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Synthesis and Iodination of Human (Phenylalanine^{13}, Tyrosine^{19}) Melanin-concentrating Hormone for Radioreceptor Assay

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This paper is dedicated to Robert C. Sheppard, Cambridge, on the occasion of his retirement.

Abstract: An analogue of human melanin-concentrating hormone (MCH) suitable for radioiodination was designed in which Tyr^{13} and Val^{19} of the natural peptide were replaced by phenylalanyl and tyrosyl residues: [Phe^{13},Tyr^{19}]-MCH. The peptide was synthesized by the continuous-flow solid-phase methodology using Fmoc-strategy and Polyhipe PA 500 and PEG-PS resins. The linear MCH peptides with either acetamidomethyl-protected or free cysteinyl residues were purified to homogeneity and cyclized by iodine oxidation, yielding the final product with the correct molecular weight of 2434.61. Radioiodination of the C-terminal tyrosine was carried out enzymatically using solid-phase bound glucose oxidase/lactoperoxidase, followed by purification on a reversed-phase mini-column and by high-pressure liquid chromatography. The resulting [^{125}I]-[Phe^{13},Tyr^{19}]-MCH tracer was the first radiolabelled MCH peptide suitable for radioreceptor assay: saturation binding analysis using mouse G4F-7 melanoma cells demonstrated the presence of 1090 MCH receptors per cell. The dissociation constant (K_D) was 1.18×10^{-10} M, indicating high-affinity MCH receptors on these cells. MCH receptors were also found in other cell lines such as mouse B16-F1 and G4F and human RE melanoma cells as well as in PC12 and COS-7 cells. Competition binding analyses with a number of other peptides such as α-MSH, neuropeptide Y, substance P and pituitary adenylate cyclase activating peptide, demonstrated that the binding to the MCH receptor is specific. Atrial natriuretic factor was found to be a weak competitor of MCH, indicating topological similarities between MCH and ANF when interacting with MCH receptors.

Keywords: Melanin-concentrating hormone; peptide synthesis; radioiodination; MCH receptor; binding analysis

Abbreviations

Acm, acetamidomethyl; ANF, atrial natriuretic factor (atriopeptin); DCM, dichloromethane; DIPC, diisopropylcarbodiimide; DTT, dithiothreitol; EDT, 1,2-ethane-dithiol; HEPES, *N*-(2-hydroxyethyl-1-piperazine *N'*-ethane)-sulphonic acid; HMPA, 4-(hydroxymethyl)-phenoxyacetic acid; HOBt, 1-hydroxybenzotriazol; MCH, (human/rat) melanin-concentrating hormone; sMCH, salmonic MCH; MSH, melanocyte-stimulating hormone; Mtr, N^G-(4-methoxy-2,3,6-trimethyl-benzene-sulphonyl); NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase activating peptide; Pmc, N^G-(2,2,5,7,8-pentamethylchroman-

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6-sulphonyl); PMSF, phenylmethylsulphonylfluoride; RP-HPLC, reversed-phase high-performance liquid chromatography; Trt, trityl.

INTRODUCTION

Melanin-concentrating hormone (MCH) is a ubiquitous vertebrate peptide which was originally isolated from teleost fish where it is produced in hypothalamic perikarya from a large precursor as a heptadecapeptide cyclized between its Cys^5 and Cys^{14} residues. It is released into the circulation when fish move from a black to a pale background, and it induces aggregation of the pigment granules within the skin melanophores [1, 2]. The mammalian form of MCH (mouse, rat, human) is a nonadecapeptide containing the same size of ring structure as the fish peptide but an altered N-terminus extended by two residues (Fig. 1). Although abundant MCH-containing cell bodies have been localized in the lateral and posterior hypothalamus of the mammalian brain, in regions commonly believed to be involved with motivational behaviours associated with arousal, eating and drinking [3], the physiological role of MCH in the mammal is not yet clear.

The identification of receptors for MCH is the most promising method to identify the sites of action of this peptide. As such studies require biologically active radiolabelled MCH, we have developed several strategies to produce MCH tracer molecules. It is known from previous studies with salmonic MCH (sMCH) that iodination of its Tyr^{11} dramatically reduces the biological activity [4] of the peptide. Modification of the N-terminus of human MCH with the Bolton–Hunter reagent or exchange of Phe^2 by a tyrosyl residue (by simultaneous exchange of Tyr^{13} by phenylalanine) produced very lipophilic molecules with a considerable tendency of non-specific binding to surfaces (Drozdz and Eberle, unpublished observations). The highly tritiated analogue [($^3H_4$)$Nva^{4,8,12,19}$]-MCH [5], although useful for qualitative binding studies, was not satisfactory for quantitative binding analyses with cells or synaptosomes containing low receptor numbers.

We now describe the synthesis of a new analogue of human/rat MCH which can be radiolabelled at its C-terminus and which retains full biological activity after iodination. With this compound it was possible to establish the first radioreceptor assay for MCH and to identify MCH receptors on melanoma and other cell lines.

MATERIALS AND METHODS

General Procedures

The general procedures for peptide synthesis described by Atherton and Sheppard [6] were rigorously applied. Solvents and coupling reagents were obtained from Millipore (Milford, MA), protected amino acid derivatives from Bachem (Bubendorf, Switzerland), Polyhipe PA-500 resin from Calbiochem-Novabiochem (Läufelfingen, Switzerland) and PEG-PS resin from Millipore. Iodine (puriss.) was purchased from Fluka (Buchs, Switzerland). Reversed-phase high-performance liquid chromatography (RP-HPLC) was carried out on a Dupont 8800 system or a PC-controlled Jasco instrument consisting of two PU-980 pumps, a 970/975 UV/vis. detector, an 851 AS autosampler and ICL 6000 software. For semipreparative HPLC, Vydac C18 columns (1.0 × 25 cm; 5 μm granulometry; 300 Å porosity) were run with 3 ml/min of eluent A (0.1%

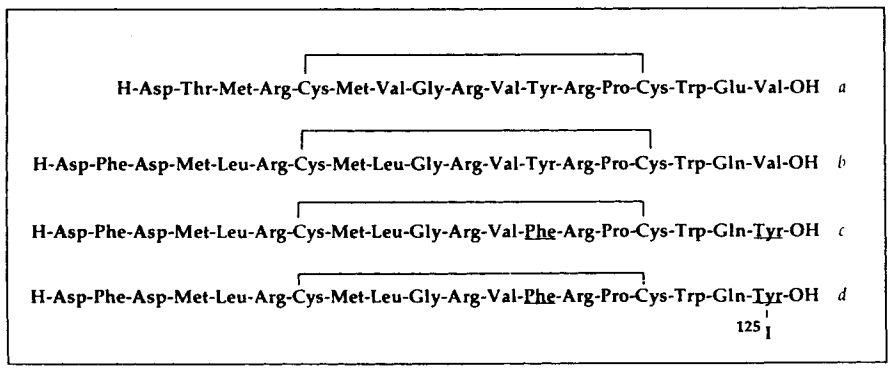

Fig. 1 Structure of different forms of MCH: (a) salmonic MCH; (b) human/rat MCH; (c) [Phe^{13},Tyr^{19}]-MCH; and (d) [^{125}I]-[Phe^{13},Tyr^{19}]-MCH.

TFA/H_2O) and eluent B (70% acetonitrile/0.1% TFA/H_2O) forming a gradient from 15 to 70% B within 40 min (monitoring at 280 nm). Analytical HPLC was performed on Vydac C18 columns (0.46 × 25 cm) at a flow rate of 1 ml/min. Amino acid analyses were performed in our laboratory on a Waters instrument using the PicoTag method. Fast atom bombardment mass spectrometry (FABMS) was done on a Kratos MS50RF instrument by Ciba-Geigy Ltd (courtesy of Dr. B. Kamber, Ciba-Geigy, Basel).

Solid-phase Synthesis of Linear [Phe^{13},Tyr^{19}]-MCH

Linear [Phe^{13},Tyr^{19}]-MCH was synthesized on a Milligen 9050 automated peptide synthesizer using the continuous-flow technology [7] and either 1.0 g Fmoc-Tyr(*t*Bu)-Polyhipe PA 500 resin (→ synthesis A) or 1.0 g Fmoc-Tyr(*t*Bu)-PEG-PS resin (→ synthesis B). Both resins contained the acid-labile hydroxymethyl-phenoxyacetyl linker (HMPA) [7]. The substitution of the resins was 0.3–0.4 meq/g (PA) and 0.15–0.2 meq/g (PEG-PS). The following sequence of operations was used: (1) flow of 20% piperidine/*N*,*N*-dimethylformamide (DMF) (7 min; cleavage of Fmoc); (2) flow of DMF (5 min; wash); (3) recirculation of Fmoc-amino acid residue (acylation; times see below); (4) flow of DMF (10 min; wash). After completion of the synthesis, the N-terminal Fmoc group was cleaved with 20% piperidine/DMF, the resin was washed with DMF and ether and then dried *in vacuo*. Samples of the resin were taken for amino acid analysis before and after cleavage of the peptide.

Synthesis A. The first synthesis was performed with acetamidomethyl (Acm)-protected cysteine residues. Coupling reactions were carried out with a twofold excess of protected amino acid residues, a twofold excess of DIPC and HOBt and the following acylation times: Fmoc-Gln(Trt)-OH (60 min), Fmoc-Trp(Boc)-OH (60 min), Fmoc-Cys(Acm)-OH (80 min), Fmoc-Pro-OH (45 min), Fmoc-Arg(Pmc)-OH (80 min), Fmoc-Phe-OH (45 min), Fmoc-Val-OH (45 min), Fmoc-Gly-OH (45 min), Fmoc-Leu-OH (45 min), Fmoc-Met-OH (60 min), Fmoc-Asp(O*t*Bu)-OH (70 min). A sample of the dried, fully protected peptide–resin (300 mg) was treated with TFA/H_2O/thioanisol 85:5:10 for cleavage from the resin using a flow-through method with a special funnel, through which 15 ml of this solution was slowly filtered within 30 min. Deprotection from acid-labile protecting groups was continued for further 90 min at room temperature. The peptide was then isolated by precipitation with cold ether, centrifugation, dilution in 20% AcOH and lyophilization, yielding 80 mg (80%) of crude [Cys(Acm)^{7,16},Phe^{13},Tyr^{19}]-MCH. Purification was carried out by semi-preparative RP-HPLC and peak fractions were re-examined by analytical RP-HPLC. Overall yield: 31% of the pure linear peptide.

Synthesis B. The second synthesis was performed with Trt-protected Cys. Coupling reactions were carried out with a fourfold excess of protected amino acid residues, a fourfold excess of diisopropylcarbodiimide (DIPC) and HOBt and the following acylation times: Fmoc-Gln(Trt)-OH (45 min), Fmoc-Trp(Boc)-OH (45 min), Fmoc-Cys(Acm)-OH (45 min), Fmoc-Pro-OH (30 min, followed by re-acylation under identical conditions), Fmoc-Arg(Pmc)-OH (45 min), Fmoc-Phe-OH (45 min), Fmoc-Val-OH (30 min, followed by re-acylation under identical conditions), Fmoc-Gly-OH (45 min), Fmoc-Leu-OH (45 min), Fmoc-Met-OH (60 min), Fmoc-Asp(O*t*Bu)-OH (45 min). The protected peptide–resin was treated with trifluoroacetic acid (TFA)/H_2O/1,2-ethane dithiol (EDT)/thioanisol 85:5:5:5 for 2 h at room temperature, as described above for synthesis A. The crude peptide was partitioned between 20% AcOH and DCM and the aqueous layer was washed further with DCM and cold ether. The ether extracts were back-washed with water and the combined aqueous solutions lyophilized. The peptide was examined with analytical RP-HPLC and purified on a Vydac C_{18} column (1.0 × 25 cm) as described above. The cleavage yielded 50 mg (60%) of HPLC-pure linear [Phe^{13},Tyr^{19}]-MCH.

Cyclization of [Phe^{13},Tyr^{19}]-MCH

Cyclization of both peptides, i.e. linear [Cys(Acm)^{7,16},Phe^{13},Tyr^{19}]-MCH and linear [Phe^{13},Tyr^{19}]-MCH with free cysteinyl groups, was achieved by iodine oxidation using a 40-fold excess of iodine [8].

Synthesis A. Iodine (35 mg, 140 μmol) was dissolved in 3.5 ml of redistilled glacial AcOH and kept under nitrogen. [Cys(Acm)^{7,16},Phe^{13},Tyr^{19}]-MCH (8 mg, 3.5 μmol) was dissolved in the same volume of AcOH and added dropwise under nitrogen to the vigorously stirred iodine solution over a period of 10 min. The cyclization solution (final volume 7 ml containing 1 mM peptide and 40 mM iodine) was stirred for another 10 min and the reaction was then stopped

by addition of approximately 0.5 ml of 10% ascorbic acid solution until it was completely colourless. Analytical RP-HPLC showed only one major peak containing the cyclic product which was eluted before Acm-protected MCH. The reaction product was purified on a Sephadex G-25 column (1.5 × 60 cm) topped with Sephadex G-10 and eluted with 15% AcOH at a speed of 24 ml/h. The fractions (2 ml) were tested by RP-HPLC and thin-layer chromatography (TLC). The appropriate peak containing the cyclic [Phe^{13},Tyr^{19}]-MCH was collected and lyophilized, yielding 4.4 mg of crude peptide. The final purification was achieved by semipreparative RP-HPLC and the peak fractions re-examined by analytical RP-HPLC. The overall yield of pure cyclic [Phe^{13},Tyr^{19}]-MCH was 10%.

Synthesis B. Linear [Phe^{13},Tyr^{19}]-MCH (10 mg, 4.2 μmol) was treated with 42.6 mg (168 μmol) of iodine as described above for synthesis A, except for a shorter reaction time (addition, 2 min; reaction, 8 min). After Sephadex G-25 chromatography, 7.8 mg of crude cyclic [Phe^{13},Tyr^{19}]-MCH were obtained which were further purified by semipreparative RP-HPLC. The final yield was 5.8 mg (58%) of completely homogeneous peptide.

Radioiodination of [Phe^{13},Tyr^{19}]-MCH

[Phe^{13},Tyr^{19}]-MCH (5 μg in 5 μl of 1 mM HCl) and 50 μl of 0.2 M Na-phosphate buffer, pH 7.2, were added to an Eppendorf tube containing 37 MBq (1 mCi) Na^{125}I. The reaction was started by the addition of 50 μl Enzymobead (BioRad, Melville NY) suspension, followed by 20 + 20 μl of 1% β-D-glucose solution. After 1 h at room temperature, the reaction was stopped by the addition of 0.5 ml of a 50 mM Na-phosphate solution, pH 7.4, containing 0.25% bovine serum albumine (BSA) and 0.02 M dithiothreitol (DTT). The beads were centrifuged off and the supernatant was applied to a reversed-phase mini-column, i.e. a 1 ml syringe packed with 0.3 g Spherisorb ODS/10 μm RP-silica [9], and washed twice with 0.6 ml of 0.25 M Na-phosphate buffer, pH 7.4. Monoiodinated [^{125}I]-[Phe^{13},Tyr^{19}]-MCH was eluted at 67–68% methanol using a stepwise gradient of methanol/0.1% TFA. The radiopeptide was then purified by analytical RP-HPLC and eluted shortly after the parent peptide, indicating that only one iodine atom had been incorporated.

Fish Melanophore Assay

The biological activity of [Phe^{13},Tyr^{19}]-MCH was determined with the microscopic melanophore assay using carp or trout scales as described by Baker *et al.* [10].

Receptor Binding Assay

The binding assay was carried out with the following melanoma cells: mouse B16-F1, G4F (originating from B16 and not expressing MSH receptors [11], G4F-7 (G4F cells with transfected human MSH receptor; constructed by Dr J. Chluba-de Tapia) as well as human RE cells (isolated in our laboratory from a metastasis). In addition, rat PC12 phaeochromocytoma cells, COS-7 cell, CHO (Chinese hamster ovary) cells and human fibroblasts were used.

Tissue Culture. The cells were grown in modified Eagle's medium (MEM) with Earle's salts (Gibco, Paisley, UK), supplemented with 10% heat-inactivated foetal calf serum (Amimed, Basel, Switzerland), 2 mM L-glutamine, 1% MEM non-essential amino acids (100*x*; Gibco), penicillin (50 units/ml) and streptomycin (50 μg/ml), using Falcon 75 and 175 cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cells were detached with 0.02% EDTA in phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.44 g $Na_2HPO_4 \times 2H_2O$ per litre). Cell numbers were determined in a haemocytometer.

Cell Binding Assay. The binding medium consisted of MEM with Earle's salts (Gibco) containing 25 mM *N*-(2-hydroxyethyl-1-piperazine-*N'*-ethane)-sulphonic acid (HEPES), 0.2% BSA, 0.3 mM 1,10-phenanthroline (Merck, Darmstadt) and 0.16 mM phenylmethylsulphonylfluoride (PMSF). The binding reaction was started by adding 0.5 ml of a cell suspension (0.5–2 × 10^7 cells/ml) to 12 × 75 mm polypropylene tubes containing 50 μl of unlabelled peptide in a 1:3 dilution series and 50 μl (200 000 c.p.m.) of [^{125}I]-[Phe^{13},Tyr^{19}]-MCH. The cells were incubated for 90 min at 10°C. Unbound radioactivity was removed by centrifugation of triplicate aliquots (150 μl) through a layer of 150 μl silicon oil in 0.4 ml polyethylene microtubes [12]. The oil was made up to a density of 1013 kg/cm^3 by mixing equal volumes of AR-20 and AR-200 silicon oil (Wacker Chemie, Munich, Germany). The radioactivity was counted in a Packard Riastar γ-counter and the binding data

were analysed with Ligand, an iterative non-linear regression program established for Mac personal computers [13].

RESULTS

Synthesis of [Phe^{13},Tyr^{19}]-MCH

The continuous-flow solid-phase synthesis of linear [Phe^{13},Tyr^{19}]-MCH (Fig. 1) was carried out on two different resins using two types of protecting groups for Cys and different acylation times and concentrations of amino acid derivatives. Synthesis A employed a high-loading Polyhipe PA-500 resin with 0.3–0.4 meq/g of C-terminal amino acid residue. Thiol groups of Cys were protected by Acm. Amino acid residues were applied in twofold excess and coupling times ranged between 45 and 80 min. Cleavage from the resin and deprotection was carried out by a 2 h treatment with TFA (85%) containing thioanisol as scavenger (10%) and water (5%). Linear [Cys(Acm)^{7,16},Phe^{13},Tyr^{19}]-MCH was obtained in good quality, but in only 31% yield after HPLC purification.

Synthesis B employed a lower-loading PEG-PS resin (0.15–0.2 meq/g) containing polyethyleneglycol spacers grafted on a gel-type support which were then derivatized to amino functions and consecutively enlarged with 4-(hydroxymethyl)-phenoxyacetic acid (HMPA) linker and Fmoc-Tyr(*t*Bu)-OH. Thiol groups of Cys were protected with Trt. The assembly of the peptide was done with a fourfold excess of individual residues in conjunction with shorter coupling times. The acylation with Fmoc-Pro-OH and Fmoc-Val-OH (steps 6 and 8) was repeated once. Linear [Phe^{13},Tyr^{19}]-MCH with free -SH groups was obtained through a 2 h cleavage in TFA (85%) containing EDT (5%) and thioanisol (5%) as scavengers as well as water (5%), followed by HPLC purification. The yield was 60%, i.e. approximately twice as high as compared to synthesis A. The amino acid analyses of both peptide resins and of the free linear peptides showed almost identical results with the correct compositions (Table 1).

Cyclization by iodine oxidation of [Cys(Acm)^{7,16}, Phe^{13},Tyr^{19}]-MCH produced cyclic [Phe^{13},Tyr^{19}]-MCH in good quality but in low yield. Air oxidation of linear [Phe^{13},Tyr^{19}]-MCH containing free –SH groups produced high amounts of polymer and degradation products; hardly any cyclic peptide could be detected. Therefore, oxidation of the linear [Phe^{13},Tyr^{19}]-MCH was also performed with iodine: cyclic [Phe^{13},Tyr^{19}]-MCH was obtained in 78% yield (after Sephadex G-25 chromatography) and 58% yield (after HPLC purification). Both preparations of cyclic [Phe^{13},Tyr^{19}]-MCH showed virtually identical HPLC profiles and amino acid compositions (Table 1) and FABMS revealed a protonated monoisotopic ion at 2435 a.m.u. (calculated mH^+ = 2435.61 a.m.u. for $C_{109}H_{161}N_{30}O_{26}S_4$). The increase in lipophilicity of cyclic [Phe^{13},Tyr^{19}]-MCH as compared to the linear peptide was demonstrated by analytical HPLC for synthesis B (Fig. 2).

Radioidoination of [Phe^{13},Tyr^{19}]-MCH

In order to minimize oxidation of the sulphides of Met^4 and Met^8 during radioiodination, [^{125}I]-

Table 1 Amino Acid Analysis of Peptide–Resins and Free Peptides Resulting from Synthesis A and B

Peptide	Amino Acid Residue											
	D (2)	F (2)	M (2)	L (2)	R (3)	C (2)	G (1)	V (1)	Y (1)	P (1)	W (1)	Q (1)[c]
Peptide-resin[a]	2.1	2.3	2.0	2.0	2.5	2.7	1.0	0.9	1.0	1.3	0.1	1.1
Peptide-resin[b]	1.8	2.1	2.0	2.2	2.3	3.2	0.8	1.0	1.1	1.1	0.1	1.0
[Cys(Acm)^{7,16},Phe^{13},Tyr^{19}]-MCH[a]	2.4	2.7	2.1	2.4	3.2	0.8	1.0	1.1	1.1	0.9	0.7	1.2
Cyclic [Phe^{13},Tyr^{19}]-MCH[a]	2.4	2.6	2.0	2.3	3.2	0.5	1.0	1.1	1.1	0.9	0.6	1.1
Linear [Phe^{13},Tyr^{19}]-MCH[b]	2.0	1.8	1.9	2.1	3.1	0.6	1.0	1.0	0.9	1.1	0.5	1.1
Cyclic [Phe^{13},Tyr^{19}]-MCH[b]	2.1	2.1	2.0	2.1	3.1	0.5	1.0	1.0	1.1	1.2	0.4	1.1

[a] Synthesis A.
[b] Synthesis B.
[c] Theoretical.
All ratios are expressed relative to Gly = 1.00. Tryptophan and cysteine were partly decomposed during hydrolysis.

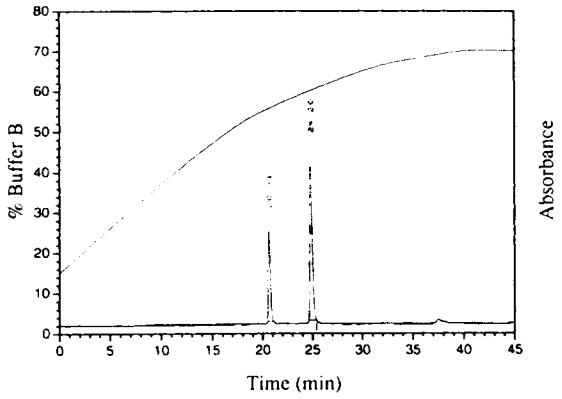

Fig. 2 Analytical RP-HPLC of linear and cyclic [Phe^{13},Tyr^{19}]-MCH of synthesis B. The two peptides (approx. 8 μg) were injected simultaneously and eluted after 20.9 (→ linear peptide) and 24.2 min (→ cyclic peptide), respectively. Conditions of chromatography are described in Materials and Methods.

[Phe^{13},Tyr^{19}]-MCH was prepared using the solid-phase coupled glucose oxidase/lactoperoxidase method (Enzymobeads) for gentle oxidation of iodine, as originally developed for α-MSH [9] and then modified for sMCH [4]. The structural difference between sMCH and human MCH necessitated several adaptations: (1) a longer reaction time (60 min), (2) the addition of a low amount (20 mM) of DTT when the reaction was terminated, (3) immediate purification of the tracer on the mini-column after the reaction. This procedure produced homogeneous [^{125}I]-[Phe^{13},Tyr^{19}]-MCH tracer which retained the biological activity.

Receptor Binding of [^{125}I]-[Phe^{13},Tyr^{19}]-MCH

When the binding properties of [^{125}I]-[Phe^{13},Tyr^{19}]-MCH tracer were tested on B16-F1, G4F and G4F-7 mouse melanoma, human RE melanoma, COS-7, PC12 and CHO cells as well as human fibroblasts, preliminary binding data indicated that the G4F-7 clone contained the highest number of MCH receptors. Therefore, the binding characteristics of the tracer were tested in detail with this cell line. Association of [^{125}I]-[Phe^{13},Tyr^{19}]-MCH to its receptor reached equilibrium after a 90 min incubation at 10°C. At 22°C or 37°C, the non-specific binding of the tracer was considerably higher, even in the presence of a set of protease inhibitors (data not shown). Specific binding to G4F-7 cells was saturable: Scatchard analysis of saturation-binding experiments using increasing concentrations (0.04–1.2 nM) of [^{125}I]-[Phe^{13},Tyr^{19}]-MCH produced a linear curve (Fig. 3) from which the following dissociation constant and receptor number were calculated: $K_D = 1.18\times10^{-10}$ M and B_{max} = 1090 sites/cell.

Competition binding experiments with the other cell lines using a constant amount (200,000 c.p.m.) of [^{125}I]-[Phe^{13},Tyr^{19}]-MCH and increasing concentrations of human MCH demonstrated the presence of low numbers of MCH receptors on B16-F1, G4F, human RE melanoma, COS-7 and PC12 cells. No MCH receptors were found on CHO cells and on human fibroblasts.

The specificity of binding of [^{125}I]-[Phe^{13},Tyr^{19}]-MCH to G4F-7 and PC12 cells was assessed in competition binding experiments using MCH, α-MSH, rANF(1–28), NPY, PACAP and substance P as competitors. With the exception of MCH and rANF(1–28), none of the peptides displaced [^{125}I]-[Phe^{13},Tyr^{19}]-MCH tracer. rANF(1–28) was found to bind to the MCH receptor on PC12 cells, but with a two- to fourfold lower affinity than MCH. In G4F-7 cells, the affinity of rANF(1–28) was even lower (data not shown). This cross-reaction may reside in a certain similarity between MCH and ANF molecules and their recognition pattern in the ligand binding site of the MCH receptor.

DISCUSSION

This paper describes the first reliable radioreceptor assay for MCH binding sites, based on a newly designed analogue of human/rat MCH which can

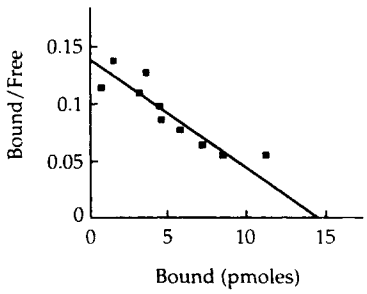

Fig. 3 Scatchard analysis of saturation binding experiments using G4F-7 cells and 0.04–1.2 nM [^{125}I]-[Phe^{13},Tyr^{19}]-MCH tracer alone (→ total binding) or in the presence of 0.7 μM MCH (→ non-specific binding). The curve represents the Scatchard transformation of specific binding data points, i.e. the difference between total and non-specific binding. The K_D was 0.118 nM and B_{max} was 1090 sites/cell.

be radioiodinated at its C-terminal end. This peptide, [Phe^{13},Tyr^{19}]-MCH, was prepared by the continuous-flow solid-phase methodology using two different resins and two ways of protection of cysteine residues. Although a precise quantitative comparison is not possible, it seems that the synthesis of this compound was faster on the new PEG-PS resin and gave higher yields after cleavage than the synthesis using Polyhipe PA-500 resin. A more important aspect, however, was the way of protection of the cysteine residues. Synthesis A, in which Acm-protected Cys was used, corresponded to a previous synthesis of sMCH [14], except for the protection of Arg (Pmc instead of Mtr) and Trp (Boc-indol instead of unprotected indol). Although the yields of linear [Cys(Acm)^{7,16},Phe^{13},Tyr^{19}]-MCH and [Cys(Acm)^{5,14}]-sMCH were very similar, the former was obtained with less side-products than the latter, mainly due to the protection by Boc of Trp-indol. On the other hand, the disulphide formation of the Acm-protected human/rat MCH sequence was even less efficient than that of sMCH. Therefore, synthesis B using Trt-protected Cys is the more favourable approach because the linear [Phe^{13},Tyr^{19}]-MCH with unprotected -SH groups can be cyclized by gentle oxidation, producing cyclic [Phe^{13},Tyr^{19}]-MCH in excellent yield (58% after HPLC purification). It is important to note that purification of the linear peptide to homogeneity is an important prerequisite for successful disulphide formation. These data prove that an efficient synthesis of MCH analogues can be obtained by carefully selecting the resins, side-chain protection, cleavage mixtures and cyclization procedures, even though the sequence is regarded as 'difficult'.

The discovery of MCH receptors on mouse and human melanoma cell lines is unexpected because of the lack of effect of sMCH on mouse melanoma cells [10], but on the other hand, not surprising in view of the physiological function of MCH in melanophores of lower vertebrates [1, 2]. Although little is known about the role of MCH in mammals, it is possible that MCH receptors on mammalian pigment cells are more than evolutionary remnants. It may turn out that in higher vertebrates, MCH is not a physiological antagonist of melanocyte-stimulating hormone (MSH) as in teleost fishes but rather shares a function with atrial natriuretic factor (ANF). Interestingly, both PC12 cells and different mouse melanoma cells express receptors for ANF as demonstrated with [^{125}I]-labelled ANF as radioligand. While in both types of cell ANF is able to displace [^{125}I]-[Phe^{13},Tyr^{19}]-MCH tracer in a competition-binding assay (to a lesser degree in melanoma cells than in PC12 cells), MCH was not able to displace [^{125}I]-labelled ANF (R. Drozdz and A. N. Eberle, unpublished observation). This indicates that there are topological similarities between the MCH and ANF molecules when interacting with MCH receptors but that the structural requirements for ligand binding of MCH receptors differ from those of ANF receptors. In view of the structural similarity of ANF and MCH and their precursors it is possible that their receptors share a certain functional relation.

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