60 YEARS OF POMC

Purification and biological characterisation of melanotrophins and corticotrophins

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Abstract

The remarkable conservation of the primary structures and anatomical location of dogfish α-melanocyte-stimulating hormone (MSH), corticotrophin-like intermediate lobe peptide (CLIP) and adrenocorticotropic hormone (ACTH) compared with mammals reinforced the tissue-specific processing hypothesis of ACTH peptides in the pituitary gland. The cloning of dogfish pro-opiomelanocortin (POMC) led to the identification of δ-MSH and simultaneously revealed the high conservation of the γ-MSH sequence during evolution. These studies have also shown that β-MSH is much less conserved during evolution and in some species is not even processed from β-LPH. Human pro-γ-MSH potentiates the corticosteroidogenic activity of ACTH and peptides generated from its N-terminal, in particular big-γ-MSH, appear to have adrenal mitogenic activity. Human big-γ-MSH (from the zona intermedia) may also cause the adrenache. The review finishes with a cautionary note with regard to the misdiagnosis of the ectopic ACTH syndrome in which partial processing of ACTH can result in large concentrations of α-MSH and CLIP, which can interfere in the performance of two-site immunoassays, and the problem of the correct disulphide bridge arrangement in synthetic N-POMC peptides is also discussed.

Key Words

- MSH
- ACTH
- LPH
- adrenal
- POMC

Introduction

I hope the reader will forgive me for the personal reflections in this article, but it is probably the last I will write on the fascinating subject of the adrenocorticotropic hormone (ACTH)/melanocyte-stimulating hormone (MSH) precursor, which we now know as pro-opiomelanocortin (POMC). Who would have predicted that starting a research career on the hormone that caused dogfish skin to darken would lead me on to unravelling the many biological activities present in the most complex multi-peptide hormone precursor involved in many aspects of human physiology and pathophysiology? I was one very lucky scientist. I will describe the characterisation of dogfish MSH and ACTH, the discovery of corticotrophin-like intermediate lobe peptide (CLIP), pro-γ-MSH and big γ-MSH and their involvement in corticosteroidogenesis and adrenal growth, and speculate on their secretion from the zona intermedia and roles in the development of both the foetal adrenal and prepubertal reticularis zones. I will finish on a note of caution when using a two-site immunometric assay in the diagnosis of the ectopic ACTH syndrome when POMC processing is similar to that in the zona intermedia.

Dogfish melanotrophin

It was 50 years ago when I was first introduced to the peptides derived from pro-opiomelanotropin. For my PhD
project, I was assigned to work on MSH from the dogfish (Squalus acanthias) by James Munro Dodd FRS, the then Professor of Zoology at the University of Leeds. This species of elasmobranch had been carefully chosen to study MSH as it was commercially available, it changed colour and its pituitary was anatomically separated into easily dissected distinct lobes. The comparatively large neurointermediate lobe that contained the MSH bioactivity was easily removed from the many heads of this cartilaginous fish that I needed to isolate enough MSH for characterisation. At that time, only the primary structures of mammalian α-MSH and β-MSH were known, although there was no associated colour change in these species, but MSH bioactivity had been detected in the pituitary glands of a number of lower vertebrates such as frogs, lizards and fish, which were adapted to the background presumably by releasing MSH bioactivity from their respective pituitary glands, which then caused dispersion of melanin in their melanophores.

**Dogfish α-MSH**

The first MSH I isolated and characterised from extracts of some thousands of dogfish pituitary glands was an α-type MSH (Lowry & Chadwick 1970a). Although the elasmobranchs diverged from the main line of vertebrate evolution 300 million years ago (Young 1962), to my surprise, the only difference between the amino acid sequence of dogfish MSH and that of mammalian α-MSH was a conservative hydrophobic amino acid substitution (methionine for valine) at the C-terminal (Fig. 1). Dogfish α-MSH however lacked the acetyl group at the N-terminal, although it did have the C-terminal amide. Circulating peptides are cleared by the kidneys recognising the free amino group at the N-terminal of the appropriate peptide (Medeiros & Turner 1994), so it would make sense for the dogfish, which would be swimming quickly from dark to light backgrounds, to secrete a melanotrophin with a much shorter half-life due to an unacetylated N-terminal amino group, resulting in more rapid colour change.

Although the amino acid sequence of mammalian α-MSH was exactly the same as the first 13 residues of ACTH, 50 years ago it was not known whether this was just by chance, whether they were a result of gene duplication, part of the same precursor caused by internal gene duplication, or whether α-MSH was derived from ACTH; peptide processing was poorly understood at that time. The incredible conservation of the α-MSH sequence over 600 million years of evolution suggested that if dogfish ACTH had the dogfish α-MSH amino acid sequence at its N-terminal with the methionine substitution at position 13, there was a distinct possibility that α-MSH was derived from ACTH (Lowry & Chadwick 1970a,b). This conservation indicated that the receptor(s) involved was also ancient and highly conserved.

**The discovery of CLIP**

Sandy Scott started his PhD studies to work on fish ACTH at St Andrews University but was seconded to St Bartholomew’s Hospital to take advantage of the ACTH antibodies, which were in routine use in radio-immunoassays. During this time, he made some interesting observations regarding the distribution of N- and C-terminal ACTH immunoreactivity in the rat pituitary, and our subsequent collaboration resulted in the discovery of the 18–39 C-terminal fragment of ACTH in the pars intermedia, which we called corticotrophin-like intermediate lobe peptide or CLIP (Scott et al. 1973). Thus, there was now a distinct possibility that ACTH was synthesised both in the corticotrophs of the pars distalis and in the cells of the pars intermedia, but that, in the latter, ACTH is further processed to form α-MSH and CLIP (Fig. 1). From research carried out by others described in this issue (Chretien & Mbikay 2016), we now know that this processing in the pars intermedia is by cleavage by prohormone-converting enzyme 2 (PC2) at the Arg17–Arg18 peptide bond, followed by removal of the resulting C-terminal basic residues 17–15 by carboxypeptidase E (CPE) and then conversion of the nascent C-terminal Gly14 residue to an amide by prohormone-amidating monoxygenase (PAM).
(Kumar et al. 2016). It should be noted that PC2 does not cleave the trypsin-resistant Arg\(^{18}\)-Pro\(^{19}\) imido peptide bond in CLIP. The same resistant imido peptide bond affects the processing of substance \(P\) from its precursor in a similar way as the sequence around the N-terminal of substance \(P\) is \(-\text{Arg}^{1}\text{-Arg}^{1}\text{-Pro}^{2}\)-, and the processing is also between the two arginine residues.

**Dogfish ACTH and CLIP**

The fact that the rostral lobe in the dogfish pituitary, which contains ACTH bioactivity, is anatomically separate from the neurointermediate lobe made the purification of dogfish ACTH and CLIP relatively easy (Lowry et al. 1974). When we found that the amino acid compositions of the purified C-terminal proteolytic fragments from both peptides were identical, complete sequence analysis of the C-terminal region of ACTH was relatively simple. The primary structures of dogfish ACTH and CLIP are shown in Fig. 1, and as expected, dogfish ACTH has the Met\(^{13}\) residue substitution. As we had hoped, dogfish CLIP occupied exactly the same position as in mammalian ACTH with an amino acid sequence similar to human ACTH particularly in the acidic residues at positions at 28–30, 33 and 38, which have been assumed to balance or interact with the basic residues that dominate the N-terminal region of the 39mer. Other substitutions also retained the overall hydrophobic/hydrophilic positions in CLIP adding strength to the importance of the whole of the ACTH primary structure.

**Dogfish γ-MSH, δ-MSH and β-endorphin**

As dogfish POMC cDNA was sequenced (Amemiya et al. 1999), it has been much easier to identify the rest of the dogfish melanotrophins and endorphin by their position in the precursor. Figure 2 was originally published by Bennett and coworkers (Bennett et al. 1974) and shows the final purification of dogfish \(\alpha\)-MSH (peaks B and C) and \(\beta\)-MSH (peak A) from an extract of neurointermediate lobes. The amino acid compositions of the 280 nm absorbing peaks, D and E, were identical, but as there was no associated MSH bioactivity, no further characterisation was carried out because manual sequencing was a laborious technique at that time. It was only after a third cryptic melanotrophin sequence (γ-MSH) was implicated in bovine POMC (Nakanishi et al. 1979) that we submitted these dogfish neurointermediate lobe peptides to a fast and simple automated sequenator (MacLean & Lowry 1981). As they had little or no melanotrophic activity (compare synthetic bovine γ-MSH), they were initially assumed to be a dogfish γ-type MSH. On examination

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**Figure 2**

Cation-exchange chromatography of the main MSH-active fractions from a previous gel filtration separation of an extract of 1000 dogfish neurointermediate lobes. The shaded areas represent MSH bioactivity as detected in an *in vitro* frog skin bioassay (Chadwick & Lowry 1970). (A) Dogfish β-MSH, (B) deamidated dogfish α-MSH, (C) dogfish α-MSH, (D) deamidated dogfish δ-MSH and (E) dogfish δ-MSH. This figure was originally published in the *Biochemical Journal* (Bennett et al. 1974). Reproduced with permission of The Biochemical Society.
of the complete sequence of dogfish POMC, the third MSH that we had sequenced did not occupy the same position as γ-MSH but occupied a fourth position named δ-MSH by Amemiya and coworkers (Amemiya et al. 1999). We thus did not appear to find any evidence for processed dogfish γ-MSH, which from its predicted amino acid sequence would have co-eluted with peaks B and C (Fig. 2). We are now therefore able to conclude that as with mammalian γ-MSH, there is little or no processing upstream of the dogfish γ-MSH sequence (note that no typical upstream double basic processing site is present), giving the naturally occurring dogfish γ-MSH peptide an N-terminal extension of some 50 residues similar to that seen in human γ-MSH (Fig. 3). There are three interesting observations to note: (1) the remarkable conservation evident when comparing the dogfish γ-MSH region with its human equivalent (-YVMGHFRWNK-amide vs -YVMGHFRWDRF-amide: Asn/Asp and Lys/Arg are both conserved, single-base substitutions), (2) the positions of the cysteine residues (and consequent disulphide bridges) in the extended N-terminal region and (3) the overall retention of positions of hydrophobic and hydrophilic residues. This lends credibility to the notion that what I now will name ‘big γ-MSH’ (i.e. N-terminally extended γ-MSH) serves an important physiological function with its own putative receptor; I will return to this later. Big γ-MSH should not be confused with pro-γ-MSH, which appears to be the main processed peptide found in the pars distalis and has the C-terminal extension containing the post-translational N-linked glycosylated moiety. Again, the position of the consensus glycosylation motif and its position C-terminal to the γ-MSH sequence are conserved in dogfish POMC (Fig. 3).

To this day, I do not know why approximately half the dogfish α- and δ-MSHs were also found in the deamidated forms. I can only conclude that it may be an extra control mechanism that the dogfish has evolved to render a peptide susceptible to carboxypeptidase action with an even shorter half-life in vivo. In our in vitro MSH bioassay, both forms of dogfish α-MSH were equi-active.

Mammalian β-MSH

Fifty years ago, the situation regarding the occurrence of a processed human β-MSH was also somewhat confused. An octadecapeptide that contained the same heptapeptide core MSH sequence (Met–Glu–His–Phe–Arg–Trp–Gly) as α-MSH had been isolated and sequenced from a number of different mammalian pituitary posterior lobes. An equivalent peptide had not been identified in extracts of murine pituitary glands, but a β-MSH-like peptide with a four-amino acid N-terminal extension (Ala–Glu–Lys–Lys) had been isolated from human pituitaries extracted at mild pH (Dixon 1960). In those mammals in which the primary structure of β-lipotrophin (β-LPH) was elucidated, it was revealed that they all contained the sequence of their appropriate β-MSHs (banded by double basic amino acids: Lys–Arg or Arg–Arg), and thus, it was naturally
assumed that β-LPH could be the precursor of β-MSH by simple proteolytic processing at the double basic residues by trypsin-like activity (Chretien & Mbikay 2016, Smyth 2016). The situation with human β-MSH was atypical, as it appeared not to be cleaved from its β-LPH at the N-terminal Lys–Lys double basic motif but in a non-trypsin-like manner at an Ala–Ala peptide bond. Moreover, when human pituitaries were extracted at strong acid pH to minimise any artificial proteolytic activity, no evidence could be found for a β-MSH-sized peptide: all the β-MSH immunoreactivity eluted on gel filtration in the position of β-LPH (Scott & Lowry 1974). However, if a human pituitary was extracted and incubated at mild pH, β-MSH-like immunoreactive material started to appear in the expected elution position of synthetic human β-MSH. Thus, we concluded that the 22-residue human β-MSH was an extraction artefact being cleaved from β-LPH if mild pH was used during the initial extraction from pituitary glands (Scott & Lowry 1974). Furthermore, when plasma from a patient suffering from Nelson’s disease was similarly submitted to gel filtration, all the immunoreactive β-MSH material eluted in the position of β-LPH (Bloomfield et al. 1974). This led to the reinterpretation of reports of β-MSH in the corticotrophs of the human pituitary and in the blood of patients with Cushing’s and Nelson’s diseases, which was in fact β-LPH. The observation could have also been due to the lack of a pars intermedia in the human pituitary gland, but there have never been any reports of β-MSH-like peptide even in extracts of foetal pituitary glands in which there is a distinct zona intermedia; it is more likely to be due to the fact that natural processing by prohormone-converting enzymes rarely takes place after Lys–Lys residues.

Nevertheless, these findings gave rise to the hypothesis that ACTH and LPH were biosynthesised in both the corticotrophs of the pars distalis and the cells of the pars intermedia. ACTH and LPH were released intact from the pars distalis under positive control from the hypothalamus but were processed further in the pars intermedia (ACTH to MSH and CLIP and LPH to β-MSH) and were under negative dopaminergic hypothalamic control (Lowry & Scott 1975).

I have mentioned that murine LPH is not processed to a β-MSH-like peptide nor is the heptapeptide core sequence conserved. The diversity of β-MSH-like sequences is perhaps demonstrated in the two species of dogfish, Squallus acantthias (Bennett et al. 1974) and Scyliorhinus canaliculus (Love & Pickering 1974). They seem to be processed to a 16mer and a 18mer, respectively, and there is little common sequence homology between them, perhaps suggesting that there is no functional pressure on this part of the precursor. However, the opiate pentapeptide sequence (YGGFM) that follows β-MSH in human POMC occupies the same position at the N-terminal of dogfish β-endorphin (Amemiya et al. 1999).

The recent proposal by Lee and coworkers that a mutation in the β-MSH region of POMC, which changes the tyrosine 221 residue to a cysteine residue, has led to the proposal that a lack of β-MSH activity is also responsible for obesity (Lee et al. 2006). As the natural processing of human LPH to the true β-MSH-18mer has never been observed in the hypothalamus either, there is an alternative explanation to this observation. One unusual feature of normal human POMC is that there is already a single cysteine residue in the joining peptide (Fig. 4), which even after processing leads to the joining peptide is a stable homodimer. This suggests that human POMC with a second cysteine residue in the β-MSH/β-LPH region (Fig. 4) would form into a second inter-chain disulphide bridge, making this mutant POMC a more stable dimer during biosynthesis. There is also the possibility of intra-chain disulphide bridge or aggregation due to tandem multiple inter-chain disulphide bridges. One or more of these scenarios could result in altered processing in the hypothalamus and thus may be responsible for the observed obesity.

**Common precursor**

I had the temerity to suggest in the review I wrote on finishing my PhD in 1969 that there could be a ‘large molecule with all three types of activity’ (namely, ACTH, MSH and LPH) (Lowry & Chadwick 1970b). Some years later, we did partially purify a potential common precursor from human pituitaries, which had both ACTH and LPH/β-MSH immunoreactivity (Lowry et al. 1976b, 1977). However, Nakanishi and coworkers put all the speculation to rest 3 years later with the cloning of bovine POMC cDNA (Nakanishi et al. 1979).

When I started working on the adrenal function in the 1970s, there seemed to be some doubt that ACTH was responsible for all aspects of adrenal physiology particularly with respect to adrenal growth (Rao et al. 1978) in which immunoneutralisation with an ACTH antiserum did not inhibit compensatory growth following unilateral adrenalectomy in rats. Adrenal growth did however seem to be linked to pituitary corticotroph function as compensatory growth was inhibited by dexamethasone treatment. Once we realised that it was β-LPH that was co-synthesised and co-secreted with ACTH, and we had sufficient purified human LPH, attempts were made to use...
it with and without synthetic ACTH to stimulate adrenal growth in hypophysectomised rats, but without any success (PJ Lowry and AP Scott, unpublished observations). Nor did β-LPH potentiate the adrenocorticosteroidogenic activity of ACTH in vitro.

Isolation and characterisation of human pro-γ-MSH

The cloning of the precursor POMC gene, which confirmed that ACTH and LPH were part of the same precursor, simultaneously provided possible evidence of a large N-terminal more complex glycopeptide, pro-γ-MSH, being co-secreted from the pars distalis corticotroph during stress. The sequence of POMC predicted that pro-γ-MSH had two disulphide bridges and an N-linked polysaccharide downstream of γ-MSH, and that removal of a 26-residue leader sequence would reveal a nascent N-terminal tryptophan on pro-γ-MSH. As tryptophan fluorescence had previously been observed in corticotrophs (Håkanson et al. 1975), this property was used to monitor the purification of the putative ‘pro-γ-MSH’ glycopeptide from human pituitary glands. In the initial extraction, we used strong acid to avoid any proteolytic activity. The glycopeptide we purified, which we called human pro-γ-MSH, proved to be a 76-residue peptide with an N-terminal tryptophan. It had two disulphide bridges between the cysteine residues at positions 2 and 24, and those at 8 and 20 (Bennett et al. 1985), and contained an N-linked polysaccharide attached to the asparagine at position 65 in the region downstream from the γ-MSH sequence (Estivariz et al. 1980). As expected, when a homologous immunoassay for human pro-γ-MSH was used to detect this relatively stable glycopeptide in unextracted plasma (Hope et al. 1981) in various pathological conditions (Cushing’s disease, Nelson’s disease, Addison’s disease and the ectopic ACTH syndrome), it mirrored the concentrations of ACTH obtained using a laborious extracted assay. Even with the advent of unextracted two-site assays for ACTH (Hodgkinson et al. 1984), given the labile nature of the latter compared with pro-γ-MSH, it is surprising to me that the potentially more robust and sensitive immunoassay for pro-γ-MSH has not replaced the ACTH immunoassay for the diagnosis of pathologies of the hypothalamic/pituitary/adrenal (HPA) axis. I will discuss some other shortcomings of the two-site ACTH immunoassay particularly with respect to diagnosing the ectopic ACTH syndrome later.

Initial experiments failed to find any activity with purified human pro-γ-MSH with respect to MSH bioactivity or adrenal growth-promoting properties (Estivariz et al. 1980). When added alone to adrenal cells in vitro, it...
did not cause steroidogenesis but did promote RNA synthesis (Al Dujaili et al. 1982). It also potentiated the steroidogenic activity of ACTH several-fold in perfused rat adrenal cells in vitro (Al Dujaili et al. 1981). This potentiation was completely abolished in the presence of actinomycin D, indicating that the increase in RNA synthesis was linked to the potentiating activity of pro-γ-MSH (Al Dujaili et al. 1982). This ability to increase ACTH-induced corticosteroidogenesis several-fold is largely ignored in the diagnosis of human adrenal pathology and can be particularly relevant in some cases of the ectopic ACTH syndrome, in which ACTH is processed mainly to desacyt yl α-MSH and CLIP, but pro-γ-MSH is still partly intact.

**Adrenal growth activity of N-POMC peptides**

Once antisera to pro-γ-MSH were available in the laboratory, we were able to show that rats treated with this antisera showed a decrease in thymidine incorporation in their adrenal glands, suggesting that pro-γ-MSH may be a mitogenic precursor (Estivariz et al. 1980). This was confirmed when either trypsinised pro-γ-MSH or purified human N-terminal POMC (N-POMC) fragments were used in vivo or in vitro (Estivariz et al. 1982). One mitogenic N-terminal pro-γ-MSH fragment that we isolated from human pituitaries, being relatively small, was easily characterised as N-POMC(1–28) from its amino acid composition and N- and C-terminal analyses (McLean et al. 1981). Given that this fragment was isolated in significant amounts from a side fraction of large-scale growth hormone purification (using mild pH throughout) and that there was no evidence for large amounts of intact pro-γ-MSH, it was concluded that it had been generated from the latter at the Leu 28/Ser 29 peptide bond. Generated from the latter at the Leu 28/Ser 29 peptide bond intact pro-γ-MSH, it was concluded that it had been generated from the latter at the Leu 28/Ser 29 peptide bond and that there was no evidence for large amounts of intact pro-γ-MSH, it was concluded that it had been generated from the latter at the Leu 28/Ser 29 peptide bond.

The presence of a C-terminal amide on big γ-MSH would suggest the action of the enzyme PAM, implicating pars intermedia-like processing (see Fig. 4). As the amounts of big γ-MSH isolated were similar to those expected for desacyt yl α-MSH found in the human pituitary gland, and the latter (and probably PAM) has been found to be mainly associated with zona intermedia tissue in the human pituitary, it is likely that big γ-MSH is a product of the same cells/tissue.

**The human pituitary zona intermedia, the adrenal foetal zone and the adrenarche zona reticularis**

In most mammals, the pars intermedia is separated anatomically from the pars distalis by a cleft and usually forms a homogeneous band of cells on the face of the neural lobe; the combination of the two is referred to as the posterior lobe. In humans, higher apes, manatees and dugongs, whales and dolphins, elephants and birds, there is no cleft and no evidence for a functional pars intermedia of the pituitary gland. In the foetal human pituitary, there is an anatomical location of a band of intermedia-like cells referred to as the zona intermedia, and there is also evidence in foetal extracts for significant concentrations of α-MSH-like and CLIP-like immunoreactive peptides (Silman et al. 1976). In a comprehensive immunocytochemical study in adult human pituitary glands, using a specific α-MSH antibody, immunoreactive α-MSH cells were found in a total of 97 out of 100 pituitary glands, but only 10 of the 97 showed a marked concentration of α-MSH cells in the area commonly seen in the pars anterior than in the zona intermedia, and in 41 cases, α-MSH cells were completely absent from the zona intermedia (Coates et al. 1986).

Secretion of all immunoreactive POMC peptides from rat pars intermedia cells is under common dopaminergic inhibitory control (Jackson & Lowry 1983). The use of
a dopaminergic antagonist provided indirect evidence for active zona intermedia-like cells in prepubertal children, as it resulted in a significant increase in plasma α-MSH-like concentrations but not in post-pubertal controls. The elution position on HPLC suggested that the immunoactive material was mainly desacetyl α-MSH (Facchinetti et al. 1995). Desacetyl α-MSH does stimulate adrenal steroidogenesis, albeit at high concentrations, but its secretion would be accompanied by significant concentrations of other potential biologically active intermedia-like processed POMC peptides, e.g. big γ-MSH.

Thus, it may not just be a coincidence that human adrenal development in the foetus and in the prepubertal child occurs exactly at the times when the zona intermedia is active. More specifically, one or more intermedia-like processed POMC peptides could be involved in controlling both the development of the adrenal foetal zone and the development of the prepubertal adrenal zona reticularis; the prime hormonal candidate is big γ-MSH.

In the pituitary glands of mammals with a distinct pars intermedia, the separate tonic inhibition by dopamine of lactotrophs in the pars distalis is anatomically feasible, but as there is no cleft separating the zona intermedia cells in the human pituitary and some appear to migrate into the pars distalis (Coates et al. 1986), differential release of big γ-MSH and prolactin would be difficult to control. Thus, the appearance of breast buds in both the neonate and prepubertal child may simply be due to the lower hypothalamic dopamine concentrations needed at these times to increase the release of big γ-MSH from active zona intermedia cells/tissue to stimulate the development of the adrenal foetal and the prepubertal reticularis zones simultaneously allowing the release of significant amounts of prolactin.

Most studies that have been carried out with the melanocortin receptors have been with relatively small synthetic γ-MSH peptides, yet the high conservation of the structures of the dogfish and human big γ-MSHs (and the potent adrenal mitogenic activity of the latter) suggests that there has been evolutionary pressure from a putative receptor for big γ-MSH.

**Immunooassay of blood samples from Cushing’s disease and the ectopic ACTH syndrome**

**Radioimmunoassay**

Originally, single-site competitive radioimmunoassays were used to detect ACTH in the plasma of patients suffering from Cushing’s disease, and antibodies raised against the 1–24 region of ACTH usually gave the best indication of biological activity. Blood samples generally needed a prior extraction technique as the high concentration of proteins in plasma interferes with the binding of radiolabelled ACTH with the limited amount of antibody used in the classical competition immunoassay format. Using ACTH antibodies directed towards the C-terminal region, the measurement of ACTH in blood samples taken from patients with pituitary-dependent Cushing’s disease usually gave similar results but with samples from cases with the ectopic ACTH syndrome, much higher readings were often recorded. This was because some ectopic tumours, with regard to processing, behave more like a pars intermedia, resulting in the N-terminal 1–24 antibody (while not reacting with smaller α-MSH-like peptides) still giving a good indication of ACTH biological activity but C-terminal antibodies giving a much higher reading as, generally, they would not distinguish between ACTH and CLIP. In the first ectopic ACTH tumour, I studied (Ratcliffe et al. 1973), >95% of the C-terminal ACTH immunoreactivity eluted in the position of CLIP (Fig. 5). After further purification, amino acid and N- and C-terminal analyses were consistent with ACTH(18–39). Only a small amount of bioactive material eluted in the position of ACTH(1–39). MSH bioactive peaks eluted in the positions of LPH and α-MSH. In the second ectopic ACTH-secreting tumour I studied, almost all the ACTH was intact (Lowry et al. 1976a). Thus, it would appear that ectopic tumours can behave as a pars distalis corticotroph in processing POMC to ACTH, or like a pars intermedia, processing POMC to mainly desacetyl α-MSH and CLIP (Fig. 4). In some ectopic tumours, there thus may be large concentrations of potentiating and mitogenic N-POMC peptides with very little corticosteroidogenic activity (small or negligible amounts of ACTH but large amounts of desacetyl α-MSH). The accompanying large amount of mitogenic N-POMC peptides would give rise to gross bilateral adrenal hyperplasia and Cushing’s syndrome with relatively normal or even low ACTH concentrations. This confusion would not be the case if an N-POMC immunoassay was used in diagnosis.

**Two-site immunometric assay**

As these assays use much higher concentrations of two complementary antibodies (one N-terminally and the other C-terminally directed) than the single antibody used in the competitive immunoassay, there is less
interference from plasma proteins allowing direct assay of samples and thus avoiding time-consuming prior extraction (Hodgkinson et al. 1984). Generally, they give reliable results with samples from patients with pituitary-dependent Cushing’s disease as the adult human pituitary gland secretes intact ACTH. Again, it is with plasma samples from patients with the ectopic ACTH syndrome that problems can be encountered.

If the ectopic tumour behaves like a pars distalis and most of the ACTH is the simple 1–39 peptide, then the result of the two-site assay will reflect the amount of ACTH biological activity in the sample. One feature of ACTH which is often ignored is that 20–30% of ACTH present in the human pituitary gland is phosphorylated at the serine residue at position 31 (Bennett et al. 1983). This does not affect its biological activity, but if this highly charged negative group interferes with the binding of the C-terminal antibody used in the two-site assay, then a lower reading with phosphorylated ACTH would result. The problem can be more extreme with some ectopic tumours in which the bulk of ACTH(1–39) can be phosphorylated (Massias et al. 1994).

The problem of getting erroneous results with the two-site assay becomes even more acute when the ectopic tumour is processing ACTH like a pars intermedia. As the two-site assay signal relies on both antibodies binding to the same contiguous peptide, the high concentrations of ACTH fragments such as CLIP that are present will use up most of the C-terminal antibody, and an inaccurate low reading of any intact ACTH(1–39) present will result. If there is little CPE processing, this would result in high concentrations of steroidogenic ACTH(1–17), which would react with the N-terminal antibody making it unavailable for any binding to intact ACTH(1–39). Processing of ACTH (by CPE and PAM) to desacetyl α-MSH still results in a full agonist at the adrenal gland ACTH receptors. Thus, with ectopic ACTH tumours, significant amounts of adrenal bioactive peptides can be present but with very little signal from the two-site ACTH assay. It is only when the tumour is removed and submitted to immunocytochemical examination that its true endocrine nature can be revealed.

It should also be remembered that when studying rats, a significant amount of its ACTH is glycosylated (Bennett et al. 1982). The reason for this is that there is a single base change in the ACTH region of the rat POMC gene, which results in the aspartate residue found at position 29 in human ACTH becoming an asparagine in

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**Figure 5**

rat ACTH. This creates the classical N-linked glycosylation motif Asn–Xxx–Ser at residues 29–31. The resulting carbohydrate moiety attached to the side chain of Asn29 would not be expected to change the biological activity at the ACTH receptor but would give the peptide a much longer half-life in vivo. The bulky carbohydrate group would however interfere with any C-terminally directed antibody giving very low signals in the two-site ACTH immunometric assay. It is not inconceivable that some humans may carry this mutation resulting in glycosylated ACTH with the same assay problems and an adrenocorticosteroidogenic agonist with a much longer half-life in their blood.

The importance of the correct disulphide bridges in N-POMC peptides

Disulphide bridge arrangement during polypeptide biosynthesis is governed by the ‘Anfinsen’s dogma’, which states that there is enough information in the polypeptide chain to direct the formation between the appropriate cysteine residues. Short synthetic peptides containing the relevant cysteine residues can however form the wrong disulphide bridges. This unfortunately seems to be the case with N-POMC peptides, as early attempts produced short synthetic N-POMC peptides, which were less potent adrenal mitogens than the purified peptide, in particular N-POMC(1–36) which was almost inactive as an adrenal mitogen (Estivariz et al. 1988). Rather than forming the natural 2–24 and 8–20 bridge configuration (Fig. 3), the N-POMC(1–36) peptide apparently formed the 2–8 and 20–24 bridges in the so-called calcitonin configuration (Henry Keutmann, personal communication). Early reports of ‘calcitonin’ immunoreactivity being part of the ACTH precursor in the pars intermedia (Deftos et al. 1985) until it was added to this confusion. It should be noted that the ‘calcitonin type’ was the first disulphide arrangement reported in human N-POMC (Seidah et al. 1981) until it was corrected (Bennett et al. 1985). The disulphide bridges in fragments of POMC(1–48) were also found to be unstable (H Bennnett, personal communication). Fortunately, Fassnacht and coworkers (Fassnacht et al. 2003) before they embarked on their adrenal mitogenic studies in vitro with N-POMC(1–28) were aware of the disulphide bridge problem but after two unsuccessful attempts (M Fassnacht, personal communication), embarked on a synthetic strategy to direct the correct alignment in their preparation of N-POMC(1–28). The same peptide preparation, however, seemed to be inactive in rescuing adrenal growth in POMC-/POMC- mice (Coll et al. 2006). Although the physiological relevance of the use of herculean doses of both N-POMC(1–28) and ACTH(1–24) could be questioned, the instability of the disulphide bridge arrangement in concentrated solutions in long-term experiments (and the effects this could have on biological activity) would lend caution to the conclusions reached from this study.

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