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Mechanisms of glucagon degradation at alkaline pH

Nicholas Caputo[a], Jessica R. Castle[a], Colin P. Bergstrom[a], Julie M. Carroll[b], Parkash A. Bakhtiani[a], Melanie A. Jackson[a], Charles T. Roberts Jr.[a,b], Larry L. David[a], W. Kenneth Ward[a,*]

[a] Oregon Health and Science University, United States
[b] Oregon National Primate Research Center, United States

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ABSTRACT

Glucagon is unstable and undergoes degradation and aggregation in aqueous solution. For this reason, its use in portable pumps for closed loop management of diabetes is limited to very short periods. In this study, we sought to identify the degradation mechanisms and the bioactivity of specific degradation products. We studied degradation in the alkaline range, a range at which aggregation is minimized. Native glucagon and analogs identical to glucagon degradation products were synthesized. To quantify biological activity in glucagon and in the degradation peptides, a protein kinase A-based bioassay was used. Aged, fresh, and modified peptides were analyzed by liquid chromatography with mass spectrometry (LCMS). Oxidation of glucagon at the Met residue was common but did not reduce bioactivity. Deamidation and isomerization were also common and were more prevalent at pH 10 than 9. The biological effects of deamidation and isomerization were unpredictable; deamidation at some sites did not reduce bioactivity. Deamidation of Gln 3, isomerization of Asp 9, and deamidation with isomerization at Asn 28 all caused marked potency loss. Studies with molecular-weight-cutoff membranes and LCMS revealed much greater fibrillation at pH 9 than 10. Further work is necessary to determine formulations of glucagon that minimize degradation and fibrillation.

1. Introduction

Glucagon is a 29-amino acid polypeptide whose major function is to increase plasma glucose by promoting glycogenolysis and gluconeogenesis [13]. Early studies with somatostatin established the role of glucagon in the minute-to-minute physiologic regulation of glucose homeostasis [7]. Its current therapeutic role is limited to rescue therapy for hypoglycemia [20].

Several recent investigations have shown the value of automated administration of glucagon with insulin in the setting of closed-loop glycemic control in persons with type 1 diabetes [4,5,10,11,29]. Because of its potency, glucagon (unlike glucose) offers the potential for delivery from a small portable pump worn on the body. However, such an application is hampered by its intrinsic physical instability and chemical degradation in aqueous solution. In aqueous environments, glucagon forms polymerized gels and fibrils, a process affected by temperature, concentration, and ionic strength [2,8,25,26]. While fibrillation occurs rapidly at acidic pH, it is greatly minimized in alkaline solutions [14,33]. Although fibrillation is minimized at a pH of 10, degradative processes such as deamidation tend to be prevalent at alkaline pH [15,22]. Other degradative processes include oxidation at Met residues, a process to which glucagon is susceptible [21].

Joshi and Kirsch extensively studied the mechanisms of glucagon degradation at acid pH and found that deamidation was caused by direct hydrolysis of the amide side-chain by water [19]. In addition, they found unexpectedly that Gln deamidation occurred more commonly than Asn deamidation [16,18]. In the current study, we explore the mechanisms of glucagon degradation in the alkaline pH range, a promising range for glucagon stability due to its low degree of fibrillation. Since the effect of specific biochemical changes such as deamidation or oxidation on receptor binding and biological activity are not always clear, we used a cell-based bioassay in the current study to measure the consequences of the aging-induced modifications. In addition, aged native glucagon was characterized by liquid chromatography coupled with mass spectrometry (LCMS). To better understand the effects of specific degradation products, the results for aged glucagon were compared to synthesized peptides that were identical to known glucagon degradation products.

Abbreviations: HPLC, high performance liquid chromatography; MS, mass spectrometry; RT, retention time; EGFP, enhanced green fluorescent protein; PKA, protein kinase A; MW, molecular weight; Da, Dalton; Fmoc, N-9-fluorenylmethoxycarbonyl; rp, reverse phase; CHO-K1, Chinese hamster ovary cells, K1 line; EC_{50}, effective concentration for 50% maximal activity.

* Corresponding author at: 3181 SW Sam Jackson Park Road, Portland, OR 97239, United States. Tel.: +1 503 494 1226; fax: +1 503 346 6951.
E-mail address: wardk@ohsu.edu (W.K. Ward).

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2. Research design and methods

2.1. Glucagon and degradation peptide synthesis

Native human glucagon and modified amino acid glucagon analogs were synthesized by AmbioPharm, Inc. (Beech Island, SC). The peptides were synthesized using standard stepwise N-9-fluorenylmethoxycarbonyl solid-phase peptide synthesis chemistry (Fmoc) and assembled in a linear fashion on Fmoc-Thr(tBu)-Wang resin [1]. The peptide was purified to >99% and shipped as a lyophilized solid.

2.2. Sample formulation and aging

Glycine buffer was prepared from solid glycine (Sigma–Aldrich, Saint Louis, MO) and brought to pH 9 or 10 with NaOH. Glucagon was added to yield a concentration of 1 mg/ml. The solutions were then passed through a 0.2 μm filter into a glass vial, sealed, and placed into a 37 °C incubator for the desired aging period. Unaged samples were immediately placed into a −20 °C freezer. After aging was completed, the samples were frozen at −20 °C until analysis.

2.3. Green fluorescent protein–protein kinase A bioassay

The glucagon bioassay employed an engineered cell line expressing the human glucagon receptor and a fluorescent protein kinase A (PKA) catalytic subunit reporter molecule. The cAMP generated from activation of the glucagon receptor redistributes fluorescent PKA from a concentrated pattern to a diffuse, cytoplasmic pattern with a reduction in fluorescent signal. CHO-K1 cells stably overexpressing the human glucagon receptor and the catalytic domain of human PKA fused to the N-terminus of enhanced green fluorescent protein (EGFP) [35] were obtained from Thermo Scientific (Pittsburgh, PA). Cells were seeded at 20,000 cells per well in a 96-well plate and cultured overnight at 37 °C/5% CO_2 in Ham's F-12 medium supplemented with 1% penicillin–streptomycin, 100 μg/ml amphotericin B, 0.5 mg/ml G418, 1 mg/ml Zeocin and 10% FBS. After overnight culture, the cells were exposed to glucagon (serially diluted in culture medium without antibiotics) for 30 min at 37 °C/5% CO_2. Cells were then fixed in 10% formalin for 20 min, washed, and subsequently labeled with 1 μM Hoechst DAPI in PBS for 30 min.

Cells were then imaged with a Marianas fluorescence microscope (Intelligent Imaging Innovations, Inc.; Denver, CO) to quantify the DAPI and EGFP signals at 10×. Images were analyzed and quantified for fluorescence using Slidebook 5.0 (Intelligent Imagining Innovations). The fluorescence data measures the cellular response to glucagon as a GFP/DAPI ratio. The data was normalized against a zero glucagon control (designated as 100% fluorescence) then plotted as a dose response curve (x = dose of glucagon in pg/ml; y = % of bioactivity loss). The loss of fluorescence is the measure of the cellular response to glucagon. Using XLfit software program V. 5.3.1.3 (IDBS, Guildford, UK), a fitted curve was created using a sigmoidal Boltzman equation $\left(y = A + \left[\frac{B-A}{1+e^{\frac{C-x}{D}}}\right]\right)$. Using the fitted curve, the 50% bioactivity level (EC_{50}, a metric of potency, in pg/ml) was determined as the midpoint between the high and low data plateaus.

2.4. Liquid chromatography–mass spectrometry

Unaged, aged, and modified peptide samples were analyzed by LCMS in the OHSU Proteomics Shared Resource. Samples were diluted to 1 μM in 1% formic acid from 287 μM (equivalent to 1 mg/ml) and placed into Agilent autosampler vials, which were held at 4 °C until analysis. Approximately 10 picomoles of protein was injected into an Agilent 1100 high performance liquid chromatography (HPLC) system fitted with a reverse-phase Agilent ZORBAX SB-C18 capillary column (Agilent Technologies, Santa Clara, CA). A mobile phase of 1% formic acid and a gradient of acetonitrile from 7.5% to 45% over 60 min were used. Mass spectrometry analysis was performed in a Thermo linear ion trap Velos instrument (Thermo Fisher Scientific, Inc., San Jose, CA). Peptide MS analysis and identification was performed using Xcalibur 2.2 (ThermoFisher Scientific Inc., San Jose, CA).

Unmodified glucagon has a monoisotopic molecular weight (MW) of 3480.62 Da. Detection of oxidized or deamidated glucagon was carried out by examining species that had a MW increase of +16 or +1 Da, respectively. Other modifications were determined from the mass spectrum using a software program (Xcalibur 2.0, Thermo-Scientific) and web based proteomics tools.

2.5. Molecular weight fractionation

Size fractionation was performed by taking 100 μl of sample and applying it to an Amicon filter (Millipore Corp, Billerica, MA) with a nominal MW limit of 100 kDa. Material was centrifuged at 14,000 RPM for 5 min to separate the low and high-MW complexes. The filtrates were analyzed by LCMS as described above. The high-MW complexes (retentates) were resolubilized using 6 M urea and shaken for 30 min. After the incubation period, the filter tube was inverted into a collection tube, briefly centrifuged, and the material sent for LCMS.

2.6. Synthesis of peptide modifications

Although LCMS and bioassay data provide clues as to the identity of glucagon degradation products, the specific nature of those products can be elusive. For this reason, we synthesized modifications of glucagon and subjected those modified peptides to the same analyses (LCMS and cell-based bioassay) as for aged native glucagon. Six modifications were chosen, including deamidation, deamidation with isomerization, isomerization alone, and oxidation. The sites of these modifications encompassed much of the glucagon sequence, with two modifications occurring at the N terminus, one more centrally located and three at the C terminus. The modifications were: Gln 3 deamidated to Glu 3; Gln 3 deamidated and isomerized (rearranged) to iso-Glu 3; Asp 9 isomerized to iso-Asp 9; Met 27 oxidized to Met oxide 27; Asn 28 deamidated to Asp 28; and Asn 28 deamidated and isomerized to iso-Asp 28. Reaction diagrams for each can be found in Fig. 4A–C and are referred to as Glu3, iso-Glu3, iso-Asp9, Met(O)27, Asp28, and iso-Asp28. Like native glucagon samples, the solutions of modified glucagon analogs were created to a concentration of 1 mg/ml before being analyzed by the glucagon bioassay and by LCMS. These samples were resuspended immediately before analysis to avoid aging in the aqueous state.

2.7. Statistical analysis

Comparisons were analyzed by a two-sided Student's t-test with significance defined as $p < 0.05$. Results are presented as mean ± SD unless otherwise specified.

3. Results

3.1. Bioassay shows loss of glucagon potency after aging

The bioassay was used to compare fresh vs aged glucagon. Fig. 1A shows that after aging glucagon at pH 10 for 7 days at 37 °C, potency declined as measured by an increase of EC_{50} by 1.6 to 4.7-fold vs fresh glucagon ($p < 0.001$, $n = 6$ for aged and fresh). Fig. 1B shows a