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MECHANISM OF α-MELANOTROPIN ACTION

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Summary

A structure-function study of α-melanotropin has shown that this tridecapeptide consists of two message sequences, (-Glu)-His-Phe-Arg-Trp- and -Gly-Lys-Pro-Val·NH_2, and a potentiator sequence, Ac·Ser-Tyr-Ser-Met-(Glu-), when acting on its melanophore receptors. The key elements of the message, -Phe-Arg- and -Lys-Pro-, do not correspond exactly to those responsible for eliciting the effect in other tissues. It appears that α-MSH contains more information than would be necessary to interact with only one complementary receptor site; therefore, the topography of the hormone exposed to the binding site may be different on contact with the receptors of different target cells. To further investigate this aspect, new methods for the isolation and characterization of functional receptors must be developed. We are investigating the use of chemically well-defined, biologically active, covalent hormone-macromolecule complexes for this purpose. Another approach utilizes model receptors with a recognition pattern similar to that of the biological receptor, as described in this communication for certain highly specific antibodies.

Zusammenfassung

Struktur-Funktions-Untersuchungen beim α-Melanotropin (α-MSH) haben kürzlich ergeben, dass dieses Tridecapeptid zwei getrennte "Befehlssequenzen", (-Glu)-His-Phe-Arg-Trp- und -Gly-Lys-Pro-Val·NH_2, und eine "Potentiatorsequenz", Ac·Ser-Tyr-Ser-Met-(Glu-), für seine Melanophoren-Rezeptoren besitzt. Die Schlüsselelemente der Befehle, -Phe-Arg- und -Lys-Pro-, entsprechen nicht genau denjenigen, welche die Wirkungen auf andere Zell-Rezeptoren (z.B. Fett- und Nebennierenrinden-Zellen, sowie Zellen des Zentralnervensystems) auslösen. Es scheint deshalb, dass α-MSH mehr Information enthält, als nötig wäre, um nur mit einem (komplementären) Rezeptor-Oberflächenbereich in Wechselwirkung zu treten. Die für die Wechselwirkung verantwortliche Topographie des Hormones könnte verschieden sein,

wenn das Hormon auf Rezeptoren verschiedener Gewebe einwirkt. Um diesen Aspekt der hormonalen Pleiotropie und der Molekül-Flexibilität zu untersuchen, müssen neue Methoden zur Isolierung und Charakterisierung von Rezeptoren entwickelt werden. Zu diesem Zwecke könnten sich vielleicht chemisch gut definierte, biologisch wirksame, kovalente Hormon-Makromolekül-Komplexe eignen; diese Möglichkeit untersuchen wir gegenwärtig. Andere Einsichten chemischer Natur könnten durch die Verwendung von "Rezeptor-Modellen" gewonnen werden, die ein ähnliches Erkennungsmuster für das Hormon aufweisen, wie die funktionellen Rezeptoren. Zu diesem Zweck untersuchen wir gewisse hochspezifische Antikörper.

1. Introduction

One of the fundamental principles of polypeptide hormone action is the transfer of information, the hormone serving as the vehicle of communication between a "sending" cell and a "receiving" cell. It is well documented that the signal produced by the hormone is received and decoded by the target cell with highly specialized elements of the cell membrane (1) which have been shown to be independent (exchangeable) functional units (2): the discriminators ("receptors") and the effectors. The discriminator can therefore be regarded as a tuner receiving encoded information from the source (the hormone-excreting cell) via a transmitter (the hormone), Figure 1. The latter is recognized and selected by the discriminator via a pattern recognition system; the topochemical pattern is "matched" to that of the discriminator. This interaction between the hormone and its discriminator is equivalent to the channel through which the signal is transferred to the discriminator part of the receptor. The signal is now tranduced to and decoded by the effector into a cell-specific form. The fact that the hormone acts as a transmitter conveying encoded information from the source to the receiver implies a "common language" for the intercellular communication. Such a hormonal language must comprise an alphabet of topochemical elements; ordered patterns of these elements (the letters) form "words" which, grouped together in the correct sequence, are capable of information transmission. Each word has its own significance: address, message, potentiation, transport label etc. (3, 4). This comparison with a simple communication system visualizes the main points of interest for our studies of the mode of action of polypeptide hormones like α-melanotropin, adrenocorticotropin, and angiotensin: (i) organization of the information within the hormone molecule (analysis of the "words"), (ii) transfer of the information from the hormone to the discriminator, (iii) degradation of the hormone after the stimulation of the receptor, (iv) localization, isolation, and characterization of the discriminator, (v) transduction of the information to the effector, (vi) cell specific form of the signal.

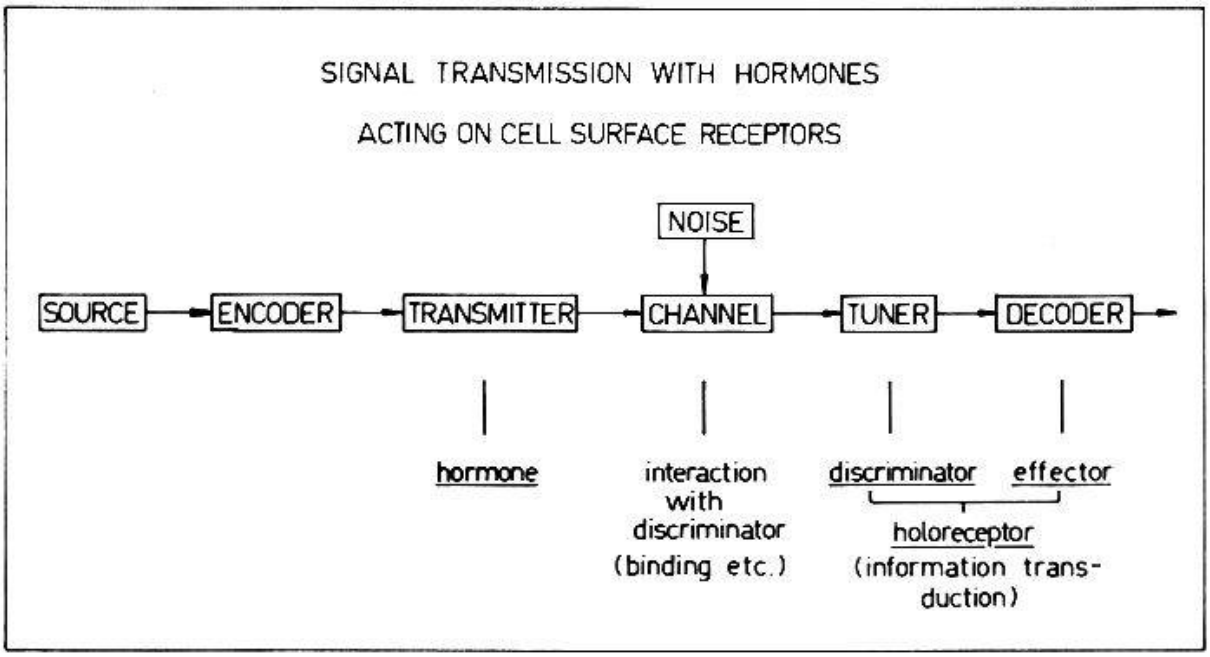

Fig. 1. Comparison of hormonal information transfer with a simple communication system.

α-Melanotropin (α-melanocyte stimulating hormone, α-MSH) is a basic tridecapeptide and is secreted in the intermediate lobe of the pituitary. In addition to its "classical" function as a pigmentary hormone of lower vertebrates it exhibits strong effects on a variety of tissues of mammals and to some extent also of man, such as proliferation of melanocytes, stimulation of pigment production in melanocytes and melanoma cells, sebum secretion, lipolysis in rabbit fat cells, actions on the central nervous system (e.g. behaviour, stimulation of dopaminergic neurons), fetal growth, and stimulation of somatotropin release (reviews 5, 6). This diversity of actions of α-MSH together with additional effects of some of its fragments - e.g. opiate receptor affinity (7, 8, 9) - provides an attractive tool for the comparative investigation of the chemical mechanisms by which a peptide hormone elicits its biological responses in different target cells.

2. Organisation of Information in α-Melanotropin

α-Melanotropin is biogenetically related with adrenocorticotropin (10, 11) and exhibits some similarities of biological action with its parent hormone. Except for the N-terminal acetyl and the C-terminal amide groups, the primary structure of α-MSH is identical with that of the N-terminal tridecapeptide sequence of ACTH. The heptapeptide sequence -Met-Glu-His-Phe-Arg-Trp-Gly- is also found in β-MSH and the lipotropins of almost all species investigated, with the exception of the dogfish β-MSH's of Squalus acanthias (-Phe-Gly-His-Phe,Arg.Trp-Gly-) (12) and Scyliorhinus canicula (-Met-Gly-His-Phe-Arg-Trp-Gly-)

(13). This "common sequence" has early been shown to be responsible for the melanotropic effects of α-MSH, β-MSH and ACTH (14, 15). It is also capable of eliciting the adipose lipolytic response (16)(in the rabbit, but not in the rat, where the adjacent sequence -Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-, an inactive, strongly binding address sequence (17) is necessary), and the adrenal steroidogenic response typical of ACTH (18). It affects the acquisition and retention of learned responses of the rat in the same manner as α-MSH and ACTH (19). Because no other active fragments outside this region had been found, it was called the message sequence (3) or active site (15) of the hormones. The N-terminal tripeptide Acetyl·Ser-Tyr-Ser- of α-MSH or H·Ser-Tyr-Ser- of ACTH enhances the melanotropic, lipolytic and steroidogenic activities of the message sequence considerably. It has therefore been called a potentiator sequence (3). Our own studies of structure-activity relationships of α-MSH were aimed at the question whether or not these three types of information-bearing sequences - address, message, potentiator - correspond to those of ACTH, whether they could be further subdivided and whether more precise statements could be made about a specific involvement of particular elements triggering the stimuli in different target cells.

We have synthesized about 60 α-MSH analogues and fragments and have tested them for melanotropic activity with an in vitro frog skin assay (20). The results of 40 of these peptides are summarized in Fig. 2 (four values from the literature are included): The zeniths of the semicircles represent the biological activity in units/mmol of the corresponding sequences, the symbols the activity of α-MSH-(1-13)-tridecapeptide derivatives with one modified residue (dotted lines indicate a simultaneous two- or threefold alteration). The analysis of this graph reveals three major facts:

1) The N-terminal tetrapeptide Ac·Ser-Tyr-Ser-Met·OH as well as the hexapeptide H·Ser-Tyr-Ser-Met-Glu-His·OH are devoid of melanotropic activity; the tetrapeptide potentiates the C-terminal nonapeptide (5-13) about 100-fold, whereas the tripeptide Ac·Ser-Tyr-Ser potentiates (4-13) only about 10-fold. The subdivision into a more hydrophobic part (Met) and a part with balanced hydrophobicity/hydrophilicity (Ac·Ser-Tyr-Ser) seems to be important. Glutamic acid[5] is most probably a quite unspecific spacer element and its assignment to either potentiator or message sequence rather arbitrary (It is, however, of great importance for triggering the lipolytic response in the rat fat cell; see Schwyzer, this volume.). In conclusion, the N-terminal part of α-MSH, Ac·Ser-Tyr-Ser-Met-(Glu-) is a potentiating sequence containing no message element to elicit the stimulus (5, 21, 22).
2) The C-terminal tripeptide, hitherto regarded as inactive per se, exhibits melanotropic activity and is therefore a message sequence (20, 21). This observation is substantiated by

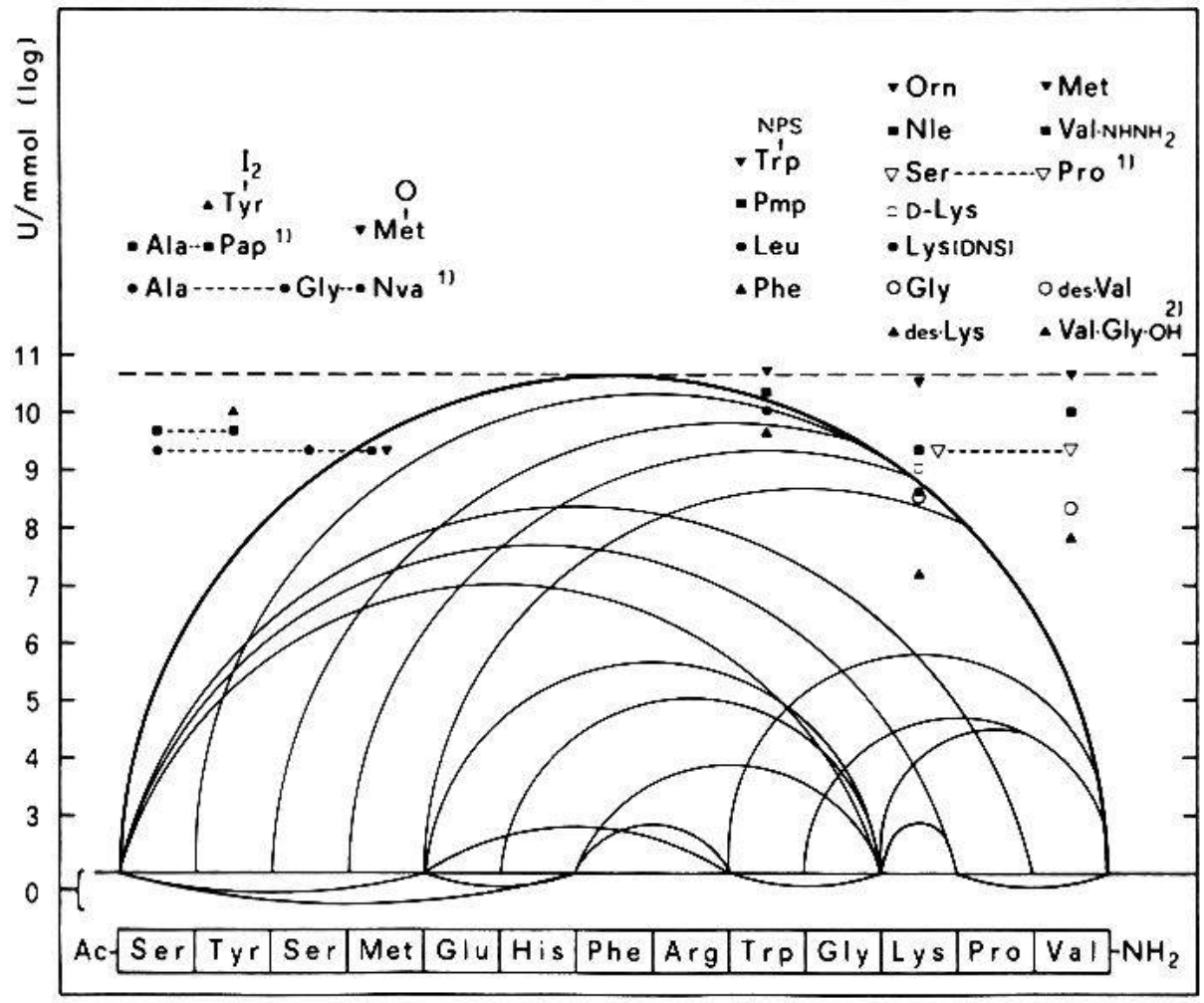

Fig. 2. Structure-activity study of α-MSH. The zeniths of the semicircles covering a certain partial sequence represent the activity of the corresponding peptide. The symbols indicate the activity of α-MSH derivatives with one single modification of a residue. 1) simultaneous two- or threefold modification. 2) ACTH(1-14)-tetradecapeptide; activity determined with an in vivo frog skin assay (35).

many assays of α-MSH fragments containing -Lys-Pro-. It is best illustrated by the fact that H·Gly-Lys-Pro-Val·NH_2 is 2.5 times more active than the "classical" α-MSH message, H·His-Phe-Arg-Trp·OH. Lysine[11] is very sensitive to alterations: protection of the side chain with bulky groups (N^{ε}-dansyllysine), omission of the amino function (norleucine) or of the whole side chain (glycine), or the change of configuration (D-lysine) all lead to a drastic loss of activity. Especially the latter finding shows that the chirality at the center of the C-terminal message sequence of α-MSH is much more important than would have been concluded from the results with randomly racemized tridecapeptides according to which the melanin-dispersing effect is prolonged and not decreased by inclusion of D-amino acids into the "classical" message sequence (23).

3) Within the "classical" message sequence, -Met-Glu-His-Phe-Arg-Trp-Gly-, the elements necessary for triggering the melanotropic, steroidogenic, and lypolytic responses are con-

tained in the tetra (penta-)peptide sequence (-Glu)-His-Phe-Arg-Trp-. Whereas His^6 and Trp^9 are particularly essential for stimulating adrenal cell and rat adipocyte receptors, they play a minor role in eliciting the melanin dispersion of melanocytes. Here, -Phe-Arg- seems to be the key fragment of the message. This is concluded from the following observations: H·Phe-Arg·CH is the shortest fragment with a small but definite melanotropic activity; it is embedded between the two inactive dipeptides H·Glu-His·OH and H·Trp-Gly·OH (the tetrapeptides H·Glu-His-Phe-Arg·OH and H·Phe-Arg-Trp-Gly·OH both exhibit biological activity). Arg^8 is very sensitive to alterations (24, 25), and replacement of Phe^7 by ar-pentafluorophenylalanine in the active tetrapeptide (7-10) leads to a complete loss of activity (5). Trp^9 can be replaced by 2-(nitrophylsulfenyl)-tryptophan, phenylalanine, ar-pentamethyl-phenylalanine, or leucine without or with only little adverse effects (5, 21, 26). His^6 is an important potentiating factor; its attachment to the N-terminus of Phe-Arg-Trp-Gly- enhances the activity at least three-fold (21).

It appears that the melanocyte α-MSH discriminator contains two message-recognizing sites, one for (-Glu)-His-Phe-Arg-Trp-, the N-terminal or first message sequence, and one for -Gly-Lys-Pro-Val·NH_2, the C-terminal or second message sequence. The two sites can - in the experimental situation - operate either alone or in combination to trigger melanin dispersion. In combination, they have a multiplicative, "cooperative" effect. Since the N-terminal tetrapeptide is a potentiating sequence, the address must be contained within the message; no separation of the two functions is possible, with the exception that the centers of the message are surrounded by rather unspecific lipophilic "contacts": the exchange of $Met^4 \rightarrow Nle^4$, $Trp^9 \rightarrow Leu^9$, or $Val^{13} \rightarrow Met^{13}$ does not influence the biological activity. The congruence of message and address in α-MSH is a major contrast to ACTH interacting with its receptors of rat adipocytes or adrenal cells (SCHWYZER, this volume). With respect to its activity on the active avoidance behaviour in rats, α-MSH seems to contain even three independent centers, each of which is capable of interacting with cells of the central nervous system (19, 27). Met-Glu-His constitutes one, Phe-Arg-Trp the second, and -Lys-Pro-Val·NH_2 the third center; essential functions are attributed to Met^4 and Phe^7. Thus, the refinement of structure-function relationships in α-MSH and ACTH has revealed that the elements mainly responsible for eliciting the effect in different target cells may differ from one another (21, 28). Most probably, different parts of one and the same molecule can interact with its discriminators with different binding constants and can produce a number of different effects. With respect to this ability, which is based upon a flexible peptide chain and a sychnological organization of the information, such hormones act as pleiotropic effectors (28, 29). This does, however, not preclude defined conformations of the hormone on contact

with the receptor surfaces. Considering the fact that in addition to the pleiotropy of the intact polypeptides also some of their fragments can adopt new functions, which may be entirely different from those of the parent molecules (e.g. lipotropin→β-melanotropin + endorphin), the difficulty of a detailed analysis of the storage of the information within the hormone and the complexity of the flux of the hormonal information within an organism become evident.

3. Covalent α-melanotropin-macromolecule complexes for the identification of receptor sites

The site of action of α-MSH on melanophores has not been identified unequivocally. It has only been shown that a Sepharose-β-MSH complex was capable of activating tyrosinase in Cloudman mouse melanoma cells (30) and that a biologically active α-MSH-ferritin-fluorescein isothiocyanate complex could not be found in intracellular vesicles of such cells by electron microscopy (31). On the other hand, an internalization of β-MSH into the cytoplasm of melanoma cells was postulated (32); it has, however, not been proved whether the intact hormone was taken up by the cell or whether it was only a hydrolysed part thereof. A definitive answer will only be possible after a thorough study of the degradation of the melanotropins near or at the site of action.

Biologically active high molecular forms of polypeptide hormones represent an attractive tool for the investigation of hormonal receptor sites. We have therefore synthesized and carefully purified a number of covalent serum albumin complexes with α-MSH and α-MSH fragments, which have been attached to the protein through a chemically defined linage, and have tested them at the "classical" site of action of this hormone, the melanophore. Table 1 lists the melanotropic activity of the free tridecapeptide, of its message and potentiator sequences (B), and of the corresponding peptide-protein complexes (A): The A/B ratios (complex/free peptide) all lie between 0.5 and 1.1. Considering the observation that the kinetics of the response are the same for all free peptides but differ from those of the complexes, the fragments of α-MSH seem to elicit their stimulus by the same mechanism as the parent hormone, namely by acting on a cell surface membrane receptor (22, 33).

Extension of these studies to larger carrier-proteins like thyroglobulin (Table 1) or tobacco mosaic virus (TMV) revealed that the activity of such peptide-protein complexes does not depend on the protein as long as the hormone is still readily accessible to the receptor. This was shown by the following experiment (Fig. 3): A Nα-bromoacetyl-α-MSH derivative was coupled to thiol-containing TMV. The complex was then purified and tested for melanotropic activity. It exhibited $\sim 10^7$ U/g. Incubation of the complex with a specific anti-α-MSH

Table 1. Biological Activity of Covalent Peptide-Albumin Complexes

	Structure	Melanotropic 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	$8 \cdot 10^8$ $2 \cdot 10^7$ [1]	$2 \cdot 10^9$	0.45
2	TMV-X-Ala-Tyr-Gly-Nva-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	$1 \cdot 10^7$ [1]	-	-
3	Albumin-X-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	$3 \cdot 10^8$	$5 \cdot 10^8$	0.6
4	Albumin-X-Glu-His-Phe-Arg-Trp-Gly-OH	$2 \cdot 10^5$	$4 \cdot 10^5$	0.5
5	Albumin-X-Trp-Gly-Lys-Pro-Val-NH_2	$7 \cdot 10^5$ $2 \cdot 10^4$ [1]	$6 \cdot 10^5$	1.1
6	Thyroglobulin-X-Trp-Gly-Lys-Pro-Val·NH_2	$8 \cdot 10^4$ [1]	-	-
7	Albumin-Ser-Tyr-Ser-Met-OMe	0	0	-
8	Albumin-Tyr-Gly-Gly-Phe-Met-NH_2	0	0	-
9	Albumin-XH, Albumin	0	-	-

[1] U/g

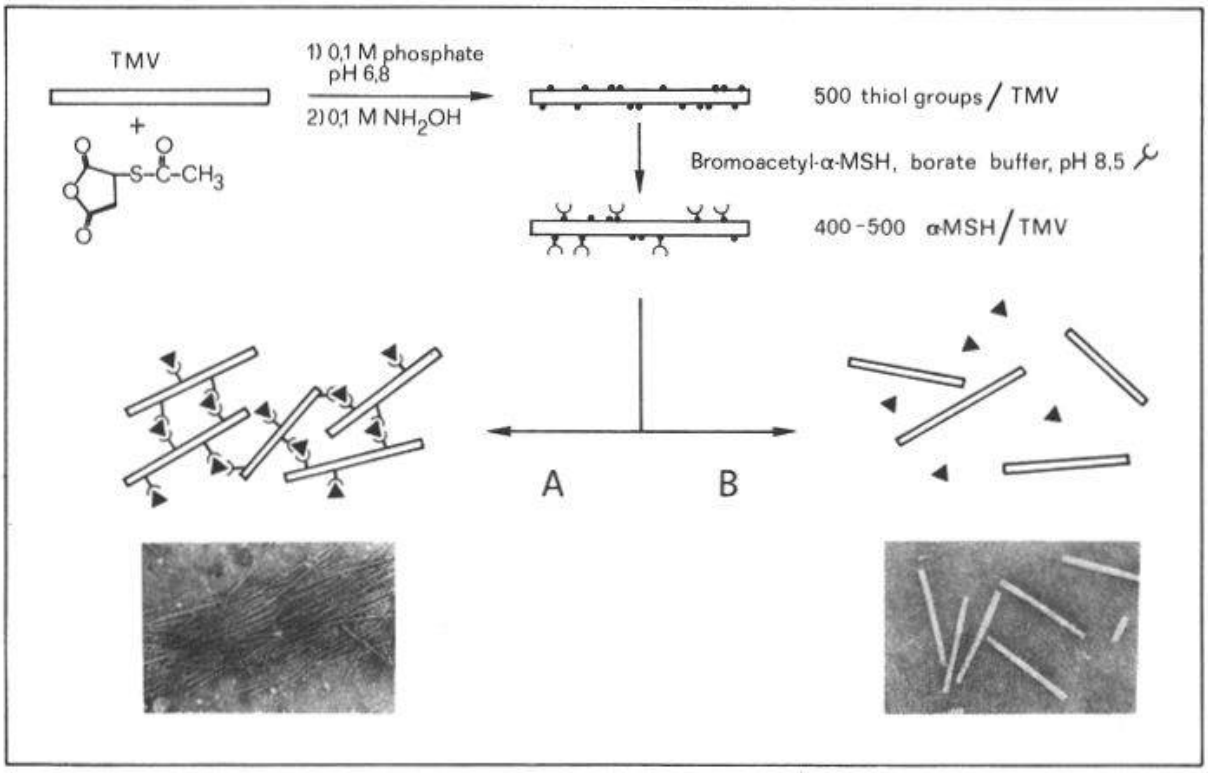

Fig. 3. Interaction of specific α-MSH antibodies with the hormone bound to tobacco mosaic virus (see text).

antiserum (34) resulted in an aggregation of the TMV rods (A). No aggregation was observed when the complex was incubated with normal serum or when the anti-α-MSH serum was mixed with native TMV (B). Thus, the specific α-MSH-antibody, which acts as a soluble receptor in this experiment, is capable of recognizing the hormone on the TMV surface. These preliminary observations are encouraging enough to pursue the investigation of covalent polypeptide hormone-protein complexes, also for other applications.

4. Specific antibodies as model receptors for α-melanotropin

The isolation and purification of α-MSH-receptor molecules from melanophores requires large amounts of cells which are not available at present. Nevertheless, it would be extremely valuable to know more chemical details of the interaction between hormone and recognizing macromolecule, in order to study the transfer of information from the peptide to the discriminator. Presently, the only way of doing this with α-MSH is to investigate a model receptor. We have therefore produced an α-MSH antibody by imminizing rabbits with a complex antigen (35). The resulting antisera had high antibody titers (up to $1 : 10^6$), and their specificity was directed towards the C-terminal part of α-MSH. The cross-reaction with ACTH was small (< 0.1 %) and negligible with other peptides, including α-MSH. The association con-

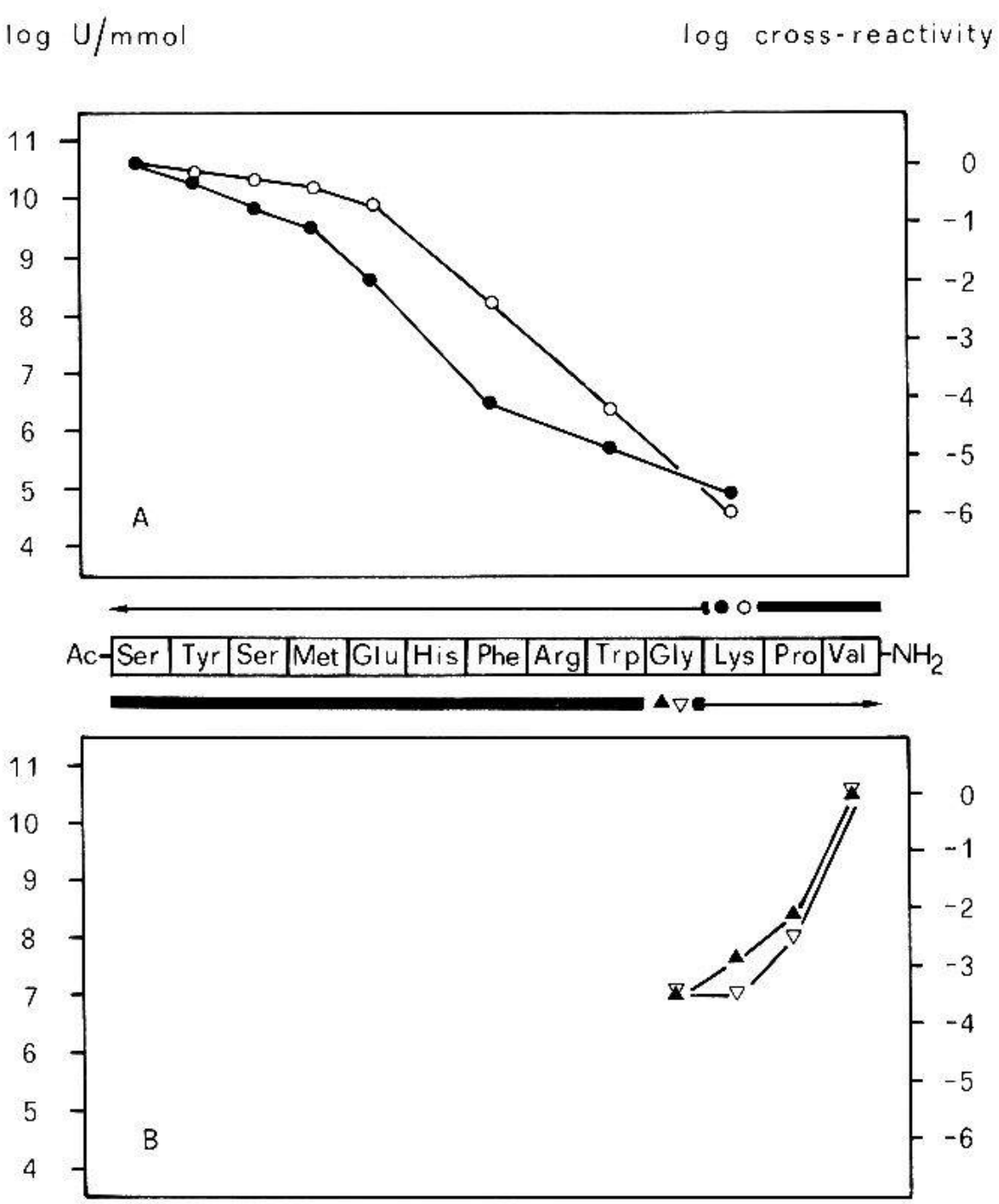

Fig. 4. Comparison of the biological activities and the immunochemical cross-reactions of synthetic α-MSH and α-MSH fragments (35). Abscissae: fragments with chain lengths extending from -Lys-Pro-Val·NH_2 to the left (N-terminal extension) in (A), and from Ac·Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly- to the right (C-terminal extension) in (B). Left ordinates: biological activity in vitro in the frog skin assay, log Units per mmol (●, ▲). Right ordinates: log cross-reactivity (○, △; cross-reactivity of α-melanotropin = 1).

stant of the α-MSH-antibody complex seems to be within the same range ($\sim 10^{10} \ldots 10^{11}$ l/mol) as that estimated for the α-MSH-receptor complexes on the melanophores. The biological activity of structural analogues and fragments of α-MSH (Figure 2) paralleled their activity in the radioimmunoassay (Figure 4). This may indicate similar structural requirements of the melanophore discriminator and antibody populations. Thus, it is possible to generate specific, "receptor-like" antibodies against α-MSH, which - after purification - may prove to be suitable substitutes of receptor proteins for a first approach.

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