^1, Gly^3, Nva^4]α-MSH (▲—▲—▲), and albumin-α-MSH_{9-13} (▽), during prolonged incubation and wash phases.

3. Results

Melanotropic potencies of the free tridecapeptide, of its message and potentiator sequences, and of the corresponding peptide–protein complexes are listed in table 1. The amino acid sequence of α-MSH was altered at its N-terminal part in order to obtain a more suitable compound for the reaction with bromoacetate: [D-Ala^1, Gly^3, Nva^4]α-MSH exhibits a biological activity of 2×10^9 U/mmol (B1). The corresponding albumin–MSH complex reaches 8×10^8 U/mmol (A1), i.e. 45% of the potency of the free peptide. Similar relationships also hold for the C-terminal nonapeptide (B2 vs. A2) and for the two message sequences, –Glu–His–Phe–Arg–Trp–Gly·OH (B3 vs. A3) and –Trp–Gly–Lys–Pro–Val·NH_2 (B4 vs. A4), this in spite of the considerably higher concentrations necessary due to the much lower potencies of the shorter peptides.

The N-terminal tetrapeptide, H·Ser–Tyr–Ser–Met·Met·OMe, HCl (B5), is devoid of melanotropic activity in the Rana as well as in the Anolis skin assays (in the latter even in very high concentrations: 10 to 100 mg/ml); no difference can be observed when the tetrapeptide is covalently attached to albumin (A5). N(α)-Acetyl-enkephalin-amide (B6) lacks any melanosome-dispersing ability either as the free peptide (B6)

Table 1
Melanotropic activities of covalent complexes between human serum albumin and α-MSH-peptides (A) and of the corresponding free, N^{α}-acetylated peptides (B). X = $-CO-CH(CH_2-COOH)-S-CH_2-CO-$, combining N^{ϵ}-amino groups of the protein (left), with the N^{α}-amino group of the peptide (right)

	Structure of the complexes	Biological activity (U/mmol)		
		A	B	A/B
1	ALBUMIN–X–Ala–Tyr–Gly–Nva–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–NH_2	8×10^8	2×10^9	0.45
2	ALBUMIN–X–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–NH_2	3×10^8	5×10^8	0.6
3	ALBUMIN–X–Glu–His–Phe–Arg–Trp–Gly–OH	2×10^5	4×10^5	0.5
4	ALBUMIN–X–Trp–Gly–Lys–Pro–Val–NH_2	7×10^5	6×10^5	1.1
5	ALBUMIN–Ser–Tyr–Ser–Met–OME	0	0	–
6	ALBUMIN–Tyr–Gly–Gly–Phe–Met–NH_2	0	0	–
7	ALBUMIN–SII, ALBUMIN	0		

or in its 'immobilised' form (A6). Free [5-leucine]-enkephalin was found to behave similarly. Human serum albumin and its thiol-containing form are inactive in the concentration range of the assay ($\leqslant 10^{-4}$ molar).

The time-response curves for the incubation with 2 units of hormonal activity (α-MSH, Ac·Lys–Pro–Val·NH_2, albumin-[D-Ala^1, Gly^3, Nva^4]α-MSH and albumin-α-MSH_{9-13}) and for the wash phase are shown in fig.1. Although the concentration of free and of covalently bound Ac·Lys–Pro–Val·NH_2 is at least 1000 times greater than that of the corresponding forms of [D-Ala^1, Gly^3, Nva^4]α-MSH, the time-response patterns of the two free peptides and of the two complexes respectively are virtually indistinguishable from one another. Significant differences are observed only between the curves of the free and protein-bound peptides, especially during the wash phase. The slower reaction during the incubation and wash phases is a strong indication that the complexes are not hydrolysed during their contact with the tissue and the melanophores.

4. Discussion

The A/B ratios (complex/free peptide) all lie between 0.5 and 1.1. Considering a factor 2 of bioassay tolerance due mostly to seasonal variations of the test animals, it can be stated that almost identical molar concentrations of both the free N^{α}-acetylated peptides and their corresponding covalent albumin complexes are necessary to elicit the same degree of melanosome dispersion. This is true for the tridecapeptide as well as for the central and the C-terminal message sequences. The N-terminal tetrapeptide, however, does not exhibit any biological activity neither as free peptide nor as peptide–protein complex. This fits well into our earlier observations that Ac·Ser–Tyr–Ser–Met·OH and H·Ser–Tyr–Ser–Met–Glu–His·OH are devoid of melanotropic activity [10,11]. Thus, α-MSH consists of two main parts: The N-terminal one, Ac–Ser–Tyr–Ser–Met–, contains no information to trigger the melanophore receptors, but potentiates the biological activity of the C-terminal nonapeptide. The latter embodies two 'active cores' or message sequences which seem to induce melanosome dispersion in the same way as their parent peptides, $\alpha\text{-MSH}_{5-13}$ and α-MSH, namely by stimulating a membrane receptor. It appears that this mechanism of biological action is much more typical of α-MSH-sequences than has been assumed in the report by Medzihradszky and Medzihradszky-Schweiger [5]: We have synthesized and tested a number of non-MSH peptides that were related to angiotensin, oxytocin, and other hormones; they were consistently found to be completely inactive in the frog skin assay. The same is true for [Leu^5]-enkephalin and N^{α}-acetyl-[Met^5]-enkephalin-amide (table 1). This agrees with the observation [12] that [Met^5]-enkephalin is devoid of melanotropic activity. We therefore conclude that, in spite of their low affinity, the shorter α-MSH peptides are still recognized by the receptor with considerable selectivity. The specificity may, of course, be relatively lower than in the case of the whole tridecapeptide: For example, H·Phe–Arg–Leu–Gly·OH is five times more active than H·Phe–Arg–Trp–Gly·OH, whereas [Leu^9]α-MSH exhibits only 25% of the potency of α-MSH [11]. On the other hand, H·Pfp–Arg–Trp–Gly·OH is inactive [2], demonstrating the importance of Phe^7 for the interaction with the receptor.

Up to now, the site of action of α-MSH on melanophores had not been identified unequivocally. It had only been shown that a Sepharose–α-MSH complex was capable of activating tyrosinase in Cloudman mouse melanoma cells [13]. Such complexes may, however, slowly be solvolysed [14], and are therefore no definitive proof of a membrane receptor. Recently, an internalization of β-MSH into the cytoplasm of melanoma cells was postulated [15], but questioned shortly afterwards [16]. Our own results with frog melanophores are consistent with the hypothesis of a cell surface receptor for α-MSH; an internalization of the protein-bound hormone seems rather unlikely. This conclusion is supported by a recent finding of DiPasquale et al. [17] that a biologically active α-MSH-ferritin-fluorescein isothiocyanate complex could not be found in intracellular vesicles of Cloudman melanoma cells by electron microscopy. It appears that protein–α-MSH complexes might well be suitable for further investigations of melanotropin receptors.

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