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ESTERASE ACTIVITY OF SYNTHETIC FRAGMENTS OF HUMAN ADRENOCORTICOTROPHIC HORMONE

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The anterior pituitary hormone adrenocorticotrophin (ACTH) has been extensively studied in terms of structure-function relationships and in vivo and in vitro activities of different synthetic fragments of ACTH have been characterized. Here we describe the ability of synthetic fragments of ACTH to hydrolyze a fluorogenic esterase substrate 4-methylumbelliferyloleate (MUBO). The measured esterase activities (in μmol 4-MU mol^{-1} s^{-1}) were 79.7 for $ACTH_{1-13}$, 385.9 for $ACTH_{3-18}$, 503.0 for $ACTH_{1-19}$, 1249.9 for $ACTH_{1-24}$ D-ser3, and 1350 for $ACTH_{1-24}$. Although the significance of the observed esterase activities in the actual molecular mechanisms of action of ACTH remains to be established it is worth noticing that the esterase activities of the different ACTH fragments closely parallel their reported ability to activate the brain lipase as well as their in vivo ability to induce steroidogenesis in adrenal cortex.

The peptide hormone adrenocorticotrophin ($ACTH_{1-39}$) possesses a number of biological activities including the induction of stereoidogenesis in adrenal cells (1), lipolysis in adipocytes (2) as well as melanotropic and behavioral effects (3). Effects of ACTH include the activation of adenylate cyclase resulting in an increase of intracellular cAMP (1, 4) and a consequent increase in steroidogenesis (5). Based on structure activity correlation studies it has been suggested that consecutive sequences in the molecule

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Abbreviations: ACTH, adrenocorticotrophic hormone; MU, methylumbelliferone; MUBO, methylumbelliferyloleate.

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represent different types of information. At least two functionally distinct sites are considered to exist in ACTH. Residues 4-10 of ACTH are common to ∂- and ß-MSH and lipotropin (ß-LPH) and thus the N-terminal decapeptide ("message segment") has been considered to represent the minimal requirement for triggering the biological responses (6). Indeed $ACTH_{4-10}$ has been shown to produce many of the responses of $ACTH_{1-39}$ (1, 7-8). The other functional sequence ("the address sequence") which is located between residues 11-24 has been considered to be important in the affinity of the hormone to its receptor and in potentiating the message.

Binding of ACTH to liposomal membranes has been demonstrated (9-11). In the interactions of ACTH with phospholipid membranes the presence of both the hydrophilic 11-24 (address) sequence and the hydrophobic 1-10 (message) sequence were required for specific membrane interactions (10-11). Based on these findings a so called primary amphiphilic structure was suggested for $ACTH_{1-24}$ (10). The binding of different ACTH fragments to lipid membranes closely parallels their biological activities (12).

ACTH has also been shown to activate 7-30 fold an acylglycerol lipase present in brain (13-14). In addition an alkaline shift in the pH optimum by ACTH was evident (13), similar to what has been observed for pancreatic lipase and co-lipase (15) as well as for lipoprotein lipase (LPL) and apolipoprotein C II (16). The activation of brain lipase by ACTH was observed also in vitro with purified enzyme preparations. It was suggested that ACTH facilitates enzyme-substrate association in a manner similar to described for pancreatic lipase and LPL activators, co-lipase and apolipoprotein C II, respectively (13, 17). The exact mechanism of activation of these lipases has remained unresolved. However, esterase activity has been described for apolipoprotein C II and co-lipase (18). This esterase activity was suggested to play a crucial role in the activation of these lipases by co-lipase and apolipoprotein C II (18-19).

We now describe esterase activity also for various synthetic fragments of ACTH. Although the biological significance of this finding is not clear it is of interest that the esterase activities of the different ACTH fragments parallel their ability to activate brain lipase, as well as their ability to induce steroidogenesis in adrenal cortex.

Materials and Methods

Chemicals

The synthetic fragments ACTH- (1-24), ACTH- (1-24 D-ser 3), ACTH- (1-19), ACTH- (3-18), and ACTH- (1-13) were generously provided by Ciba-Geigy (Paris, France). The modified hog insulin and glucagon were purchased from Novo (Denmark). Subtilisin BPN' (bacillus amyloliquefaciens), α-chymotrypsin, and pancreatic lipase (from bovine pancreas) were all obained from Sigma and were used without further purification. Lipoprotein lipase was purified from bovine skimmed milk (20).

Esterase assay

Hydrolysis of 4-methylumbelliferyloleate (MUBO) was determined by following the appearance of the hydrolytic fluorescent product 4-methylumbelliferone (4-MU) (21). Fluorescence intensities were measured with Kontron SFM 23 spectrofluorometer using excitation and emission wavelengths of 360 and 450 nm respectively. Assays were performed in magnetically stirred quartz cuvettes thermostatted to 30 °C. A 5 mM stock solution of 4-methylumbelliferyloleate (Koch-Light laboratories, Buckinhamshire, England) was prepared in a nonionic detergent ethylene monomethyl ether. This was stored at - 20 °C and used within 24 h of preparation. The final reaction system contained in a total volume of 2.0 ml from 6.5 to 200 nmol of 4-MUBO as indicated, and from 0.03 % to 1 % (vol/vol) of monomethyl ether in 50 mM Tris/HCl buffer pH 8.5. Corrections for background hydrolysis were made by recording the baseline fluorescence for a minimum of 5 minutes. The assay was calibrated using a standard curve of 4-methylumbelliferone (Sigma) at pH 8.5. Reactions were started by adding the indicated amounts of the peptides.

Results and Discussion

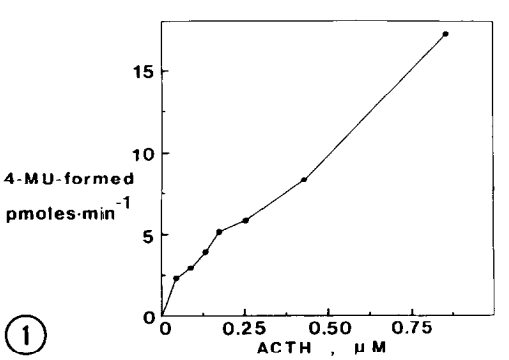
In the course of our studies on the mechanism of peptide hormone action we observed the ability of ACTH to produce hydrolysis of a synthetic fluorogenic esterase substrate, methylumbelliferyloleate (MUBO). These esterase activities of synthetic ACTH fragments are summarized in Table I. The measured hydrolytic activities of all the synthetic peptides studied increase linearly both with increasing

Table I. Esterase activities of synthetic fragments of ACTH measured as described under materials and methods using 1-5 µg per assay each of the above peptides

ACTH FRAGMENTS	Catalytic activity µmol 4-MU mol^{-1} s^{-1} (mean + s. d. n= 4-6)
1-13	79.7 ± 18.4
3-18	385.9 ± 45.4
1-19	503.0 ± 8.9
1-24 D-ser 3	1249.9 ± 50.2
1-24	1350.4 ± 50.2

peptide concentration and with increasing substrate concentration the latter exhibiting apparent saturation, illustrated in Fig's 1 and 2 for $ACTH_{1-24}$.

Comparison of the esterase activities of the different fragments reveals a significant increase in the catalytic rate upon the addition of the pentapeptide to produce $ACTH_{3-18}$ from $ACTH_{1-13}$ as shown in Table I. Both $ACTH_{3-18}$ and $ACTH_{1-19}$ possess considerable esterase activity. This activity is likely to be located in the so called "basic core" lys15 lys16 arg17 arg18 (22). However, the esterase activity is further increased upon completion of the synthetic polypeptide chain to produce the fragment $ACTH_{1-24}$, Table I.



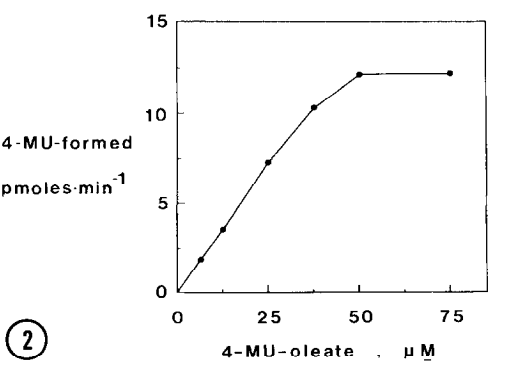


Fig. 1. The liberation of the fluorescent hydrolytic product 4-methylumbelliferone (4-MU) as a function of $ACTH_{1-24}$ concentration. Concentration of the substrate was 25 µM.

Fig. 2. The formation of methylumbelliferone as a function of the substrate 4-methylumbelliferyloleate concentration. The concentration of $ACTH_{1-24}$ was 0.25 µM.

Table II. Esterase activities of some peptide hormones compared to those of hydrolytic enzymes

	Catalytic activity μmol 4-MU mol^{-1} s^{-1}
ACTH (1-24)	1350
Insulin	191
Glucagon	233
Lipoprotein lipase	10450
Pancreatic lipase	6583
Chymotrypsin	6516
Subtilisin	52596

Activities were measured using 1-5 μg per assay each of the above peptides or protein. Substrate concentration was 37.5 μM.

Therefore the possibility should also be considered that a conformational change takes place in the tertiary structure of the peptide molecule upon increasing the chain length of a given fragment, subsequently resulting in increased esterase activity. This is supported by the finding that both the hydrophobic (1-10) and hydrophilic (11-24) sequences were required for the specific interactions of ACTH with phospholipid membranes (10).

For comparison the esterase activity of two other peptide hormones, semisynthetic human insulin and porcine glucagon, was also tested for hydrolytic activity against MUBO, Table II. Both of these hormones show esterase activity although the activities were clearly lower than those measured for ACTH. Activities of 191 and and 233 μmol 4-MU mol^{-1} s^{-1} were observed for human insulin and porcine glucagon respectively. In Table II are also shown esterase activities for several other proteins and hydrolytic enzymes. It is noteworthy that the hydrolytic rates of the true lipolytic enzymes against MUBO are also comparatively low. Thus the activities of pancreatic lipase and lipoprotein lipase were 6583 and 10450 μmol 4-MU mol^{-1} s^{-1} respectively. The recorded low rates of catalysis are likely to be due to the large difference between MUBO and the physiological substrates for these lipases, tri- and diacylglycerols. The highly unnatural character of the fluorogenic substrate used by us must be emphazised. Thus the highest catalytic activity was observed for subtilisin, the most nonspecific of the enzymes studied. MUBO was chosen as it allows the detection of very low amounts of esterase

activity. However, it is of interest that the catalytic activity observed for $ACTH_{1-24}$ is comparable to those of the so called catalytic antibodies (23).

The esterase activity described here could be considered non-spesific. However, it is not a general property of proteins. Accordingly no esterase activity against MUBO was found in phosvitin, calmodulin, human apolipoproteins E and B or poly-L-serine (18). Furthermore, it also worth noticing that $ACTH_{1-13}$ possessed only minimal esterase activity but the addition of pentapeptide to produce $ACTH_{3-18}$, which is only three amino acids longer, enhanced the esterase activity approximately five fold (Table I).

The findings in this paper indicate that ACTH should have a of reactive site capable of producing hydrolysis of MUBO. The specificity of this activity cannot be judged by the results of the present study. Yet, it is worth noticing that the measured rates of hydrolysis of MUBO by different synthetic fragments of ACTH parallel the reported biological activities as well as their ability to adsorb on lipid membranes (10-11). Furthermore, immunoreactive and bioreactive ACTH (ACTH-like activity) has been demonstrated in the brain of normal and also hypophysectomized rats (24-25). It has been considered somewhat unlikely that this activity of ACTH would be mediated by cAMP dependent processes (17). Direct activation of brain lipase by ACTH has been demonstrated and a co-lipase like role was suggested for ACTH in this activation process (14,17). Therefore, the role of ACTH in the activation of brain lipase could perhaps be similar to that described for apolipoprotein C II in the activation of lipoprotein lipase and co-lipase in the activation of pancreatic lipase (18-19). In the activation of these lipases it was suggested that apolipoprotein C II acts on an acyl-enzyme intermediate and cleaves the hydrolytic product fatty acid from the serine residue of the enzyme active site, thus generating a free enzyme (18-19). Similarily, the action of ACTH on brain lipase could involve a hydrolytic step. This is supported by the present findings that ACTH possesses esterase activity and furthermore that the esterase activities of different synthetic fragments of ACTH closely parallel their reported ability to activate brain lipase (13, 17).

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