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Enzymes

Identification of α_2 Macroglobulin as a Major Serum Ghrelin Esterase**

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Obesity or excessive body weight leads to a morbidity, known collectively as metabolic syndrome, and afflicts around one billion people worldwide.[1] Reduction in calorific input continues to be the most effective means of treatment, with approaches that range from simple dieting to the extreme of bariatric surgery.[2] Regulating the desire to eat through pharmaceutical intervention is a lofty goal with a troubled history, mostly because of an inability to separate regulation of satiety from the body's fundamental reward system. The end result, depending on the mechanism, is that potential therapeutic agents become drugs of abuse or lead to depression.[2b]

The profound role ghrelin plays in feelings of satiety and the fact that it is considerably upstream of the body's reward system has lead to intense interest in the biochemical pathway of this hormone. Logically, therapeutic intervention in ghrelin regulation has attracted the interest of the pharmaceutical industry in the pursuit of anti-obesity agents.[2b,3]

Ghrelin is an appetite-stimulating hormone secreted from endocrine cells in the stomach. Ghrelin was first identified in 1999 by Kojima et al. as the endogenous ligand for the growth-hormone receptor 1a (GHSR1a), a G-coupled receptor.[4] It has been well established that the potent growth hormone secretagogue plays an important metabolic role in regulating food intake and energy homeostasis;[5] more recently, ghrelin has been implicated in the regulation of glucose metabolism.[6] Ghrelin is synthesized as a 117 amino acid polypeptide (preproghrelin), which is translocated through the endoplasmic reticulum membrane followed by proteolytic cleavage of the N-terminal 23 amino acid signal sequence to produce proghrelin (94 amino acids). Subsequent posttranslational modification events include cleavage of proghrelin after residue Arg28 and octanoylation of residue Ser3 with an eight-carbon fatty acid chain to generate the mature ghrelin protein, which is 28 amino acids in length. Ghrelin is the only known protein to contain an *n*-octanoyl moiety and, importantly, this posttranslational modification is essential for binding to and activation of GHSRla (Figure 1 a).

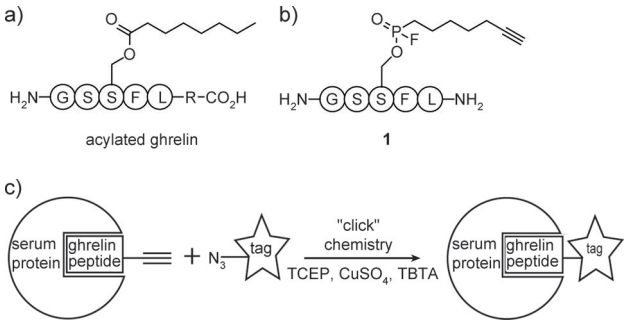

Figure 1. a) Mature ghrelin (R = 23 amino acids). b) Ghrelin-activity-based probe **1**. c) Labeling of serum proteins by a copper(I)-catalyzed azide–alkyne cycloaddition reaction. G = glycine, F = phenylalanine, L = leucine, S = serine. TCEP = tris(2-carboxyethyl)phosphine, TBTA = tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine.

Under fasting conditions, ghrelin production is increased and correlates with a higher level of circulating ghrelin in serum; conversely, serum levels of ghrelin decrease immediately after food intake. The enzyme that is responsible for the posttranslational acylation and activation of ghrelin was recently identified as the acyltransferase enzyme ghrelin O-acyltransferase.[7] However, the enzymes and pathways involved in ghrelin deacylation and proteolysis are poorly understood.

Ghrelin has been reported to be deacylated and proteolyzed into smaller peptide fragments by serum and tissue homogenates.[8] Two enzymes have been proposed to participate in the deacylation of ghrelin in serum, liver carboxylesterase (rat serum) and butyrylcholinesterase (human serum).[8] Additionally, acyl-protein thioesterase 1/lysophospholipase I has been isolated from both rat stomach homogenates and bovine serum; a recombinant form of this enzyme deacylates ghrelin in vitro.[9]

The above-mentioned studies relied on the identification of potential deacylating enzymes from analytically separated serum or homogenated fractions, followed by analysis of the proteins present in the active samples. Our laboratory pursued a different approach by use of a mechanism-based probe that could irreversibly bind to ghrelin-deacylating enzymes directly within serum. Upon conjugation of a

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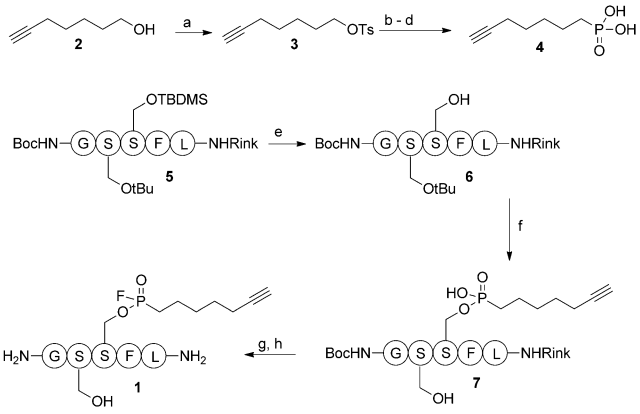
reporter tag to the now enzyme-bound probe, deacylating enzymes could be identified by proteomics.

Within serum, inactivation of ghrelin is thought to occur predominately by way of deacylation. In addition, the work of De Vriese et al., in which the stability of ghrelin in serum was monitored in the presence of a variety of known hydrolase inhibitors, strongly implicated serine hydrolases as the dominant participants in ghrelin degradation.[8] The serine hydrolases are a large family of enzymes that represent more than 2% of the eukaryotic proteome and share a common chemical mechanism involving an activated serine as part of a catalytic diad or triad. These enzymes hydrolyze ester and amine bonds in small-molecule and protein substrates. Carboxylesterases and cholinesterases also belong to the serine hydrolase family.

By virtue of their catalytic mechanism, serine hydrolases are susceptible to the phosphonofluoridate warhead when it is incorporated within a chemical structure with sufficient recognition elements for the target enzyme to attempt to hydrolyze it. Thus, phosphonofluoridates have found wide use in the design of irreversible inhibitors of serine hydrolases and have become a valuable tool in the production of activity-based probes for this family of enzymes.[10] By use of this approach, we generated a "bait" ghrelin-like molecule that contains a phosphonofluoridate warhead to capture serum-based serine hydrolase enzymes that participate in the deacylation of circulating ghrelin.

To design an appropriate ghrelin analogue, we noted that the amino acid sequence of ghrelin is highly conserved among mammals and the first ten amino acids are identical. Short truncated peptides that contain the first four to five residues of ghrelin are potent agonists of GHSR1a with efficiencies similar to the full-length protein; the tetrapeptide Gly-Ser-Ser(*n*-octanoyl)-Phe is considered to be the active core of ghrelin.[11] However, while the activities of the truncated tetra- and pentaghrelin peptides are essentially equivalent, the binding affinity of the tetrapeptide is increased approximately 16-fold by the addition of the fifth residue, Leu.[11] The octanoyl chain of ghrelin also undergoes an important binding interaction with GHSR1a and is accommodated in a hydrophobic pocket of the receptor.[12] Therefore, ghrelin probe **1** was designed as a short peptide that contains the first five amino acids of ghrelin with a phosphonofluoridate warhead at the Ser3 residue in place of the physiological *n*-octanoyl modification (Figure 1b). A key feature of the probe is the conservation of the hydrocarbon chain length within **1**, this factor may also be important for the binding of and selectivity toward ghrelin deacylating enzymes. Lastly, an alkyne group at the end of the hydrocarbon chain allows the conjugation of an azide reporter tag by a copper(I)-catalyzed azide–alkyne cycloaddition reaction, or "click" chemistry, for subsequent analysis and protein identification (Figure 1c).[13]

The chemical synthesis of **1** was achieved as shown in Scheme 1. Primary alcohol **2** was prepared from 2-heptyn-1-ol by following the procedure of Li and O'Doherty,[14] and subsequent tosylation gave **3**.[15] The tosyl group of **3** was displaced with LiBr to give the intermediate bromide, which was, without further purification, heated with tris(trimethylsilyl) phosphite to give phosphonic acid **4** after hydrolysis of



Scheme 1. Synthesis of phosphonofluoridate **1**. Reagents and conditions: a) TsCl (1.1 equiv), NEt_3 (2.1 equiv), DMAP (catalytic), CH_2Cl_2, 0°C to RT, 16 h, 80%; b) LiBr (4 equiv), acetone, 14 h; c) $P(OSiMe_3)_3$ (1.0 equiv), 150°C, 8 h; d) H_2O, RT, 4 h, 22% (2 steps); e) 0.1 M TBAF, DMF, RT, 2×15 min; f) **4** (5.5 equiv), DIC (12 equiv), DIPEA (5 equiv), DMAP (5.5 equiv), DMF, RT, 2 days; g) DAST (4.0 equiv), CH_2Cl_2, RT, 2 h; h) 95% TFA, 2.5% TIPS, 2.5% H_2O. DAST = (diethylamino)sulfur trifluoride, DIC = diisopropylcarbodiimide, DIPEA = diisopropylethylamine, DMAP = 4-dimethylaminopyridine, DMF = *N*,*N*-dimethylformamide, TBAF = tetra-*n*-butylammonium fluoride, TFA = trifluoroacetic acid, TIPS = triisopropylsilyl, Ts = toluene-4-sulfonyl.

the trimethylsilyl group. The TBDMS protecting group of peptide **5**, which was still attached to the resin from the solid-phase synthesis, was selectively removed by using TBAF, followed by Mitsunobu coupling of partially deprotected peptide **6** with phosphonic acid **4** to give resin-attached phosphonic acid modified peptide **7**. Compound **7** was then converted to the phosphonofluoridate with DAST, followed by TFA-mediated cleavage from the resin to give crude phosphonofluoridate-modified peptide **1** after precipitation by diethyl ether. While phosphonofluoridate **1** could be purified by preparative HPLC, all attempts to concentrate the HPLC fractions that contained the phosphonofluoridate product resulted in hydrolysis to the corresponding phosphonic acid modified peptide. As a result, crude phosphonofluoridate **1** was used for all labeling experiments.

For the enzyme capture phase, probe **1** was incubated with albumin-depleted rat serum for 90 min at 37°C to allow cross-linking between the mechanism-based probe and reactive serum enzymes. The samples were then subjected to a cycloaddition reaction with a rhodamine–azide tag (RhN_3), and the labeled proteins within the serum sample were subsequently separated by SDS-PAGE and visualized by fluorescence imaging (Figure 2a).[13b] Two dominate bands were excised and in-gel trypsin digested, and the resulting peptide fragments were analyzed by nanoLC-MS/MS. The results were searched against protein databases and the two labeled proteins were putatively identified.

One of the proteins identified was rat liver carboxylesterase (E.C.3.1.1.1). Notably, this enzyme has been proposed previously to play a major role in the deacylation of ghrelin in rat serum.[8] Studies conducted with commercially purified porcine liver carboxylesterase validated the ability of this enzyme to catalyze the deacylation reaction.[8] The identifi-

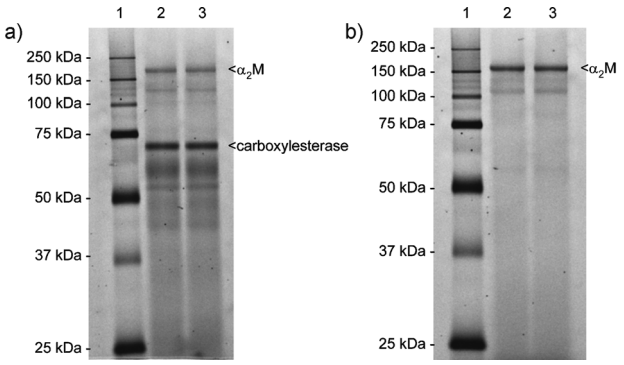


Figure 2. Protein labeling with probe **1**. a) Serum samples or b) purified human α_2M were incubated with **1** and reacted with RhN_3 by a cycloaddition reaction after which the reactions were analyzed by SDS-PAGE and visualized by in-gel fluorescence scanning. Lane 1: molecular weight marker, lanes 2 and 3: duplicate labeling reactions.

cation of liver carboxylesterase in our study illustrated the proof-of-concept and we were encouraged by this positive result. The second protein that was identified was α_2 macroglobulin (α_2M), which is a large homotetrameric plasma glycoprotein with a molecular weight of approximately 725 kDa for the human form.[16] α_2M can inhibit all mechanistic classes of proteinases through a serpin-like mechanism that involves proteinase-mediated proteolysis of a "bait" region within α_2M, followed by immediate and major conformational changes of α_2M, thus ultimately "trapping" the enzyme.[17] This proteinase-induced conformational change then triggers the exposure of binding sites for proteins such as cytokines, growth factors, and hormones.[17] Additionally, the hidden receptor-binding domain of α_2M is revealed, thus leading to receptor-mediated endocytosis and rapid clearance of the complex from circulation.[17] In general, α_2M plays an important role in the regulation of numerous biological processes.

The capture of α_2M from rat serum by probe **1** (Figure 2a) suggests that α_2M possesses a ghrelin recognition site that contains an appropriately positioned reactive serine residue. However, to the best of our knowledge such hydrolytic activity has never been identified for α_2M. To further delineate this previously undocumented esterolytic activity, we employed our previously reported HPLC-based ghrelin hydrolase assay.[18] The substrate for this assay is a full-length acylated ghrelin that contains the fluorophore 7-methoxycoumarin-4-acetic acid (MCA) at the C terminus of the protein. Solutions containing purified human α_2M (2 μM) and low micromolar concentrations of the ghrelin–MCA substrate (ca. 3–15 μM) demonstrated that α_2M can indeed catalyze the deacylation of ghrelin. To further characterize this reaction, steady-state kinetic studies were conducted with α_2M and varying concentrations of ghrelin–MCA. Typical Michaelis–Menten kinetics were observed with α_2M, which displayed a Michaelis constant (K_m) of (24 ± 3) μM and a rate constant (k_{cat}) of $(2.3 \times 10^{-2} \pm 0.1 \times 10^{-2})$ min^{-1}. Probe **1**, which was used to initially isolate the α_2M protein from the labeling studies, was further investigated as an inhibitor of this reaction. Studies were conducted at approximately the K_m value of ghrelin–MCA with varying concentrations of **1**; a dose-dependent inhibition response was observed, thereby verifying that this fluorophosphonate pentapeptide probe was an effective mimic of the acylated ghrelin substrate. Additionally, labeling studies that involved purified human α_2M and probe **1** further validated the ability of the human protein to react with the acylated ghrelin mimic (Figure 2b).

A number of protease inhibitors that are known to inhibit hydrolytic enzymes in serum were evaluated.[8] The mechanism-based serine hydrolase probes phenylmethylsulfonyl fluoride (PMSF), 4-(aminophenyl)methanesulfonyl fluoride (*p*-APMSF), and bis(*p*-nitrophenyl) phosphate (BNPP) were evaluated as inhibitors of the deacylating catalytic activity of α_2M. Other compounds such as ethylendiaminetetraacetate (EDTA), eserine, and NaF were not expected to inhibit the deacylating activity of α_2M, thereby providing additional evidence that α_2M is a serine hydrolase. All compounds were tested at approximately the K_m value of the substrate. Only two compounds displayed significant inhibition at a concentration of 1 mM or less after preincubation for 30 min. BNPP, a carboxylesterase inhibitor, showed 40% inhibition at 1 mM and 0% inhibition at 100 μM. *p*-APMSF, a serine protease inhibitor, showed 100% inhibition at 50 μM and 80% inhibition at 5 μM, thus making *p*-APMSF the most potent inhibitor of the screen. *p*-APMSF is an irreversible inhibitor that is mechanistically analogous to phosphonofluoridate compounds. While it was not explicitly characterized, we can place a lower limit on the *p*-APMSF/α_2M specificity constant of $470\ M^{-1}s^{-1}$ (see the Supporting Information).

Irreversible inhibitors can titrate the number of catalytic sites present in an enzyme sample, thus making them a reliable method for quantitating the amount of active enzyme present in a sample.[19] The instability of ghrelin probe **1** made its precise quantitation difficult, therefore we selected *p*-APMSF as a titrant to identify the number of catalytic sites within the α_2M tetramer. The protein α_2M is a homotetramer and, in principle, each subunit could contain a functional active site. Titration experiments conducted at a concentration of 2 μM tetramer/8 μM monomer of α_2M and varying concentrations of *p*-APMSF (0–10 μM) are shown in Figure 3. Extrapolation of the velocity to the x-intercept gives an active site concentration of approximately 6 μM. Within experimental uncertainty, there are four active sites per α_2M tetramer.

Could the α_2M–ghrelin esterase activity be of physiological importance? The plasma half-life of ghrelin has been

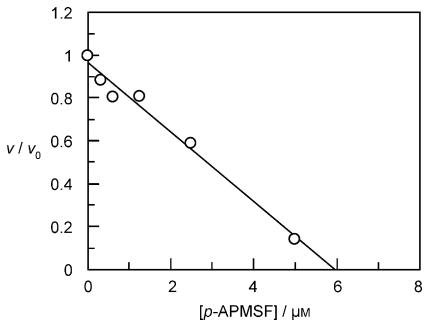


Figure 3. Titration studies with the inhibitor *p*-APMSF and α_2M. v = velocity, v_0 = velocity at [*p*-APMSF] = 0.

estimated to be 240 min, thus affording a first-order rate constant of 0.003 min^{-1}. Therefore, the physiological concentration of ghrelin must be low relative to the K_m values of any preferred substrates for reported esterases.[8] As such, each esterase is operating under substrate limiting (first-order) conditions. The overall half-life of ghrelin is then governed by the sum of the concentrations of each participating enzyme multiplied by its $(k_{cat}/K_m)_{ghrelin}$. The concentration of plasma α_2M has been reported in the range of 0.25 gL^{-1} to 2 gL^{-1}. Taking a midrange value of 1 gL^{-1} gives a plasma concentration of 1.4 μM, which when coupled with the above-mentioned ghrelin specificity constant gives a plasma first-order rate constant of 0.0014 min^{-1}, which accounts for half of the total rate. This calculation thus places α_2M as an important participant in ghrelin metabolism.

Mechanism-based probes offer an objective approach to the identification of physiologically relevant enzyme activities. The method confirmed the alternative approach of De Vriese et al. by capturing carboxylesterase.[8] On the other hand, the capture of α_2M by ghrelin probe **1** and the subsequent discovery of ghrelin esterase activity of α_2M were unanticipated and unprecedented, as there are no prior reports of α_2M to possess esterase activity. The studies reported here constitute one step toward the goal of better understanding nutrient–hormone interactions that contribute to metabolic disease states.

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