Enzyme linked spectroscopic assays for Glyoxylate: The use of Peptidylglycine alpha-Amidating Monoxygenase for the discovery of Novel alpha-Amidated hormones

Sarah Elizabeth Carpenter
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Enzyme Linked Spectroscopic Assays for Glyoxylate; The Use of Peptidylglycine
alpha-Amidating Monoxygenase for the Discovery of Novel
alpha-Amidated Hormones

by

Sarah Elizabeth Carpenter

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy Department of Chemistry College of Arts and Sciences University of South Florida

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Keywords; glyoxylate, platform technology, mouse joining peptide, glycolate oxidase, chemi-luminescence, calcitonin gene related peptide, high performance liquid chromatography

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Dedication

I would like to dedicate this dissertation to Mrs. Charlotte R. Carpenter.
Acknowledgements

I would like to acknowledge the help and support of my colleagues Neil R. McIntyre and Edward W. Lowe Jr., and my parents Melissa A. Leffler, Herbert L. Carpenter and Allan T. Leffler, M.D. I would also like to acknowledge the support of my major professor David Merkler Ph.D., and my committee members for the guidance and direction provided to me as a graduate student. In conclusion, I would also like to acknowledge the following people: Terrance C. Owen Ph.D., Angelo P. Consalvo, Ted Gauthier Ph.D., Patricia Mueisner Ph.D., Ellen Verdel Ph.D., Julie Harmon Ph.D., Brian Space Ph.D., James Garey Ph.D., Steven Grossman Ph.D., the USF Chemistry Department Faculty and Staff, and Unigene Laboratories, Inc.
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Enzyme Linked Spectroscopic Assays For Glyoxylate; The Use of Peptidylglycine alpha-Amidating Monooxygenase for the Discovery of Novel alpha-Amidated Hormones

Sarah Elizabeth Carpenter

ABSTRACT

Peptide hormones are responsible for cellular functions critical to the survival of an organism. Approximately 50% of all known peptide hormones are post-translationally modified at the C-terminus. Enzymatic oxidative conversion of C-terminal glycine extended peptide precursors results in an α-amidated peptide and glyoxylate. Peptidylglycine α-amidating monooxygenase (PAM) is the single known enzyme responsible for catalyzing this reaction. PAM is an O₂, Cu(II), and Zn(II) dependent bifunctional enzyme. Initially, PAM hydroxylates the glycy1 α-carbon followed by dealkylation of the hydroxylated intermediate to an α-amidated product and glyoxylate. PAM is also responsible for the conversion of glycine extended fatty acids to fatty acid amides and glyoxylate. PAM catalyzes the activation of all glycine-extended prohormones including biomolecules ranging from neuro to physio-homeostatic hormones. Identification of α-amidated hormones from a biological source has been severely hindered by the lack of a specific assay for this distinctive class of biological
hormones, indicating that numerous α-amidated hormones remain undiscovered. Based on the selective in situ chemistry of PAM, a novel and specific assay was developed for the discovery of α-amidated hormones. The identification of novel α-amidated hormones will lead to an increased understanding of post-translational modifications and will pioneer a new understanding of α-amidated hormone biosynthesis, regulation, and bioactivity. Discovery of novel α-amidated biomolecules could also lead to their use as pharmaceuticals as there are several currently marketed α-amidated peptide based pharmaceuticals.

Inhibition of PAM in cell culture leads to the accumulation of glycine-extended hormones in the conditioned medium. The medium was fractionated by chromatographic techniques and each specific fraction was then assayed by the newly developed platform technology for the presence of α-amidated hormones. For every α-amidated hormone synthesized by PAM, glyoxylate is also formed. Based on this 1:1 molar ratio, several novel spectrophotometric, fluorescent, and chemi-luminescent enzyme linked assays for glyoxylate were developed, which when utilized on cell culture fractions proved positive for the identification of α-amidated hormones. Each novel spectroscopic assay was independently verified by a variety of known methodologies. Moreover the assay was utilized to identify two known α-amidated hormones accumulated from cell culture, which were further verified by Mass Spectral analysis.
Chapter One

Peptidylglycine α-Amidating Monooxygenase (PAM)

Introduction

The Catalytic Role of Peptidylglycine α-Amidating Monooxygenase

Peptidylglycine α-amidating monooxygenase (PAM; E.C. 1.14.17.3) is the sole known enzyme responsible for the bioconversion of inactive glycine-extended prohormones to their bioactive α-amidated product. PAM exhibits a broad range of substrate specificity; the enzyme can catalyze the post translational α-amidation of both glycine-extended fatty acids, and glycine-extended peptides. Ultimately, it is the C-terminal glycyl residue which is necessary for PAM catalysis.

PAM is a bifunctional enzyme comprised of two catalytically independent domains, which synergistically convert a glycine extended substrate to an α-amidated product and glyoxylate (Fig.1). Peptidylglycine α-hydroxylating monooxygenase (PHM) is an O₂, Cu(II), and ascorbate (reductant) dependent domain, which removes the pro-S hydrogen for the hydroxylation of the glycyl α-carbon. The second catalytic domain, peptidylamidoglycolate lyase (PAL), is a Zn(II) dependent enzyme which dealkylates the
hydroxyglycine intermediate to the \(\alpha\)-amidated product and glyoxylate. PAM is the only known enzyme that catalyzes this unique form of post-translational modification; glycine extended prohormones generally remain inactive prior to \(\alpha\)-amidation. Several alternately spliced isoforms of PAM exist within a single organism. Often, in more primitive organisms PAM is found as two catalytically independent domains PHM and PAL, and in some cases only PHM is found [1].

PAM is both the rate determining, and last step in the catalytic cascade of events for the synthesis of bioactive \(\alpha\)-amidated hormones [2, 3] Published data has demonstrated that the inhibition of PAM in rats, and in cultured mammalian cells leads [4, 5] to a decrease in \(\alpha\)-amidated peptide formation, resulting in the accumulation of the glycine-extended precursors.

**Peptide \(\alpha\)-Amidation Reaction as Catalyzed by PAM**

![Peptide amidation reaction catalyzed by PAM](image)

**Figure 1. Peptide amidation reaction catalyzed by PAM.** The bifunctional enzyme is comprised of two separate catalytic domains: peptidylglycine \(\alpha\)-hydroxylating monooxygenase (PHM) and peptidylamidoglycolate lyase (PAL).
α-Amidated Peptide Hormones; Biosynthesis, Detection, and Applications

α-Amidated Peptide Hormones and their Biosynthesis

Conversion of a glycine-extended hormone to the α-amidated product is in most cases necessary for full potency or activation of a peptide hormone [6]. Peptide hormones containing a C-terminal α-amide functionality are widely important, found in mammals [7, 8, 9], insects [10, 11], cnidarians [12], and plants [13]. Although, reports of α-amidated peptides in plants exist, (TRH-like tripeptide, pyroGlu-Tyr-Pro amide, [13]) unpublished work from the Merkler lab indicates that a PAM-like enzyme does not exist in plants (Carpenter and Merkler, unpublished). The absence of a PAM-like enzyme (sequence, and catalytic similarity) suggests that plant α-amidated peptides are produced via a PAM-independent pathway.

A catalytic cascade of sequence specific proteolytic events takes place to produce the glycine-extended prohormone from a larger polypeptide. A C-terminal glycine extended prohormone is generally excised from a larger peptide precursor. Peptides whose fate is to become glycine-extended have a pair of basic amino acid flanking the internal glycine. The collective endoproteolytic activity of substilin-like proprotein convertase (SPC), and carboxypeptidase E (CPE) liberate the once internal glycine by removing the juxtaposed basic residues, thereby producing the glycine-extended peptide.
The Rate Limiting Role of PAM

Approximately 50% of all mammalian peptide hormones have an α-amide functionality at their C-terminus [8], in several cases conversion of the glycine-extended peptide (PAM) to the α-amide is required for bioactivation. To further this study Merkler and Kreil defined this by a numerical value the potency ratio [6, 9] as the bio-activity contribution of the mature α-amidated peptide as compared to the non-amidated pro-hormone (see Table 1). Evidence to support this includes the identification of several glycine-extended peptide precursors from cell homogenates, in contrast to small quantities of the α-amidated peptides [6, 7]. Moreover, glycine-extended adrenomedulin from human plasma was found to be 5.4-fold higher in concentration as compared to the mature α-amidated adrenomedulin [14]. Accumulation of the PAM substrates defines PAM as the rate limiting step in the biosynthetic cascade of α-amidated peptide formation. Refer to Table 1, for several examples of alternate peptide hormones, their potency ratios, and length.

Further studies on the role of PAM in α-amidated peptide physiology, utilized a cell line known to express PAM. This cell line when treated with PAM anti-sense mRNA resulted in the under-expression of PAM. PAM under-expression resulted in a peptide-NH$_2$ / Peptide-Gly ratio of ~0.3 as compared to the wild type ratio of wild ~ 1.0. These
results provide further evidence that PAM is involved in catalyzing the rate limiting step in α-amidated peptide biosynthesis [15].

### An Abbreviated list of α-Amidated Mammalian Peptide Hormones

<table>
<thead>
<tr>
<th>Peptide Hormone</th>
<th>Length</th>
<th>C-terminus</th>
<th>Potency Ratio a</th>
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<tbody>
<tr>
<td>Thyrotropin-Releasing Hormone (TRH)</td>
<td>3 amino acids</td>
<td>Pro-NH₂</td>
<td>4,400</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>32 amino acids</td>
<td>Pro-NH₂</td>
<td>1,700</td>
</tr>
<tr>
<td>Corticotropin-Releasing Factor (CRF)</td>
<td>41 amino acids</td>
<td>Ala-NH₂</td>
<td>1,000</td>
</tr>
<tr>
<td>Lutenizing Hormone-Releasing Factor (LHRH)</td>
<td>10 amino acids</td>
<td>Gly-NH₂</td>
<td>1,000</td>
</tr>
<tr>
<td>Adrenomedulin</td>
<td>52 amino acids</td>
<td>Tyr-NH₂</td>
<td>&gt; 330</td>
</tr>
<tr>
<td>Substance P</td>
<td>11 amino acids</td>
<td>Met-NH₂</td>
<td>100-1,000</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>36 amino acids</td>
<td>Tyr-NH₂</td>
<td>&gt; 225</td>
</tr>
<tr>
<td>Secretin</td>
<td>27 amino acids</td>
<td>Val-NH₂</td>
<td>10</td>
</tr>
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Table 1. An abbreviated list of mammalian α-amidated peptide hormones, their length, C-terminus, and potency ratio [6, 9]. The potency ratio is a numerical value defined as the contribution of the C-terminal amide to the activity of the peptide hormone. aThe potency ratio is defined as the activity of α-amidated peptide divided by the corresponding C-terminal amino acid analog. For example, the activity of TRH-pro-NH₂ divided by the activity of TRH-pro is 4,400.
Current Methodologies Used for the Identification of α-Amidated Hormones

Method of Tatemoto and Mutt

Figure 2. Tatemoto & Mutt Methodology. Procedure for the identification of novel α-amidated peptides developed by Tatemoto & Mutt.
The majority of α-amidated peptides have been isolated, to date, utilizing bioassays for identification. The use of bioassays for the discovery of α-amidated peptides is not uniquely specific to α-amidated peptides, and is both laborious and expensive. For example, the α-amidated peptide neuromedulin C was isolated using a uterine contractile assay and monitoring platelet cAMP concentrations on a set of column fractions. The use of bioassays is not limited to α-amidated peptides only and due to these limitations other chemical based assays have been developed.

The most successful approach for the identification of novel α-amidated hormones was developed by Tatemoto & Mutt [16]. Tatemoto & Mutt used their procedure to isolate and identify a number of α-amidated peptides such as galanin [17], pancreastatin [18], PHI [19] and PYY [20]. The key to the Tatemoto-Mutt procedure is the proteolytic fragmentation of a target peptide (Fig. 2). Proteolytic fragmentation of a target peptide resulted in a degradation mixture of peptides, amino acids, and a single C-terminal amino acid amide which could be chemically identified. This degradation mixture was subjected to dansylation, and the resultant hydrophobic dansylated-amino acids were extracted into an organic solvent. Two-dimensional TLC was then employed to isolate the peptides and dansylated amino acids, with a detection limit of approximately 1 nanomole. Since the development of the Tatemoto & Mutt procedure in the 1970’s, improvements in the separation and detection of derivitized amino acid amides have been made, [21, 22, 23, 24] with an increased detection limit in the picomole range [25].
Despite improvements the Tatemoto & Mutt procedure is extremely laborious and inefficient for a variety of reasons. Incomplete proteolytic fragmentation coupled to inefficiencies in the extraction and labeling procedures proved deleterious to assay sensitivity as compared to model studies. This methodology has not been widely used, and is especially non-amenable to high-throughput analysis. Consequently, Tatemoto & Mutt have been the primary users of this technology for the discovery of novel α-amidated peptides, while most others have adapted the procedure to test for and identify the α-amidated C-terminus in an otherwise purified, bioactive peptide.
Method of Hill and Flannery.

Hill & Flannery Procedure for α-Amidated Peptide Discovery

![Diagram of Hill method for the discovery of α-amidated peptides. Outline of the technique designed by Hill et al. for the identification of α-amidated peptides utilizing chemical dyes.]

Figure 3. Hill method for the discovery of α-amidated peptides. Outline of the technique designed by Hill et al. for the identification of α-amidated peptides utilizing chemical dyes.

Hill and Flannery [36] developed a more chemical approach for the identification of α-amidated peptides from a mixture of peptides purified from a biological source. Acetylation of peptide free amines followed by conversion of the N-acetylamides to amines by a Hoffman rearrangement allows for detection of the resultant amine by ninhydrin (Fig. 3). Amide derived amines separated into column fractions can be correlated to the presence of a C-terminal glycine by conversion of glycine to 2-thiohydantoin. This assay is based on the presumption that the C-terminal glycine and the amide will co-elute, and that their co-elution is strongly indicative for the presence of an α-amidated peptide. Overall, this assay is only sensitive to the millimole range, and is completely reliant on the co-elution of two species which may or may not co-elute in most cases. Furthermore, this assay will always be fraught with false positives for any
Asn and Gln containing peptides. Adapting the assay for improved sensitivity by the use of other amine dyes [25] may improve sensitivity; however it is unlikely that this procedure could ever find a widespread use. The Hill & Flannery procedure has never been successfully used to identify an α-amidated peptide, and a Web of Science search indicates that this paper has only been cited once since Feng & Johnson [25].
**Immunological Detection of α-Amidated Peptides**

A library of antibodies could theoretically be used to exploit the physiological difference between a glycine-extended peptide and an α-amidated peptide. This approach would necessitate the use of collection of 20 antibodies, each one specific for a particular amino-acid amide. The Grimmelikhuijzen group has used a similar approach by generating an antibody against the dipeptide amide, Arg-Phe-NH$_2$ to discover α-amidated peptides in cnidarians [25]. This approach would require a collection of 400 antibodies in order to test all of the possible di-peptide permutations of the 20 common amino acids.

**Computer Based Analysis for α-Amidated Peptides**

With the advent of the genomic database and specific peptide sequence information, it has been suggested that the search for α-amidated peptides become a computer-based dry technique, which could be followed by wet chemistry. Unfortunately, without a defined model peptide sequence to search for, database searching would produce ambiguous results. Recall, that the only defining factor for a peptide whose fate is to become α-amidated, is the basic residues flanking the initially internal glycine. The outcome of a computer based search on different permutations of the basic amino acid residues would yield results entirely too non-specific and numerous.


**Therapeutic Uses of the Known α-Amidated Peptides**

<table>
<thead>
<tr>
<th>Drug Name (Company)</th>
<th>Compound</th>
<th>Delivery</th>
<th>Dose</th>
<th>Indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lupron (TAP)</td>
<td>Nonapeptide analog of LHRH</td>
<td>Subcutaneous, daily</td>
<td>1.0 mg daily</td>
<td>Advanced prostate central, precocious puberty</td>
</tr>
<tr>
<td>DDAVP (Sanofi-Aventis)</td>
<td>analog of 8-Arg vasopressin</td>
<td>Oral tablets</td>
<td>0.1 to 0.8 mg daily</td>
<td>Central diabetes insipidus, primary nocturnal enuresis</td>
</tr>
<tr>
<td>Cortrosyn (Organon)</td>
<td>α-(1-24)-corticotropin</td>
<td>i.m. or i.v. injections or i.v. infusion</td>
<td>250 μg</td>
<td>Diagnostic agent for adenocortical deficiency</td>
</tr>
<tr>
<td>Sandostatin (Novartis)</td>
<td>Cyclic octapeptide analog of somatostatin</td>
<td>s.c or i.v. injections</td>
<td>50-1500 μg daily</td>
<td>Acromegaly, carcinoid tumors, VIPomas</td>
</tr>
<tr>
<td>Thyrel TRH (Ferring)</td>
<td>Synthetic tripeptide</td>
<td>i.v. injection</td>
<td>500 μg</td>
<td>Diagnostic assessment of thyroid function</td>
</tr>
<tr>
<td>Miacalcin (Novartis)</td>
<td>Salmon calcitonin</td>
<td>s.c. injection</td>
<td>100 I.U. daily</td>
<td>Postmenopausal osteoporosis, Paget’s disease, hypercalcemia</td>
</tr>
<tr>
<td>Miacalcin Nasal (Novartis)</td>
<td>Salmon calcitonin</td>
<td>Nasal spray</td>
<td>200 I.U. daily</td>
<td>Postmenopausal osteoporosis</td>
</tr>
<tr>
<td>Geref (Serono)</td>
<td>GHRF(1-29)-NH₂</td>
<td>s.c. injection</td>
<td>30 μg/kg daily</td>
<td>Pediatric, idiopathic growth hormone deficiency</td>
</tr>
<tr>
<td>Acthrel (Ferring)</td>
<td>Ovine CRF(1-41)-NH₂</td>
<td>i.v. injection</td>
<td>1.0 μg/kg single injection</td>
<td>Differentiates pituitary and ectopic production of ACTH in Cushing’s syndrome</td>
</tr>
<tr>
<td>Secretin-Ferring (Ferring)</td>
<td>Porcine secretin(1-27)-NH₂</td>
<td>Slow i.v. injection</td>
<td>1-2 CU/kg</td>
<td>Testing for pancreatic function and gastrinoma</td>
</tr>
<tr>
<td>Byetta (Amylin)</td>
<td>Exenatide(1-39)NH₂ (Exendin-4)</td>
<td>s.c injection</td>
<td>5-10 μg b.i.d.</td>
<td>Adjunctive therapy in Type 2 diabetes</td>
</tr>
<tr>
<td>Fuseon (Roche)</td>
<td>T-20 peptide</td>
<td>s.c. injection</td>
<td>90 mg daily</td>
<td>Treatment of HIV-1 infection in combination with antiretroviral agents</td>
</tr>
<tr>
<td>Symlin (Amylin)</td>
<td>Analog of Amylin(1-37)NH₂</td>
<td>s.c. injection</td>
<td>15-60 μg for Type 1 and 60-120 μg preprandial for Type 2 diabetes</td>
<td>Adjunct treatment for Type 1 and Type 2 diabetes</td>
</tr>
<tr>
<td>Fortical (Upsher-Smith)</td>
<td>Salmon calcitonin</td>
<td>Nasal Spray</td>
<td>200 I.U. daily</td>
<td>Postmenopausal osteoporosis</td>
</tr>
<tr>
<td>Forteo (Lilly)</td>
<td>Parathyroid hormone(1-34)</td>
<td>s.c. injection</td>
<td>20 μg daily</td>
<td>Postmenopausal osteoporosis with high risk of fracture</td>
</tr>
</tbody>
</table>

**Table 2. Examples of currently marketed peptide hormones.** A summary of the currently available therapeutic α–amidated peptide hormones, their manufacturers and pharmacological usage.
Several α-amidated peptide hormones have already been discovered, and a few have proven to be useful pharmaceutical therapeutics (Table 2). Considering the large marketability of the known α-amidated peptides, the likelihood that undiscovered α-amidated peptide hormones could lead to the development of novel diagnostics and pharmaceuticals is of extremely high probability.

Based on the literature it is evident that some tissues known to express high levels of PAM [27], do not produce similar quantities of known α-amidated hormones (exocrine), and vice versa (autocrine). Autocrine cells both synthesize and utilize the machinery necessary for their cell growth. Conversely, exocrine cells generally synthesize materials for exportation to other cells, and acquire materials via a receptor mediated pathway. PAM is localized in the secretory vesicles, and has been linked to both autocrine growth factor loops [28, 29], such that PAM is both highly expressed and functional within the same tissue, and exocrine growth loops [30, 31]. Glycine-extended hormones may therefore be expressed in cells which do not also express PAM, and conversely cells known to express large quantities of PAM may not co-express any glycine-extended hormones. Thus, it is likely that several undiscovered α-amidated hormones exist both in tissues known to express large quantities of PAM, and in tissues which do not express large quantities of PAM. Cultured cell lines known to express large quantities of PAM, yet not known to express many α-amidated peptides could serve as an initial source material for the discovery of novel α-amidated hormones. It is possible that many cell lines which do not express PAM, may express glycine-extended hormones. There are many tissues to search for glycine-extended peptides, without a specific assay in place for
their discovery, the probability that several $\alpha$-amidated peptides remain to be discovered is highly likely.

A review on the literature of PAM strongly suggests that the main physiological function of PAM is to hydroxylate (PHM) extended prohormones (glycine-extended peptides, fatty acyl-glycines), and to further dealkylate the $\alpha$-hydroxyglycyl intermediate (PAL). Currently, no data exists to suggest an alternate physiological function of PAM, or to provide reason for high levels of PAM in tissues not known to produce and/or utilize $\alpha$-amidated peptides. This fact combined with the large amounts of PAM in certain tissues and lack of a glycine-extended hormone assay provides impetus for the assertion that several $\alpha$–amidated hormones exist which have yet to be discovered.
Impetus for the Design of a Novel, Broadly Applicable Assay for the Discovery of α-amidated Peptide Hormones

Utilizing PAM as a General Tool for α-Amidated Hormone Discovery

From a chemical perspective there is little to differentiate an α-amidated hormone from a non-amidated hormone. Any assay developed on the basis of an amide specific reagent would be fraught with high backgrounds and false-positives due to the cross reactivity with any Asn or Gln containing peptides. It is evident in the literature on α-amidated peptides and their discovery methods, that no single methodology has proven effective as a general and specific assay for the presence of an α-amidated peptide/hormone. The lack of a general assay for the discovery of α-amidated peptides has left a void in the knowledge of α-amidated peptide hormones as a whole, their biosynthesis, tissue distribution, and physiological role. Capitalizing on the very unique role of PAM and the stoichiometric production of glyoxylate:α-amidated peptide, identification of PAM produced glyoxylate could lead to the identification of yet to be discovered α-amidated hormones. Utilization of enzymatic assay systems for the detection of glyoxylate allows for a signal specific assay, and decreases the loss of sensitivity due to high backgrounds created by non specific interactions. As previously discussed, other α-amidated peptide discovery methods to date have been fraught with non-specific / insufficient interactions for α-amidated peptide detection. Quantitative analysis of PAM produced glyoxylate is both stoichiometric for the presence of α-amidated peptides and their glycine-extended
prohormone precursors. Ultimately this assay has one very positive feature in that it is non-specific for a particular class of PAM substrates, thereby increasing the application of this assay to not only the discovery of several α-amidated peptides, but also novel α-amidated fatty acid amides.
The utilization of PAM is paramount to the development and use of a robust and specific assay for biologically generated α-amidated hormones. Capitalizing on the unique chemistry of PAM allows for the generation of an unequivocal route to both purify and discover novel α-amidated hormones from a biological source. The key to designing an assay for α-amidated peptides utilizing the unique physiological role of PAM is glyoxylate. For every α-amidated peptide produced via PAM, a molecule of glyoxylate results (Fig. 4). Employing glyoxylate as a signal molecule for the discovery of novel peptides leads to the design of an assay system enriched with a specific yet general method for detection of α-amidated peptides. As mentioned, several tissues known to express PAM at high levels are not known to produce correspondingly high levels of α-amidated peptide hormones [30, 32].
Cell lines generated from tissues known to express high levels of PAM will serve as an initial source material for the discovery of novel $\alpha$-amidated hormones. These cells cultured in the presence of a PAM inhibitor accumulate the glycine-extended hormone precursors. Mains and Eipper [4, 33] demonstrated that the glycine-extended peptide mouse joining peptide (mJP-Gly) did in fact accumulate when grown in the presence of a PAM inhibitor. Glycine-extended precursors accumulated from cell culture when HPLC purified [4, 34], result in a series of semi-purified fractions containing the glycine-extended hormones. Treatment of the semi-purified fractions with PAM results in glyoxylate production only in the presence of a PAM substrate. Namely, fractions positive for PAM produced glyoxylate, are also positive for the glycine-extended precursor. This unique method allows for the identification of any glycine-extended molecule permitting the identification of a broad range of novel PAM substrates. A sensitive high-throughput assay for glyoxylate provides a signal that is independent of the peptide (or acyl group) upstream of the terminal glycine. All fractions positive for glyoxylate can be further analyzed for content, and the structure of novel substrates determined by mass spectrometry. Comparison of the cells treated with the PAM inhibitor to non-treated cells for glyoxylate content removes the interference of any intrinsic glyoxylate resultant of other metabolic processes.
Development of a Series of Glyoxylate Assays; Glyoxylate as a Signal Molecule for the Detection of α-Amidated Hormones

A series of enzyme linked glyoxylate assays have been designed and coupled to spectrophotometric, fluorescent, and chemi-luminescent detection. Each assay was developed with the interest of ease of use, sensitivity, specificity, and high-throughput analysis. Based on the absolute necessity of a specific and sensitive technique, the establishment of robust glyoxylate assays is the foundation of this novel platform technology. Three spectrophotometric, two fluorescent, and two chemi-luminescent for a total of seven novel enzymatic assays were created each able to detect PAM produced glyoxylate. Each of these assays will be discussed in detail in the following chapters. Enzymes provide a degree of selectivity far greater than that of typical chemical assays, based on their inherent substrate specificity. The glyoxylate assays constructed for this platform technology were designed on the premise of specificity and as a result all newly developed assays are based on the enzymatic detection of glyoxylate. Utilizing nature’s tools provides an unrivaled degree of sensitivity as compared to organic / chemical methods for glyoxylate detection.
Prior Art for the Detection of Glyoxylate; A Literature Review

The Rimini-Schryver Chemical Spectrophotometric Assay for Aldehydes

Detection of PAM produced glyoxylate is the nexus of a definitive and sensitive assay for α-amidated peptides. Moreover, the ability to detect glyoxylate is the foundation on which this platform technology resides. A great deal of effort has been made towards the production of glyoxylate assays which are amenable to high throughput analysis, feasible within the working conditions of many laboratories, and foremost sensitive detection without ambiguity.

Methodologies utilizing chemical detection rely on the reactivity of a specific functionality constituent of the analyte of interest. Often a chemical detection assay design, when applied to biochemical analyses results in large background signals as a result of the increased complexity of biochemical solutions. Detection of analytes from biochemical samples requires analyte specific detection (enzymatic detection), as biochemical samples are often grossly impure. Specifically, the Rimini-Schryver colorimetric reaction was developed as a method for the detection of formaldehyde [35]. Originally developed by Rimini the assay was developed to detect aldehydes in the presence of phenylhydrazine and sodium hydroxide, it was later adapted by Schryver [36] to increase sensitivity by the addition of ferricyanide (Fe(III)) in the presence of
hydrochloric acid (Fig. 5). The assay has in most part been utilized to detect allantoin content in various biological fluids [37, 38], allantoin is indicative of purine metabolism as it is the end-product of purine metabolism (Fig. 6) [38].

Rimini-Schryver Chemical Assay for Glyoxylate

![Chemical reaction diagram]

Figure 5. Rimini-Schryver chemical assay for glyoxylate. The condensation reaction developed by Rimini and adapted by Schryver, for the detection of the colorimetric condensation adduct of glyoxylate and phenylhydrazine, glyoxylic acid phenylhydrazone respectively [35].

An excerpt of the AMP Catabolic Pathway as it Applies to Allantoin Metabolism

![Pathway diagram]

Figure 6. An excerpt of the AMP catabolic pathway as it applies to allantoin metabolism. A portion of the AMP catabolism cascade displays the enzymatic cascade for the production of glyoxylate from allantoin. Determination of allantoin content in blood samples was of interest in early metabolic investigations of purine metabolism.
The following is a list of molecules which in the presence of phenylhydrazine also produce a hydrazone colorimetric adduct detectable at the same wavelength as the glyoxylate: phenylhydrazone adduct: xanthine, hypoxanthine, glycerol, pyruvic acid, malic acid, tartaric acid, alloxan, aloxantin, glycine, chloral, lactic and uric acids, and aldehydes in general [35]. Alternately, 2,4-dinitrophenylhydrazine can also be used to produce the condensation product glyoxylic acid 2,4-dinitrophenylhydrazone (glyoxylate 2,4-DNPH) [39]. Production of keto-acid 2,4-DNPH adducts has long since been used as a method to characterize both aldehydes, and oximes by melting point and color.

In addition to the non-specificity, of the Rimini-Schryver method, analyses are laborious, not very sensitive (~ 10μM), time consuming, and not completely amenable to high-throughput analysis as the colorimetric product fades quickly. Although the Rimini-Schryver method has been used to detect PAM activity, its use is only amenable to the detection of glyoxylate formed with highly purified enzyme. In addition, this assay is not useful in the detection of PAM activity in non-pure extracts, resulting in a very limited use of this technique.
Determination of Glyoxylate by Capillary Electrophoresis

Calcium oxalate is the major constituent of kidney stones [40] and ~50-60% of urinary oxalate ("OOC-COO") is derived from the enzymatic oxidation of glyoxylate (HCO-COO⁻) [41] As a consequence of the metabolic importance and role of glyoxylate in kidney stone formation, a number of assays have been developed for glyoxylate. Existing assays for the determination of glyoxylate include colorimetric methods [36, 42-46], fluorometric methods [47, 48], the iodometric or potentiometric titration of the bisulfite adducts [49] and the use of capillary electrophoresis with direct UV detection [50, 51]. Generally, these are insensitive, non-specific, or both. Capillary electrophoresis has been the detection method of choice for the separation and quantification the organic acids contained in urine. Capillary electrophoresis (CE) separation is based on the movement of an analyte velocity within an electric field. The inherent velocity of an analyte is a function of its electrophoretic mobility in relation to an applied voltage coupled to spectrophotometric detection. Nishijima et al. [50] developed a CE separation on a polyamide fused silica column at a constant voltage of -30kV. Urine derived organic acids including glyoxylate elute over an eight minute period, with a detection limit for glyoxylate of 8 - 9μM [50]. Although separation and quantification can be achieved by the Nishijima [50] CE method, the practicality of this technique for the quantification of glyoxylate from alternate sources is of limited value [52]. In addition to
the limited utility a detection method based on the inherent spectrophotometric absorption of glyoxylate itself results in a limited degree of sensitivity.
The Fluorescent / Spectrophotometric Adduct of Glyoxylate and Resorcinol

An alternate spectrophotometric assay for glyoxylate was developed by Zarembski and Hodgkinson in 1965 [53], based on the colored product produced by the reaction of glyoxylate and resorcinol in the presence of an acid extract of *Psuedomonas oxalaticus*. The glyoxylate : rescorcinol adduct is spectrophotometrically visible with a $\lambda_{\text{max}}$ of 490nm, and detection limit of thirteen micro-molar. In addition the colored product is also visible by fluorescence within a pH range of 7 to 9. This procedure while not explicitly stated in the reference is dependent upon the enzymatic activity of glyoxylate dehydrogenase which is a metabolic product of the growth of *Psuedomonas* only under high oxalate conditions. The Zarembski method is reliant on the oxalate supported *Psuedomonas* extract, an extract which is extremely time and preparation intensive to produce in any appreciable quantities. The Zarembski method has not found general use, and has been cited only once since its 1965 publication.

**Assay for Glyoxylate as Described by Zarembeski**

![Diagram of Assay for Glyoxylate as Described by Zarembeski](image)

*Figure 7. Assay for glyoxylate as described by Zarembeski.* Proposed reaction catalyzed in the presence of Psuedomonas oxalaticus for the formation of a glyoxylate dependent spectrophotometricly active adduct. The proposed glyoxylate-resorcinol adduct was verified by Vieles and Badre [54].
Authentication the Proposed Platform Technology

Authentication of the Newly Designed Glyoxylate Assays

A variety of techniques were necessary to prove the usefulness and feasibility of all the newly developed assays intended for the platform technology. Exigent to the use of a glyoxylate assay as a paragon for the identification of novel α-amidated hormones was the external standardization of each developed assay via an independent confirmed methodology. This served as a reference comparison for each assay in order that all newly developed techniques could be referenced against a known control. Furthermore, all PAM produced glyoxylate is a consequence of an enzymatic reaction with a 1:1 ratio of glyoxylate : α-amidated peptide; measurement of produced α-amidated peptide as compared to glyoxylate should result in a ratio of 1. All PAM produced glyoxylate determinations were indexed against α-amidated peptide concentrations assayed via well-established methodologies. Once the assay was standardized against known procedures, it was then utilized for determination of unknown glyoxylate, and PAM produced glyoxylate. Several techniques were utilized as standardization procedures, each to ascertain the ratio of glyoxylate: amidated peptide. This analysis has proven invaluable in the determination of PAM produced glyoxylate concentrations, in context to the affirmation of newly developed techniques. Details of all authentication procedures will be fully outlined in the following chapter.
Authentication for the PAM Dependent Discovery of Novel $\alpha$-Amidated Peptides

Prior to the utilization of the assay to discover novel hormones from a biological source, the assay was initially used to demonstrate its ability to identify an already known $\alpha$-amidated hormone (mouse joining peptide, mJP-Gly). It was imperative to the utility of the proposed assay to demonstrate this new methodology could be used to identify a known $\alpha$-amidated hormone from cell culture prior to employing the assay for the discovery of novel $\alpha$-amidated hormones. A mouse corticotropic cell line known to produce mouse joining peptide (mJP-Gly), and accumulate mJP-Gly upon the inhibition of PAM as outlined by Mains et. al. [4] provided the necessary data for the verification of the newly developed platform technology. The demonstration of the platform technology with a known glycine-extended peptide served both as an internal control and also as a template for the empirical design of future assays for the discovery of novel $\alpha$-amidated hormones.

Delineating the assay feasibility from tissue culture to the utility of glyoxylate as a signal for a known $\alpha$-amidated peptide by mass spectrometry is discussed in detail in this work. A comprehensive discussion of all authentication methodologies, enzymatic spectral assays for glyoxylate, and the use of these assays towards the platform technology are outlined in the following chapters, preceded by a thorough discussion of the newly designed platform technology.
Chapter Two

Glyoxylate as a Signal Molecule for the Identification of $\alpha$-Amidated Peptides

Introduction

*PAM Activity as a Key to the Discovery of $\alpha$-Amidated Peptides*

The use of glyoxylate as indicative for the presence of $\alpha$-amidated hormones is truly innovative. Development of a glyoxylate based route for $\alpha$-amidated hormone detection exploits the inherent biochemistry of their production. This was the method of choice for a variety of fundamental reasons. Often the discovery of novel enzyme substrates is hindered by the lack of a general non-substrate dependent assay. Glyoxylate is always a product of PAM catalysis. This feature imparts an “analytical” quality to PAM which had not been exploited to its full potential, prior to the development of this platform technology. Capitalizing on this “tool-like” quality brings a novel functionality to PAM beyond typical mechanistic biochemistry. Moreover, PAM directs both the qualitative and quantitative discovery of $\alpha$-amidated peptides via the use of glyoxylate as a signal. Simple chemical techniques lack this unique culmination of qualities, resulting in their definite inferiority.
Identification of PAM Produced Glyoxylate

Manipulating the biochemical role of PAM for the discovery of α-amidated peptides mandates the facile detection of glyoxylate as a PAM product. This feature requires that glyoxylate assays be amenable to PAM reaction conditions. As a result, prior to the analysis of glyoxylate, a series of sample pre-treatment procedures was necessary pending the glyoxylate detection system. In this chapter, the development of several spectrophotometric assays is detailed, followed by the development of more sensitive fluorescent and chemi-luminescent assays in chapter three.

Reaction Stoichiometry of α-Amidated Peptide Production as Catalyzed by PAM

![Figure 8. Reaction Stoichiometry of α-amidated peptide production as catalyzed by PAM. The stoichiometric production of glyoxylate : peptide-NH$_2$ is the central dogma of the developed platform technology.](image)

A series of novel spectrophotometric (Chapter 2), fluorescent (Chapter 3), and chemi-luminescent (Chapter 3) assays have been developed for the detection of standard glyoxylate, PAM produced glyoxylate, and α-amidated peptides. The differences between standard glyoxylate detection and PAM produced glyoxylate detection is a ramification of the necessary additional reagents used for PAM catalysis. Detection of PAM produced glyoxylate necessitates the consideration of several factors relating to the
tandem activity of PAM, and glyoxylate consuming enzymes. Although glyoxylate consuming enzymes do not need to be catalytically active in tandem with PAM, they must be able catalytically active in the presence of the necessary PAM reagents. This is a direct consequence of this novel platform technology, such that glycine-extended peptides are discovered through the use of PAM and glyoxylate consuming enzymes as analytical tools. PAM is a redox active, copper-dependent monooxygenase that relies on the presence of a reductant for reduction of Cu(II) for catalytic turnover. Additionally, PAM requires the presence of a radical/peroxide scavenger to protect the enzyme from ·OH radicals, and H₂O₂, produced as a result of the Fenton chemistry between Cu(II) and ascorbate (reductant). Each glyoxylate assay must be designed with the presence of these molecules in mind.

Circumvention of the tandem catalysis of PAM and glyoxylate consuming enzymes was readily achieved. Utilizing PAM first for the production of all glyoxylate from the glycine-extended substrates allows for inactivation of PAM after catalysis, followed by optimization of reaction conditions for glyoxylate consuming enzymes. Inactivation of PAM is necessary such that reaction conditions may be altered to optimize for glyoxylate detection. It is necessary to remove PAM co-substrates, adjust pH conditions, and / or add cofactors for the subsequent detection of glyoxylate. Ascorbate inhibits many glyoxylate consuming enzymes and interferes with methodologies utilized to detect their activity. Therefore it is imperative that ascorbate be removed after PAM catalysis. This is accomplished by the oxidation of ascorbate by the enzyme ascorbate oxidase. Likewise, in some cases addition of different buffering reagents is required to facilitate
enzymatic detection of glyoxylate. Manipulation of the PAM produced glyoxylate conditions was particular to the enzyme(s) utilized for glyoxylate detection and will be discussed as they apply to each particular glyoxylate assay.
Introduction of Spectrophotometric Enzyme Dependent Assays for Glyoxylate

Enzyme linked spectrophotometric assays were developed around two chemistries: the glyoxylate-dependent oxidation / reduction of NADPH / NAD⁺ and subsequent formazan dye production, and the glyoxylate dependent formation of hydrogen peroxide (H₂O₂).

Spectrophotometric Assays for Glyoxylate

Figure 9. Spectrophotometric assays for glyoxylate. Overview of the spectrophotometric assays developed for the analysis of glyoxylate. (A) The malate synthase / malate dehydrogenase assay, detection is based upon the concomitant reduction of NADH and oxidation PMS to yield a tetrazolium dye. (B) The glycolate oxidase assay for the stoichiometric production hydrogen peroxide based on glyoxylate consumption. (C) The glyoxylate reductase dependant glyoxylate reduction upon NADPH oxidation visible at 340nm.
**Introduction to the Malate Synthase / Malate Dehydrogenase Glyoxylate Consuming Enzymes**

Malate synthase catalyzes the condensation of glyoxylate and acetyl-Co-A to produce malate, and is an enzyme of the glyoxylate cycle of eubacteria, plants, and fungi [55]. The glyoxylate cycle is involved in the regeneration of three carbon molecules which become depleted during the TCA cycle [56]. Malate synthase activity can be spectrophotometrically measured by the release of free CoA upon condensation. Free CoA reacts with 5′,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB / Ellman’s reagent) to form a TNB anion (thio-bis-2-nitrobenzoate) and a CoA-TNB adduct with a $\lambda_{\text{max}}$ of 412nm and a literature extinction value of $\varepsilon = 13.6 \text{ mM}^{-1}\text{cm}^{-1}$[57].

Malate dehydrogenase is a member of the citric acid cycle; it catalyzes the last reaction of the TCA cycle via production of oxaloacetate and NADH from malate and NAD$^+$. Malate dehydrogenase activity is thermodynamically regulated and plays a major role in the regulation of NADH production. In the forward direction the malate synthase oxidation of malate to oxaloacetate coupled to the reduction of NAD$^+$ to NADH, is a highly unfavorable reaction, with a $\Delta G^o = +29.7 \text{ kJmol}^{-1}$
The concomitant oxidation of malate and reduction of NADH necessary for glyoxylate detection is a non-spontaneous reaction of malate dehydrogenase \textit{in vitro}. The large $\Delta G^\circ'$ value for the reduction of NAD$^+$ by malate dehydrogenase necessitates the use of an electron shuttling reagent for the catalytic activity in the forward direction \textit{in vitro}. An electron shuttling reagent is a catalytic redox active cycling reagent, responsible for affecting the equilibrium of a reaction by consumption of the product. An electron shuttling reagent is the terminal electron acceptor, which upon reduction can auto-catalytically become re-oxidized. This catalytic mechanism allows a single shuttling molecule to shuttle numerous times, thereby imparting a significant effect on the turnover of the terminal oxidant (NADH / NAD$^+$) (Fig. 10).

Utilization of an electron shuttling reagent for NAD$^+$ reduction by malate dehydrogenase is a manifestation of Le Chatlier's principle. For malate dehydrogenase activity, the use of an electron shuttling reagent is essential to shift the equilibrium in favor of product formation (NADH). This affect is a ramification of the instantaneous removal (via oxidation) of any NADH production. In the absence of an electron shuttling reagent the malate dehydrogenase forward reaction is catalytically incompetent as a consequence of thermodynamic regulation. Several molecules including, cysteine, quinones and phenazines play the role of electron shuttling reagents in biochemical systems. A major
prerequisite for a functional electron shuttling reagent is that it must have the correct redox potential to be catalytically competent in a given system. Definitively, the electron shuttling reagent redox potential ($E^{o'}$) must be a value between the $E^{o'}$ of the reductant and the oxidant [58].

**Catalytic Role of PMS**

![Diagram of the Catalytic Role of PMS](image)

**Figure 10. Catalytic role of PMS.** The electron shuttling pathway for the NADH dependent stoichiometric reduction of MTS, the reduced MTS, formazan, is an intensely colored product visible at $\lambda_{\text{max}}$ 490nm.
Figure 11. Malate synthase / malate dehydrogenase glyoxylate assay. The malate synthase / malate dehydrogenase coupled assay for glyoxylate dependent upon the stoichiometric reduction of a tetrazolium dye.
The electron shuttling reagent chosen for the stoichiometric NADH formation and subsequent tetrazolium dye (MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) reduction was phenazine methosulfonate (PMS) [59]. PMS is also known as pyocyanin an electron shuttling pigment of the facultative anaerobe Psuedomonas aeruginosa [58]. PMS cycles electrons from NADH to a tetrazolium dye via 2, 1e⁻ processes (Fig. 10). The reduced tetrazolium dye formazan product has a \( \lambda_{\text{max}} \) of 490nm and a molar extinction coefficient of \( \varepsilon = 25\text{mM}^{-1}\text{cm}^{-1} \) [59]. This formazan dependant spectrophotometric assay is 4 fold more sensitive than the analysis of NADH production based on extinction coefficient alone. Moreover, malate dehydrogenase is catalytically inactive in the absence of phenazine methosulfonate, which drives the reaction towards concomitant glyoxylate oxidation and NAD⁺ reduction (Figs.10, 11).
Introduction to the Glyoxylate Consuming Enzyme Glycolate Oxidase

Glycolate oxidase, (E.C.1.1.3.15) is a flavin dependent, oxidoreductase enzyme found in plants [60], protists [61], and mammals [62, 63, 64].

**Enzymatic Reaction of Glycolate Oxidase**

\[
\text{glycolate} + \text{O}_2 \xrightarrow{\text{glycolate oxidase}} \text{glyoxylate} + \text{H}_2\text{O}_2
\]

*Figure 12. Enzymatic reaction of glycolate oxidase.* The oxidation of glycolate and concomitant reduction of bimolecular oxygen as catalyzed by the enzyme glycolate oxidase.

Glycolate oxidase is a peroxisomal enzyme, which in plants is part of the photosynthetic oxidative C\(_2\) cycle. The main function of the plant C\(_2\) cycle is for the complete oxidation of C\(_2\) units to carbon dioxide and water. The mammalian enzyme is involved in the production of oxalate from carbohydrates and serine [65]. Glycolate oxidase catalyzes the oxidation of both glyoxylate and glycolate, utilizing O\(_2\) as the electron acceptor [63] and has a pH range between 8.0 and 8.5 [66]. At pH values above 3.5 glyoxylate is found in the hydrated gem diol form, HC(OH)\(_2\) – COOH, in aqueous solutions [67, 68]. The glyoxylate gem diol is oxidized by glycolate oxidase to oxalate as dioxygen is reduced to hydrogen peroxide (Fig. 12).
The Glyoxylate Dependent Production of Hydrogen Peroxide

Several techniques exist for the spectrophotometric detection of hydrogen peroxide [69, 70, 71, 72]. The glycolate oxidase mediated conversion of glyoxylate to hydrogen peroxide was chosen as the basis for a novel spectrophotometric glyoxylate assay. The horse radish peroxidase (HRP), catalyzed oxidation of substrates 3-methyl-2-benzothiazolinone hydrazone (MBTH), and 3-(dimethylamino)benzoic acid (DMAB), in the presence of hydrogen peroxide produces an indamine dye adduct, \(47 \text{mM}^{-1}\text{cm}^{-1} \leq \epsilon \leq 53 \text{mM}^{-1}\text{cm}^{-1}\) [73, 74] (Fig. 13).

The MBTH / DMAB / HRP assay for hydrogen peroxide detection was chosen over other methods as a few key characteristics provided this assay with higher sensitivity, and ease of use. Several alternate HRP substrates, (homovanillic acid, resorufin, etc) undergo auto-oxidation at rates appreciably higher than that of the indamine dye production. Auto-oxidation of HRP substrates leads to the generation of background values approaching levels sufficiently large to affect the dynamic linear range of analyte detection. Spectrophotometric detection is fundamentally limited at high concentrations on account of light scattering due to precipitation (solubility issues at high analyte concentration), and by incomplete percent transmission of highly colored solutions (\(\text{Abs} = 2 \cdot \log \% \ T\)). Auto-oxidation becomes increasingly limiting for end-point enzymatic assays as long incubations at 37°C are required for reaction completion. Particularly, in the case of glycolate oxidase which has an incubation period of 40
minutes for glyoxylate detection (Fig. 14). Collectively, these issues significantly affect the both the limit of detection and dynamic linear range of analyte detection as compared to the DMAB / MBTH / HRP assay where auto-oxidation is comparably much less. Moreover, the DMAB / MBTH / HRP assay has a comparably large extinction coefficient. A glyoxylate dependent stoichiometric assay utilizing glycolate oxidase and the DMAB/MBTH peroxidase detection system has been developed based on this chemistry.

**H₂O₂ Dependent Indamine Dye Production**

![Chemical structure](image)

*Figure 13. H₂O₂ dependent indamine dye production.* The mechanism for hydrogen peroxide detection, based upon the HRP dependent oxidative coupling of DMAB and MBTH to produce the blue colored indamine dye adduct.

The glycolate oxidase assay for glyoxylate based on stoichiometric hydrogen peroxide detection is an enzyme coupled assay reliant on catalytically competent HRP. In order for the assay to be stoichiometric (linear over a defined range) for glyoxylate, the rate of HRP dye production *must* be on the order of at least 10-fold higher than glycolate oxidase activity. Such that color formation is directly proportional to the glyoxylate dependent
hydrogen peroxide production, and not affected by the rate of indamine dye formation. Such an assay requires the $v_{\text{max}}$ of HRP to far exceed that of the glycolate oxidase, for an almost instantaneous color formation upon hydrogen peroxide production.

**Glycolate Oxidase Based $H_2O_2$ Assay**

![Glycolate Oxidase Based $H_2O_2$ Assay Diagram]

$O_2 + \overset{\text{glyoxylate hydrated}}{\text{glycolate oxidase E.C.1.1.3.15}} \overset{\text{pH=8.0}}{\rightarrow} \overset{\text{H}_2\text{O}_2}{\text{H}_2\text{O}_2} + \overset{\text{oxalate}}{\text{oxalate}}$

$\overset{\text{MBTH}}{3\text{-methyl-2-benzothiazolinone hydrazone}} + \overset{\text{DMAB}}{3\text{-}(\text{dimethylamino) benzoic acid}}$

$\overset{\text{HRP}}{\text{HRP}}$

$\overset{\text{H}_2\text{O}_2 \text{ dependant MBTH/DMAB Oxidative Coupling}}{\overset{\lambda_{\text{max}} = 590\text{nm}}{\overset{\varepsilon = 53,000\text{Mcm}^{-1}}{\text{H}_2\text{O}_2}}} \overset{\text{Oxidative Coupling}}{\lambda_{\text{max}} = 590\text{nm}} \varepsilon = 53,000\text{Mcm}^{-1}$

*Figure 14. Glycolate oxidase based $H_2O_2$ assay. The glycolate oxidase assay developed for glyoxyxylate detection coupled to the peroxidase indamine dye detection system.*
**Introduction to the Glyoxylate Consuming Enzyme Glyoxylate Reductase**

Glyoxylate Reductase (GR) is a NADPH dependant reductase, and catalyzes the reduction of glyoxylate to glycolate with the concomitant oxidation of NADPH (or NADH) (Fig. 15). Glyoxylate reductase was first isolated from the leaves of *Spinacea oleracea* by Zelitch and Goto in 1962 [75]. Glyoxylate reductase is also found in bacteria [76], green algae [77], protists [78], yeast [79] and humans [80]. Liver metabolic studies on glyoxylate reductase have shown that alteration in glyoxylate reductase activity in vivo has been linked to hyperoxaluria [81], a ramification of altered glyoxylate and oxalate metabolism. In plants, glyoxylate reductase is an enzyme of the photo-respiratory cycle, Kleczkowski and Blevins defined the kinetic constants of the spinach isozyme, as having a higher affinity for glyoxylate (K_m=0.085 mM) in the presence of NADPH, as compared to NADH (K_m=1.10 mM) [82].

**Enzymatic Reaction of Glyoxylate Reductase**

![Enzymatic Reaction of Glyoxylate Reductase](image)

**Figure 15. Enzymatic reaction of glyoxylate reductase.** Oxidation of NADPH drives the reduction of glyoxylate to glycolate.
**Independent Verification of Glyoxylate Concentration as a Reference Standard to Newly Developed Assays**

The development of novel assays requires an independent verification of analyte concentration. The PAM dependent cleavage of the substrate glycyl C$_{\alpha−}$.N bond results in a 1:1 ratio of glyoxylate and amidated product [83, 84]. Each novel glyoxylate assay developed herein, was compared to the production of the corresponding amide. Independent analysis functions to both validate each glyoxylate, as well as demonstrate the utility of each assay for the discovery of novel α-amidated peptides.

Three reference assays were used to validate glyoxylate production; (a) the PAM catalyzed oxidation of N-dansyl-Tyr-Val-Gly to N-dansyl-Tyr-Val-NH$_2$ and glyoxylate, (b) the PAM catalyzed oxidation of hippurate (N-benzoylglycine) to benzamide and glyoxylate, and (c) the base-catalyzed dealkylation of α-hydroxyglycine to benzamide and glyoxylate (Fig. 16).

For each reference reaction, a RP-HPLC separation of substrate and amide product was developed and used to determine the concentration of amide formed. The concentration of the amide product was then compared to the glyoxylate concentration, as determined using the newly developed assays.
Reference Reactions for the Independent Analysis of the Developed Glyoxylate Assays

Figure 16. Reference reactions for the independent analysis of the developed glyoxylate assays. Three reference reactions for the independent analysis of glyoxylate utilized as a standard for all newly developed glyoxylate assays. (A) The PAM catalyzed oxidation of the dansylated tri-peptide substrate, amide product quantified by RP-HPLC coupled to fluorescent detection. (B) The PAM catalyzed oxidation of hippurate to benzamide and glyoxylate, benzamide product quantified by RP-HPLC coupled to spectrophotometric detection. (C) The base catalyzed dealkylation of α-hydroxyhippurate, product quantified by RP-HPLC separation and UV analysis.
Materials and Methods

Materials

*B. stearothermophilus* malate synthase, porcine heart malate dehydrogenase, *Cucurbita* ascorbate oxidase, *Spinacea oleracea* glycolate oxidase, acetyl-CoA, MBTH, DMAB, HRP, lactate dehydrogenase, nicotimamide adenine dinculeotide, 5’,5’-dithio-bis-(2-nitrobenzoic acid) (DTNB), NADH, NADPH, FAD, FMN, ammonium sulfate, glyoxylate, and PMS were purchased from Sigma, α-hydroxyhippuric acid was from Aldrich, Mimetic blue was from Prometic, MTS was a gift from Dr. T. C. Owen, and recombinant rat peptidylglycine α-amidating monooxygenase (PAM) was a gift from Unigene Laboratories, Inc. All other reagents were of the highest quality commercially available.
Methods

*Malate Synthase / Malate Dehydrogenase (MS / MD Assay) Spectrophotometric Assay for Glyoxylate*

*Standardization of the MS / MD Dependent Formazan Assay with NADH.*

A working stock solution of 1500 μM MTS / 82.5 μM PMS was prepared in water, stored at 4°C, and small aliquots removed as needed to reduce light and air exposure [59]. NADH production is frequently linked to tetrazolium reduction because the detection limit for the resulting formazan is lower than that possible for the spectrophotometric determination of NADH [85]. To verify that NADH will drive the conversion of MTS to a formazan, MTS reduction was initiated by the addition of NADH to a solution of 100 mM triethanolamine-HCl pH 7.8, 150 μM / 8.25 μM MTS/PMS, and 0-50 μM NADH to a final volume of 1mL. After a 5 min. incubation at 37 °C in the dark, the absorbance at 490 nm was measured using a Jasco Model V-530 UV-VIS spectrophotometer equipped with the Spectra Analysis software package. The small amount of MTS reduced for the zero NADH control was subtracted from that obtained in the presence of NADH. Reaction conditions (pH, temperature, and choice of buffer) were chosen to minimize the pH dependent spontaneous reduction of MTS in the absence of NADH [59, 86] and the optimal pH values for malate synthase and malate dehydrogenase [87, 88].
Standardization of the MS / MD Assay with Glyoxylate

The enzyme-coupled assay for glyoxylate was initiated by the addition of malate synthase and malate dehydrogenase. The assay contained a standard solution of 100 mM TEA-HCl pH 7.8, 150μM / 8.25μM MTS/PMS, 10mM MgCl₂, 400μM acetyl-CoA, 500μM NAD⁺, 0-50μM glyoxylate, 6U/mL malate synthase, and 6U/mL malate dehydrogenase in a final volume of 1mL. The absorbance at 490 nm was measured after 1 hr incubation at 37°C in the dark. The small amount of MTS reduced for the zero glyoxylate control was subtracted from that obtained in the presence of glyoxylate.

Measurement of the Base-Catalyzed Production of Benzamide and Glyoxylate from α-Hydroxyhippurate Utilizing the MS / MD Assay

Glyoxylate is a product of the base-catalyzed N-dealkylation of carbinolamides [89]. Incubation of 2.5mM α-hydroxyhippurate (C₆H₅-CO-NH-CH(OH)-COO⁻) in 1.0M NaOH for 12 hr at 37°C resulted in the conversion of α-hydroxyhippurate to benzamide as determined by reverse-phase HPLC. The resultant glyoxylate concentration was determined via the enzyme-coupled assay after appropriate dilution with H₂O to a final glyoxylate concentration of ≤40μM.
Measurement of PAM Produced Benzamide and Glyoxylate from Hippurate Utilizing the MS / MD Assay

Hippurate (C₆H₅-CO-NH-CH₂-COO⁻) is a PAM substrate which is amidated to produce benzamide and glyoxylate [83, 84]. Hippurate amidation at 37°C was initiated by the addition of PAM (0.6 mg) to 0.5mL of 100mM MES pH 6.0, 2.0 μM Cu(NO₃)₂, 1.0mM ascorbate, and 3.5mM hippurate. At 10 min intervals, a 45μL aliquot was removed and added to 10μL of 6% (v/v) TFA to terminate the reaction. Percent conversion of hippurate to benzamide was determined at each time interval by C₁₈ RP-HPLC.

Approximately 20 nanomoles of glyoxylate was added to a 0.9mL solution containing the necessary components for the glyoxylate assay, excluding the malate synthase, malate dehydrogenase, and MTS/PMS. Ascorbate was eliminated from all samples, prior to glyoxylate determination, with 1 hour incubation in the presence of 2U/mL of ascorbate oxidase at 37°C. Ascorbate, a co-substrate for the PAM reaction, readily reduces MTS and must be removed prior to addition of the MTS/PMS reagent. After ascorbate elimination, addition of a 100μL solution of 10X concentrated malate synthase, malate dehydrogenase, and PMS/MTS resulted in a final 1.0mL reaction at standard conditions. The glyoxylate concentration was determined by measuring the absorbance increase at 490nm after incubation at 37°C for 1 hr (ε₄₉₀nm = 25.6mM⁻¹cm⁻¹). The small amount of MTS reduced for a control lacking hippurate was subtracted from each time point.
**Glycolate Oxidase / HRP (GO / HRP) Spectrophotometric Assay for Glyoxylate**

**Standardization of the Glycolate Oxidase / Horseradish Peroxidase Assay with H₂O₂**

A standard working solution of 0.6mM MBTH, 30mM DMAB, and 50U/mL HRP was prepared and stored under N₂ in the absence of light. The working solution must be prepared fresh daily to avoid blank values of high absorbance. The quantification of a standardized hydrogen peroxide solution was carried out in 80mM phosphate buffer pH 7.8. The reaction was initiated by the addition of 72μL of the working solution in a final volume of 250μL. The formation of the blue colored indamine dye was followed at 595nm. Linear regression analysis of the obtained data was analyzed against literature regression curves for extinction coefficient analysis.
Standardization of the Glycolate Oxidase / Horseradish Peroxidase Assay with Glyoxylate

Following standardization of the MBTH / DMAB / HRP dependent system for the quantification of hydrogen peroxide, the detection system was applied to the quantification of glyoxylate production. A standard glyoxylate solution was utilized to develop a standard curve for the detection of glycolate oxidase produced hydrogen peroxide. All reactions were performed in 80mM phosphate buffer pH 7.8, 0.1mM FAD, 0.48U/mL glycolate oxidase, and 0 – 4μM glyoxylate at a final volume of 250μL. Reactions were allowed to proceed for forty minutes at 37°C prior to spectrophotometric analysis at 595nm.
Standardization of Glycolate Oxidase / Horseradish Peroxidase Assay for PAM Produced Glyoxylate

PAM reactions were carried out in 100mM MOPS pH 7.1 containing 10U/mL HRP, 1mM sodium ascorbate, 1 μM Cu(NO₃)₂, 20 μM dansyl-Tyr-Val-Gly with 3μg/mL PAM, the reaction proceeded for one hour at 37°C. The resultant concentration of the amidated PAM product was measured by RP-HPLC to quantify the exact concentrations of [dansyl-Tyr-Val-NH₂] and [glyoxylate] (Chapter 3, dansyl assay procedure). Aliquots of the PAM reaction pertaining to variable concentrations (1, 3, 5, 7, and 9μM) of glyoxylate were taken for analysis by the MBTH / DMAB / HRP assay. Prior to analysis of glyoxylate by the afore described assay, the PAM reaction underwent a sample pre-treatment procedure to remove no reacted (non-oxidized) ascorbate. An aliquot of 2U/mL ascorbate oxidase was added to the PAM reaction, incubated for a one hour time period at 37°C prior to glyoxylate analysis. Analysis of PAM produced glyoxylate was carried out under the described conditions for 40 minutes.


*Glyoxylate Reductase Spectrophotometric Assay for Glyoxylate*

**Purification of Glyoxylate Reductase from Spinach**

Glyoxylate reductase was purified from spinach leaves by the methods of Kluczkowski and Blevins [82]. The enzyme was partially purified by a 49/60 ammonium sulfate precipitation. The enzyme was homogenized in an extraction buffer containing 40 mM Tricine pH 7.8, 2mM MgCl₂, 1mM EDTA, 1mM benzamidine, 5μM leupeptin, and 14mM β-mercaptoethanol. The specific activity and protein concentration of the resuspended ammonium sulfate pellet was analyzed by the method of Kluczkowski [82].
Standardization of Glyoxylate Reductase Assay for Glyoxylate

A standard and PAM produced glyoxylate solution was utilized to develop a standard curve for the glyoxylate reductase detection of glyoxylate. All reactions were performed in 100mM MOPS buffer pH 7.1, 0.2mM NADPH. 0.4mg of enzyme at a final volume of 1mL. Glyoxylate dependent loss of NADPH was monitored at $\lambda=340\text{nm}$ on a Jasco Model V530 spectrophotometer equipped with a Spectral Analysis software package. Reactions were initiated by the addition of substrate (1, 3, 5, 7, and $9\mu\text{M}$ glyoxylate) and the $\Delta A_{340\text{nm}}$ was obtained after 10 minutes at $25^{\circ}\text{C}$. All $\Delta A_{340\text{nm}}$ measurements were obtained by the NADPH dependent loss of $A_{340\text{nm}}$ in the presence of glyoxylate, subtracted from the loss $A_{340\text{nm}}$ in the presence of NADPH without substrate.
Standardization of Glyoxylate Reductase Assay for PAM Produced Glyoxylate

A standard solution of PAM produced glyoxylate was produced as described (chapter 2). The solution was utilized for standard curve analysis of the glyoxylate reductase dependent analysis of PAM produced glyoxylate.
Independent Analysis; $C_{18}$ Reverse-Phase HPLC Separations for the Quantification of Benzamide / Glyoxylate

$C_{18}$ Reverse-Phase HPLC Separation of dansyl-Tyr-Val-Gly and dansyl-Tyr-Val-NH$_2$

HPLC assays were performed with a Hewlett-Packard 1100 liquid chromatograph equipped with a quaternary solvent delivery system, a heated column compartment, an auto-sampler, and an auto injector. Analytes were detected at $\lambda_{\text{excitation}} = 300\text{nm}$, and $\lambda_{\text{emission}} = 380\text{nm}$ using an in-line Gilson model 121 fluorometer equipped with appropriate filters. Separations were achieved using a Keystone ODS Hypersil C$_{18}$ column (100 $\times$ 4.6 mm, 5 $\mu$ particle size). Dansyl-Tyr-Val-Gly (retention time = 1.2 min), dansyl-Tyr-Val-Gly (retention time = 2.1 min) were resolved at 50 °C using an isocratic mobile phase of 100mM sodium acetate pH 6.0 / acetonitrile (52/48) at flow rate of 1.0mL/min [90]. The percent conversion of dansyl-Tyr-Val-Gly $\rightarrow$ dansyl-Tyr-Val-NH$_2$ was calculated based on peak height values obtained using a Hewlett Packard HP3392A integrator. The ratio of nmoles/peak area for dansyl-Tyr-Val-Gly, and dansyl-Tyr-Val-NH$_2$ was obtained from linear standard curves generated from 0-50 nanomoles of each. Data resulted in the ultimate determination of the quantity of PAM produced $\alpha$-amidated product and glyoxylate (Fig. 17).
C18 Reverse-Phase HPLC Separation of Hippurate, α-Hydroxyhippurate, and Benzamide.

HPLC assays were performed with a Hewlett-Packard 1100 liquid chromatograph equipped with a quaternary solvent delivery system, a heated column compartment, an auto sampler, and an auto injector. Analytes were detected at 254nm using an in-line variable wavelength UV / VIS spectrophotometer. Separations were achieved using a Keystone ODS Hypersil C18 column (100 × 4.6 mm, 5 μm particle size). Hippurate (retention time = 3.1min), α-hydroxyhippurate (retention time = 2.9 min), and benzamide (retention time = 5.9 min) were resolved at 50 °C using an isocratic mobile phase of 100mM sodium acetate pH 6.0 / acetonitrile (82/18) at flow rate of 1.1mL/min. The percent conversion of hippurate → benzamide or α-hydroxyhippurate → benzamide was calculated based on peak area values obtained using a Hewlett Packard HP3392A integrator. The ratio of nmoles/peak area for hippurate, α-hydroxyhippurate, and benzamide was obtained from linear standard curves generated from 0-50nmoles of each (Fig. 17, & Table 3).
Results and Discussion

Malate Synthase / Malate Dehydrogenase Spectrophotometric Assay for Glyoxylate

MS / MD Assay for Chemically and Enzymatically Produced Glyoxylate

The increase in absorbance at 490nm from the NADH-dependent reduction of MTS (at limiting NADH) was linear with the NADH concentration and yielded an \( \varepsilon_{490nm} = 0.0282 \text{M}^{-1}\text{cm}^{-1} \) (data not shown). The value was within 10% of that reported by Debnam & Shearer [86], 0.0256\text{M}^{-1}\text{cm}^{-1}. The sequential actions of malate synthase and malate dehydrogenase convert glyoxylate and acetyl-CoA to oxaloacetate and NADH (Fig. 9 A). PMS-dependent NADH oxidation drives the reduction of MTS to a purple-colored formazan that produces an increase in absorbance at 490nm proportional to [glyoxylate] (Fig. 10). The extinction coefficient derived from these data, \( \varepsilon_{490nm} = 0.0239 \text{M}^{-1}\text{cm}^{-1} \), was within 7% of the published value (Fig. 17).
Carbinolamides undergo base-catalyzed N-dealkylation to produce an amide and glyoxylate \([89]\) 
\[ R\text{-CO-NH-CH(OH)-COO}^- + \overset{\cdot}{\text{OH}} \rightarrow R\text{-CO-NH}_2 + \text{HCO-COO}^- + \text{H}_2\text{O} \] (Fig. 16 C). The N-dealkylation of \(\alpha\)-hydroxyhippurate provided a test for the newly developed glyoxylate assay. Percent conversion analysis of \(\alpha\)-hydroxyhippurate to benzamide by HPLC served as an independent test for the [glyoxylate] values. The N-dealkylation of 2.5mM \(\alpha\)-hydroxyhippurate with NaOH (as described in the Materials and Methods) resulted in a solution of 0.6mM unreacted \(\alpha\)-hydroxyhippurate, 1.9mM benzamide, and 1.9mM glyoxylate (76% conversion) (Fig.17 B). Analysis of this solution using the enzyme-coupled glyoxylate assay yielded an \(\varepsilon_{490nm} = 0.0277\text{M}^{-1}\text{cm}^{-1}\) (Fig. 17 A).
Table 3.

Ratio of [Glyoxylate] Produced to [Benzamide] Produced by the PAM Treatment of Hippurate

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Glyoxylate Produced (mM)</th>
<th>Benzamide Produced (mM)</th>
<th>[Glyoxylate]/[Benzamide]</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>0.69</td>
<td>0.58</td>
<td>1.2</td>
</tr>
<tr>
<td>50</td>
<td>0.67</td>
<td>0.70</td>
<td>0.96</td>
</tr>
<tr>
<td>60</td>
<td>0.71</td>
<td>0.80</td>
<td>0.89</td>
</tr>
<tr>
<td>70</td>
<td>0.75</td>
<td>0.90</td>
<td>0.83</td>
</tr>
<tr>
<td>80</td>
<td>0.77</td>
<td>0.98</td>
<td>0.79</td>
</tr>
<tr>
<td>90</td>
<td>1.3</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>100</td>
<td>1.3</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>110</td>
<td>1.3</td>
<td>1.3</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Average ± standard deviation = 1.0 ± 0.16

Note. Reactions were initiated by the addition of PAM to 2.5 mM hippurate as described in the Materials and Methods. At the indicated time, an aliquot was removed and assayed for benzamide by HPLC and glyoxylate using the malate synthase/malate dehydrogenase/MTS/PMS system.

Table 3. Comparison of PAM Produced Glyoxylate to Benzamide Production. The ratio of PAM produced glyoxylate as quantified by the newly developed MS/MD assay as compared to PAM produced benzamide analyzed by RP-HPLC.

PAM-catalyzed cleavage of the glycyl Cα-N bond of glycine to form an amide and glyoxylate requires O₂ and ascorbate as co-substrates [83, 84]. Ascorbate, a common contaminant of biological samples, reduces MTS [91] and must be eliminated in order to use the enzyme-coupled glyoxylate assay to measure PAM activity. Ascorbate was effectively removed by treatment of PAM produced glyoxylate-containing samples with 2U/mL ascorbate oxidase. The PAM-catalyzed amidation of hippurate produced glyoxylate and benzamide at a ratio of 1.0 ± 0.16 [glyoxylate] / benzamide] (Table 3), within experimental error of the expected stoichiometry of 1:1.
Thus, the developed malate synthase / malate dehydrogenase assay effectively quantifies PAM produced glyoxylate. For the MTS-derived formazan, this translates into a detection limit of ~2μM glyoxylate ($Δε_{490nm} = 0.05-0.06$). The enzyme-coupled glyoxylate assay can be adapted to a microtiter plate format with a final assay volume of 0.1mL providing a detection limit of ~0.2nmoles of glyoxylate.

In conclusion, an enzyme-based method that links glyoxylate oxidation to tetrazolium reduction has been described. Tetrazolium reduction produced a colored formazan that enabled the spectrophotometric determination of glyoxylate. Methods to eliminate ascorbate enabled the successful determination of glyoxylate in samples initially contaminated with excess ascorbate. The enzyme-coupled glyoxylate assay is amenable for use in 96-well microplate format that will increase the sensitivity of detection and may facilitate a high throughput analysis for glyoxylate.
**Glycolate Oxidase / Horse Radish Peroxidase Linked DMAB / MBTH**

**Assay for Glyoxylate**

**Glycolate Oxidase/Horse Radish Peroxidase Linked DMAB / MBTH / HRP Detection of H₂O₂**

An increase in absorbance at 595nm from the H₂O₂ driven HRP dependent oxidative coupling of DMAB / MBTH was linear with the H₂O₂ concentration and yielded an ε₅₉₅nm = 0.0456 M⁻¹ cm⁻¹ (Fig. 18). This value was within 4.20% of that reported by Ngo & Lenhoff [73] ε₅₉₅nm = 0.0476 M⁻¹ cm⁻¹ at pH 6.5, and within 16.2% of the value reported by [74] ε₅₉₅nm = 0.0530 M⁻¹ cm⁻¹ at pH 4.5. The HRP dependent oxidative coupling of DMAB/MBTH was essentially instantaneous.
Figure 18. Linear regression of the DMAB / MBTH / HRP detection of $\text{H}_2\text{O}_2$. Peroxide dependant formation of an indamine dye as analyzed at an absorbance of 595nm by a Bio-Rad Model 550 micro-plate reading spectrophotometer.
Glycolate Oxidase Dependent DMAB / MBTH / HRP Detection of Standard Glyoxylate

Glycolate oxidase in the presence of the glyoxylate gem diol form and O₂ produces a stoichiometric quantity of H₂O₂ and oxalate (Fig. 9 B). Oxidation of the glyoxylate gem diol drives the H₂O₂ HRP dependent oxidative coupling of MBTH and DMAB to produce an increase in absorbance at 595nm proportional to [glyoxylate] (Fig. 13). The extinction coefficient derived from these data, ε₅₉₅nm = 0.0450M⁻¹cm⁻¹, was within 3.2% of the Ngo & Lenhoff [73] published value (Fig. 19).

Linear Regression of the DMAB / MBTH / HRP Detection of Glyoxylate

![Linear regression graph](image)

**Figure 19.** Linear regression of the DMAB / MBTH / HRP detection of glyoxylate. Calibration of glycolate oxidase activity with glyoxylate as a substrate, demonstration for the use of glycolate oxidase as an enzyme to detect stoichiometric quantities of glyoxylate.
Glycolate Oxidase Dependent DMAB / MBTH / HRP Detection of PAM-Produced Glyoxylate

The PAM-catalyzed amidation of dansyl-Tyr-Val-Gly proceeded to approximately 100% as analyzed by RP-HPLC product formation. Thus, PAM produced a stoichiometric quantity of \([\text{glyoxylate}]_{f} = [\text{dansyl-Tyr-Val-Gly}]_{i}\) for the glycolate oxidase analysis. The glycolate oxidase assay resulted in a ratio of \(1.0 \pm 0.17\) (\(\varepsilon_{595\text{nm, glyoxylate}} = 0.0450\text{M}^{-1}\text{cm}^{-1} / \varepsilon_{\text{glyoxylate, PAM produced}} = 0.0375\text{M}^{-1}\text{cm}^{-1}\)), for the compared values of standard glyoxylate vs. PAM produced glyoxylate, a value within experimental error of 1 : 1 stoichiometry (Fig. 20).

Linear Regression of the DMAB / MBTH / HRP Detection of PAM-Produced Glyoxylate

\[
y = 0.0375x + 0.0072 \\
R^2 = 0.9953
\]

Figure 20. Linear regression of the DMAB/MBTH/HRP detection of PAM-produced glyoxylate. Stoichiometric detection of PAM produced glyoxylate generated from a glycine-extended peptide substrate.
The developed glycolate oxidase assay effectively quantifies PAM produced glyoxylate. For the H$_2$O$_2$ produced indamine, this translates into a detection limit of ~0.5μM glyoxylate ($\Delta\varepsilon_{595nm} = 0.05$-0.06). The enzyme-coupled glyoxylate assay was adapted to a micro-titer plate format with a final assay volume of 0.25mL providing a detection limit of ~125 picomoles of glyoxylate.
**Glyoxylate Reductase Dependent Assay for Glyoxylate**

**Glyoxylate Reductase Detection of PAM-Produced Glyoxylate**

The standardized solution of PAM produced glyoxylate as analyzed by the oxidation of NADPH resulted in a $\Delta\epsilon_{340\text{nm}}=0.0063 \text{ M}^{-1}\text{cm}^{-1}$. This $\epsilon$ value is within 1% of the reported $\epsilon_{340\text{nm}}$ value for NADPH of $\epsilon_{340\text{nm}}=0.0062 \text{ M}^{-1}\text{cm}^{-1}$ (Fig. 21).

**Glyoxylate Reductase Determination of PAM Produced Glyoxylate**

![Graph](image)

**Figure 21. Glyoxylate Reductase Determination of PAM Produced Glyoxylate.** The glyoxylate reductase dependent assay for glyoxylate determination.

$y = 0.0063x + 0.0002$

$R^2 = 0.9912$
In conclusion, two enzyme based systems have successfully been developed for the stoichiometric detection of PAM produced glyoxylate. Both assays provide novel methodologies for the sensitive detection of glyoxylate, glycine-extended peptide, and α-amidated peptides. It is important to note that the glycolate oxidase assay is also amenable to the sensitive spectrophotometric detection of glycolate as well. Both assays have proven to be valid techniques for the detection of PAM activity and have provided a framework for the design of other more sensitive novel techniques for the stoichiometric detection of α-amidated peptides.
Chapter Three

Fluorescent and Chemi-Luminescent Assays for Glyoxylate

Introduction

The spectrophotometric assays described in Chapter 2 are robust, stoichiometric assays for glyoxylate and subsequently PAM activity. However, more sensitive assays for glyoxylate are required for the development of a platform technology to discover $\alpha$-amidated peptides. Several methods exist for the sensitive detection of enzyme catalysis. In particular, fluorescent and chemi-luminescent assays for hydrogen peroxide have detection limits in the femtomole range. To take advantage of these assays, the PAM-dependent production of glyoxylate must be linked to hydrogen peroxide production. This can be accomplished utilizing two enzymes, glycolate oxidase (chapter 2, Fig. 9 B) and glyoxal oxidase (Fig. 22). As described in this chapter, both were used for the successful detection of femtomole levels of PAM produced glyoxylate.

Reaction Catalyzed by Glyoxal Oxidase

![Reaction catalyzed by glyoxal oxidase](image)

**Figure 22.** Reaction catalyzed by glyoxal oxidase. The glyoxal oxidase dependent production of hydrogen peroxide from glyoxylate.
Fluorescent Assays for the Detection of Hydrogen Peroxide

Several fluorophores exist for the fluorescent determination of hydrogen peroxide generated in solution. Fluorescence dyes for hydrogen peroxide determination have been designed as substrates for the enzyme horseradish peroxidase. For example, horseradish peroxidase (E.C. 1.11.1.7.) utilizes Amplex Red® as an electron donor in a disproportionation reaction of hydrogen peroxide to water and molecular oxygen (Fig. 23). Amplex red becomes oxidized to the intensely fluorescent compound resorufin, in a stoichiometric ratio to hydrogen peroxide consumption. Amplex red can be utilized in a continuous assay format for enzymatic activity assays which produce stoichiometric quantities of hydrogen peroxide. Amplex Red® has a quantum yield ($\Phi$) at pH = 9 ($\Phi = \text{photons absorbed / photons emitted}$) of 0.75, rendering Amplex Red® a highly fluorescent fluorophore [91].

Fluorescent Analysis for H$_2$O$_2$ Quantification

Figure 23. Fluorescent analysis for H$_2$O$_2$ quantification. The production of the highly fluorescent dye resorufin, is dependent upon the horseradish peroxidase oxidative catalyzed oxidation of amplex red. Resorufin production is stoichiometric to glyoxylate consumption.
Enhanced Sensitivity of Amplex Red Fluorometric Detection

Spectra of the Fluorophore Resorufin

Figure 24. Spectra of the fluorophore resorufin. The Stokes shift excitation and emission spectra for the oxidized Amplex Red (resorufin) fluorophore [84].

Resorufin fluorescence is typically measured with an excitation $\lambda$ of 560 nm and emission $\lambda$ of 589 nm. The sensitivity of Amplex Red, like all fluorophores, can be compromised in a biochemical assay format as a result of a high signal / noise ratio. Fluorescence is a process allowing for greater sensitivity as result of the Stokes’ shift (Fig. 24). The Stokes’ shift describes the distance of the red shift exhibited by a fluorophore from a shorter excitation wavelength to the longer emissive wavelength. Quantification of the
emission, measures an observable event from no light emission (excitation) to emission resulting in a smaller signal / noise ratio, thereby allowing for a greater degree of sensitivity. Biochemical based fluorescent assay fluorescent detection can be compromised by auto-oxidation of the fluorophore caused by enzymes and / or enzyme cofactors and substrates. This phenomenon will be discussed as it applies to the two enzymatic based assay systems for glyoxylate described in the results section of this chapter.
Fluorescent Assays for Glyoxylate

The Flavin Dependent Glyoxylate Consuming Enzyme Glycolate Oxidase

Glycolate oxidase (hydroxyacid oxidase, E.C. 1.1.3.15) is an oxidoreductase enzyme that typically catalyzes the oxidation of a primary alcohol in the presence of O₂ to a ketone and hydrogen peroxide (chapter 2, Fig. 9 B). Glycolate oxidase is a flavoenzyme, an enzyme that requires a flavin prosthetic group for electron transfer from donor to acceptor (Fig. 25). Classically, glycolate oxidase has been known as a FMN (flavin mononucleotide) dependent flavoprotein, however published data provides evidence for the ability of this enzyme to be catalytically competent in the presence of alternate flavin prosthetic groups, namely, FAD [93]. The flavin-binding domain of glycolate oxidase is deeply seated within the interior of the enzyme, and tightly bound to the interior flavin binding domain with dissociation constants of $10^{-8}$ to $10^{-10}$M [94]. Riboflavin is the simplest of all the flavins in that it contains the basic isoalloxazine “flavin” domain and a D-ribitol group. FMN is riboflavin with a free phosphate attached to the D-ribitol, and, lastly, FAD is a conjugate of FMN containing an AMP moiety via a phospho-diester bond (Fig. 25).
Figure 25. Flavins. The structure of flavin cofactors FMN, FAD, and riboflavin, glycolate oxidase catalysis is dependent upon the presence of either FMN or FAD.
Utilization of Glycolate Oxidase as a Fluorescent Assay for Glyoxylate.

Fluorescence assays are limited in most part by signal/noise, due to the spontaneous oxidation (or reduction) of fluorophores resulting in significant background fluorescence. In addition, fluorescence detection can be further compromised in biological samples by the intrinsic fluorescence of biological molecules (peptides, proteins, etc), thus further affecting signal/noise. In the presence of FMN, Amplex Red non-enzymatically oxidizes to resorufin to the extent that glycolate oxidase activity cannot be measured via an HRP-dependent Amplex Red assay. However, non-enzymatic oxidation of Amplex Red is significantly reduced in the presence of FAD. FAD is a cofactor that completely supports glycolate oxidase activity [93]. In order to link glycolate oxidase activity to an HRP/Amplex Red based assay, the cofactor must be FAD (Fig.26).
Amplex Red Fluorescent Detection of H₂O₂

![Chemical reaction diagram]

Figure 26. Amplex Red fluorescent detection of H₂O₂. The fluorescent Amplex Red dependent assay system for glyoxylate, as catalyzed by glycolate oxidase, and glyoxal oxidase.
The Lignolytic Degrading Enzyme Glyoxal Oxidase

Glyoxal oxidase (GLOX) (Fig. 20) is a basidiomycete fungal enzyme, one of the three enzymes used in the degradation of lignin. The fungal lignin degradation pathway plays a major role in the decomposition of detritus, an integral part of the global carbon cycle [95]. Lignin is the second most abundant substance on the planet second only to cellulose, and forms the “woody” tissue of plants. Collectively, lignin is comprised of several monolignols, namely \( p \)-coumaryl, sinapyl, and coniferyl alcohols which compromise the basic set of monomers for the lignin polymer [96]. Degradation of lignin is imperative to the regeneration of carbon, eventually producing atmospheric CO\(_2\). The catalytic role of glyoxal oxidase is the oxidation of aldehydes to carboxylic acids coupled to the concomitant reduction of dioxygen to hydrogen peroxide [97]. GLOX is a copper metalloenzyme, containing a free radical-coupled copper active site. The radical-copper catalytic motif comprises the two-electron redox active site. More importantly, GLOX is isolated in the reduced form. Activation of the reduced enzyme requires oxidation via treatment with a strong oxidant such as Ir(IV) or Mo(V), or the presence of lignin peroxidase (LiP) or horseradish peroxidase (HRP) [97, 98, 99] for catalytic activity.
Utilization of Glyoxal Oxidase for the Quantification of Glyoxylate

Glyoxal oxidase has broad substrate specificity among simple aldehydes (table 4). Methylglyoxal, the preferred substrate, has a $K_m = 0.64\text{mM}$ as compared to glyoxylic acid $K_m = 2.5\text{mM}$, and a $k_{cat}/K_M$ ratio of 12.4% of the activity for glyoxylate compared to methylglyoxal [100] (Table 4). Moreover, GLOX has an acidic pH optimum (Fig. 27) which when applied to the Amplex Red detection system (Fig. 26), compromises detection limits because the reduced $\Phi_{resorufin}$ at pH 6.0 from 0.75 to 0.11, an overall 85% $\Phi_{resorufin}$ reduction at pH 6.0.

**Glyoxal Oxidase pH Profile**

![Glyoxal Oxidase pH Profile](image)

*Figure 27. Glyoxal oxidase pH profile.* pH profile of glyoxal oxidase shown in this figure was adapted from Kersten and Kirk [99].

GLOX functions in the detection system of glyoxylate like glycolate oxidase: the oxidation of the glyoxylate gem diol to oxalate with the concomitant production of $H_2O_2$. $H_2O_2$ production is coupled to Amplex Red oxidation to resorufin as catalyzed by HRP (Fig. 26).
Table 4.
Kinetic Constants for Alternate Aldehyde Substrates for Glyoxal Oxidase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_M$, mM</th>
<th>$k_{cat}$, s$^{-1}$</th>
<th>$k_{cat}/K_M$, M$^{-1}$ s$^{-1}$</th>
<th>% relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylglyoxal</td>
<td>0.64</td>
<td>198</td>
<td>309,600</td>
<td>100</td>
</tr>
<tr>
<td>Glyoxylic acid</td>
<td>2.5</td>
<td>96</td>
<td>38,200</td>
<td>12</td>
</tr>
<tr>
<td>Glycoaldehyde</td>
<td>8.3</td>
<td>208</td>
<td>25,000</td>
<td>8.1</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>8.3</td>
<td>148</td>
<td>17,800</td>
<td>5.7</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>23</td>
<td>254</td>
<td>11,000</td>
<td>3.6</td>
</tr>
<tr>
<td>Glyoxal</td>
<td>12</td>
<td>118</td>
<td>10,400</td>
<td>3.3</td>
</tr>
<tr>
<td>Dihydroxyacetone</td>
<td>38</td>
<td>188</td>
<td>5,000</td>
<td>1.6</td>
</tr>
<tr>
<td>DL-Glyceraldehyde</td>
<td>42</td>
<td>148</td>
<td>3,600</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table 4. Kinetic constants for several aldehyde substrates of glyoxal oxidase. Figure adapted from Kersten (1990) PNAS. Vol. 87 pp. 2936-2940 [97].

GLOX is isolated in the catalytically inactive, reduced form requiring the presence of an oxidant for catalysis. However, the use of strong oxidants must be avoided as this results in the spontaneous oxidation of Amplex Red. Thus, HRP has a dual role in this detection system as it both activates the reduced glyoxal oxidase, and converts Amplex Red to resorufin via a stoichiometric reaction with $H_2O_2$ (Fig. 25).
Chemi-luminescent Assays for Hydrogen Peroxide

Chemi-luminescence is a similar process to that of fluorescence however excitation to the excited state is dependent upon a chemical reaction rather than incident light ($\lambda_{ex}$ i.e. fluorescence) (Fig. 28).

A Jablonski Diagram

![Jablonski Diagram](image)

**Figure 28. A Jablonski diagram.** Display of the different types of emission from an electronically excited state to the ground state. Blue describes absorption, green fluorescence, and red phosphorescence.

Fluorescence and chemi-luminescence, undergo excitation, internal conversion, followed by an observable emission. Internal conversion is a non-photo-emissive transition of electrons between two states of the same spin number (Fig. 28), and relates to the quantum yield of a fluorophore ($\Phi$). Phosphorescence also undergoes a non-emissive transition, however initially it is between two different spin states. This type of non-emissive transfer is described as intersystem crossing and is followed by internal
conversion. Chemi-luminescence has been utilized for biochemical applications much like fluorescence due to its enhanced sensitivity.

Luminol in the presence of an oxidative (1 or 2 $e^-$ oxidant) metal catalyst such as Mn (II) [102], Cu(phen)$_3^{2+}$ [103], HRP; (Fe(III)) [104,105,106,107,108,109], K$_4$Fe(CN)$_6$•3H$_2$O; (Fe(III)) [110], and Co(II) [109] and hydrogen peroxide, becomes chemically excited to a triplet state (Fig. 29). Excitation is followed by internal conversion to a singlet state, and lastly photo-emission. The amount of luminol photo-emission is proportional to the concentration of hydrogen peroxide present. Luminol prior to becoming excited must be in the doubly de-ionized form requiring that luminol chemi-luminescence be carried out in a basic environment. Of all possible metal oxidants it was found that use of HRP resulted in the greatest light emission by luminol [109, 106].
Mechanism of Luminol Chemi-luminescence

Figure 29. Mechanism of luminol chemi-luminescence. Initially, luminol is oxidized by a metal catalyst, the oxidized luminol then reacts with hydrogen peroxide to produce the excited aminophthalate anion. The amino-phthalate undergoes relaxation emitting blue light with a wavelength maximum at 425 nm. The amount of light given off by the excited amino-phthalate is proportional to the concentration of hydrogen peroxide consumed.
Materials and Methods

Materials

Amplex red, resorufin, and luminol were purchased from Molecular Probes, (Eugene, OR); glycolate oxidase, HRP, FMN, FAD, MES, sodium glycolate, N-dansyl-Tyr-Val-Gly, were purchased from Sigma-Aldrich; recombinant rat PAM was a gift from Unigene Labs, Inc. (Fairfield, NJ); and glyoxal oxidase (source: Phanerochaete chryosporium) was a gift from Dr. James Whittaker (OGI School of Science and Engineering, Oregon Health and Science University, Beaverton, OR). Black flat-bottom, and U-shaped well plates were purchased from Corning. All other reagents were of the highest quality commercially available.
Methods

Standardization of the Fluorophore Resorufin

A standard solution of resorufin was initially used to standardize the micro-plate fluorometer for resorufin fluorescence. Concentrations ranging from 30nM to 9 μM of were analyzed for fluorescent response at $\lambda_{\text{ex}} = 530\text{nm}$ and $\lambda_{\text{em}} = 584\text{nm}$ to generate a resorufin standard curve. Samples were analyzed in black U-shaped microplates, in a Fluoroskan II microplate reading fluorometer equipped with the MTX software analysis package.

Standardization of $H_2O_2$ Produced Fluorescence

A standard hydrogen peroxide solution was utilized for the preparation of a hydrogen peroxide standard curve for resorufin production. Concentrations ranging from 30nM to 9μM peroxide were analyzed in a solution containing, HRP (1U/mL) and 50μM Amplex Red in 50mM phosphate buffer pH 6.0, and pH 8.0.
**Glyoxal Oxidase (GLOX) Fluorescent Assay for Glyoxylate**

*Standardization of the Fluorescent GLOX Assay with Standard Methyl Glyoxal and Glyoxylate.*

The GLOX assay consisted of a standard solution of 50 mM sodium phosphate pH 6.0, 50µM Amplex red, HRP (1U/mL), and either 0.03 – 2.3µM methyl glyoxal or 0.3 – 1.7 µM glyoxylate. The reaction was initiated by the addition of GLOX (final concentration = 0.4mg/mL) and fluorescence was determined after 1 hour at 37°C in the dark for the glyoxylate substrate and after a 30 min 37°C incubation for methyl glyoxal (λ<sub>ex</sub> = 530nm and λ<sub>em</sub> = 584nm). The fluorescence produced from Amplex red oxidation in the absence of glyoxylate or methyl glyoxal was subtracted from that obtained in the presence of glyoxylate/methyl glyoxal.
Standardization of the Fluorescent Glyoxal Oxidase Assay for PAM Produced Glyoxylate

Glyoxylate production was initiated by the addition of PAM (15U/L) to a solution containing 40mM MES / NaOH pH 6.0, 10U/mL HRP, 1.0mM catechol, 0.5μM Cu(SO₄), 20μM dansyl-Tyr-Val-Gly, the reaction proceeded for one hour at 37°C. It is necessary to note that catechol was used as the reductant to support PAM catalysis, as the Fenton chemistry produced by ascorbate in the presence of copper (generation of ·OH, and H₂O₂) resulted in the complete auto-oxidation of Amplex Red. The complete PAM dependent conversion of 20μM N-dansyl-Tyr-Val-Gly to N-dansyl-Tyr-Val-NH₂ and glyoxylate was verified by RP-HPLC to quantify the exact concentrations of [N-dansyl-Tyr-Val-NH₂] and [glyoxylate] (100% conversion, 20μM N-dansyl-Tyr-Val-NH₂ and glyoxylate). Aliquots of the PAM produced glyoxylate pertaining to variable concentrations (0.3, 0.7, 1.6, and 2.3μM) were utilized for analysis by the GLOX assay. Aliquots of glyoxylate were added to a solution of 50mM sodium phosphate pH 6.0, 50μM Amplex red, at HRP (1U/mL) and a final concentration of 0.4mg/mL GLOX. All samples were incubated at 37°C for one hour (as described chapter 3).
**Glycolate Oxidase (GO) Fluorescent Assay for Glyoxylate**

**Standardization of the Fluorescent Glycolate Oxidase (GO) Assay with Glycolate and Glyoxylate.**

The glycolate oxidase assay consisted of a standard solution of 70mM sodium phosphate pH 7.8, 50μM Amplex red, 0.1mM FAD, 1U/mL HRP, and either 0 – 10μM glycolate, or 0 – 10μM glyoxylate in a final volume of 300μL. Commercially available glycolate oxidase as purchased from Sigma contains 2mM FMN. Excess FMN was removed from the enzyme solution by dialysis against 3L of 10mM Tris-HCl pH 7.4. 0.75mM FAD was then added to the apo-enzyme, and pre-incubated for at least 1 hour in order to be fully catalytically competent in the glyoxylate assay. It is necessary to note that the enzyme must be pre-incubated with the FAD cofactor prior to its use. The reaction was initiated by the addition of glycolate oxidase (final concentration = 0.2mg/mL) and fluorescence determined after 1 hour at 37°C for the glyoxylate substrate, and after a 20 min. incubation for the glycolate substrate. Fluorescence produced from the Amplex Red oxidation in the absence of glyoxylate or glycolate was subtracted from that obtained in the presence of glyoxylate / glycolate.
Standardization of the Fluorescent Glycolate Oxidase (GO) Assay for PAM Produced Glyoxylate

Glyoxylate production was initiated by the addition of PAM (0.015U/mL) to a solution containing 40mM MES/NaOH pH 6.0, 10U/mL HRP, 1.0mM catechol, 0.5μM Cu(SO₄), 20μM dansyl-Tyr-Val-Gly, the reaction proceeded for one hour at 37°C. The PAM dependent conversion of 20μM N-dansyl-Tyr-Val-Gly to N-dansyl-Tyr-Val-NH₂ and glyoxylate was verified to be 100% by a RP-HPLC assay (chapter 2), to ensure a 20μM PAM produced glyoxylate solution. Aliquots of the PAM produced glyoxylate pertaining to variable concentrations (1.0, 3.0, 5.0, 7.0 and 9.0μM) were taken for analysis by the glycolate oxidase assay. The aliquots of glyoxylate were added to a solution of 70mM sodium phosphate pH 7.8, 50μM Amplex red, and HRP (1U/mL) in a final volume of 300μL. All fluorescent reactions were initiated by the addition of 0.2mg/mL GO, 0.1mM FAD (final concentration). All samples were incubated for 1 hour at 37°C prior to fluorescent analysis of resorufin production.
**Chemi-Luminescent Assays for Glyoxylate;**

**A Hydrogen Peroxide Chemi-Luminescent Standard Curve**

A standard working solution of 0.1mM Luminol in 0.1M NaHCO₃ pH 10.5 was prepared and purged in an atmosphere of N₂, (20 min. N₂/100mL solution) and stored at 4°C in the dark, the stock was prepared fresh daily. Black flat-bottom 96 well microplates were prepared by the addition of 8μL HRP (11mg/mL) and 67μL of sodium bicarbonate (0.4M), microplates were incubated for at least 45 min. in the dark prior to luminetric analysis. A Berthold / Tropix TR-717 binary injector microplate luminometer, with a 500μL dead volume was utilized for all microplate luminescent assays. The luminometer dead volume is defined as the volume of sample retained in-line after injection prior to reaching the detector for measurement. The primary injector was programmed to inject 200μL of the sample (hydrogen peroxide) and the offset injector was programmed to inject 25μL of the luminol stock. Thus, each well contained a final concentration of 0.3μg/μL HRP, 8.0μM Luminol, 0.1M NaHCO₃ pH 10.5, in a final volume of 300μL.

Standard hydrogen peroxide concentrations (5nM, 7nM, 10nM, 20nM, 50nM) were prepared within a 600μL final sample volume for luminetric analysis. Microplates were prepared such that the injection of 200μL peroxide, 25μL luminol, in addition to the HRP / NaHCO₃ prepared plates resulted in a final well volume of 300μL. Chemi-luminetric
measurements (RLU = Relative Luminescence) were obtained in the flash kinetic mode, with a 1.6sec. time delay between sample and luminol injection.

All measurements were obtained immediately following luminol injection for a time duration of 10 seconds. Three sequential 200μL injections of the standard were necessary to analyze the total 600μL hydrogen peroxide sample. A water blank control was injected prior to and in between each standard H₂O₂ solution in order to define a baseline background RLU chemi-luminescent signal, for signal / noise analysis. A standard curve was generated from the total RLU generated per standard peroxide solution.
Chemi-Luminescent Glycolate Oxidase Assay with Glyoxylate

Following standardization of the chemi-luminescent dependent system for the quantification of hydrogen peroxide, the detection system was applied to the quantification of glyoxylate dependent $\text{H}_2\text{O}_2$ production. A standard glyoxylate solution (7nM, 20nM, 50nM, 80nM, 200nM) was utilized to develop a standard curve for the detection of glycolate oxidase produced hydrogen peroxide. All glycolate oxidase reactions were performed in 100mM phosphate buffer pH 7.8, with a final concentration of 0.48U/mL glycolate oxidase, 0.2mM FMN in a final volume of 600μL. It was not necessary to dialyze the FMN containing enzyme as FMN does not interfere with the chemi-luminescent detection system. Microplates were prepared with 8μL HRP (11mg/mL) and 67μL of sodium bicarbonate (0.4M), incubated for one hour, prior to the analysis of glyoxylate dependent $\text{H}_2\text{O}_2$ production. Each 600μL reaction was injected over three wells as 200μL aliquots per well. In total, the addition of sample (200μL), luminol (25μL), HRP (8μL) and NaHCO$_3$ (67 μL), resulted in a final concentration per well of 0.3μg/μL HRP, 8.0μM luminol, 0.1M NaHCO$_3$ pH 10.5, in a final volume of 300μL. The background RLU was determined by the addition of all reagents excluding the glyoxylate, and the blank was injected prior to and between each standard glyoxylate concentration. A standard curve was generated by the addition of RLU response per well for each standard glyoxylate sample. The detection limit was defined as the signal over background which fit a linear regression of RLU vs. [glyoxylate].
Chemi-luminescent Glycolate Oxidase Assay for PAM produced Glyoxylate

Preparative RP-HPLC for the Collection of the PAM substrate N-dansyl-Tyr-Val-Gly;
An Empirical Trial for the Use of the Platform Technology to Detect a Glycine-Extended Peptide

A 20μM sample of the PAM substrate N-dansyl-Tyr-Val-Gly was injected onto a Keystone ODS Hypersil C_{18} column (100 × 4.6 mm, 5 μ particle size) RP-HPLC column, equipped with a Bio-Rad Model 1200 in-line fraction collector at 1.0 min intervals. The analyte was separated and collected by an isocratic mobile phase of 100 mM sodium acetate pH 6.0 / acetonitrile (52/48) at flow rate of 1.0mL/min [90]. Fractions were collected over the 4 minute separation, lyophilized to dryness, and reconstituted in PAM assay conditions consisting of 40mM MES-NaOH pH 6.3, 1mM sodium ascorbate, 0.5μM CuSO_{4}, 10U/mL HRP, 0.015U/mL PAM in a final volume of 300μL. The PAM reaction proceeded for 1 hour at 37°C, 10μL aliquots per fraction were removed for percent conversion analysis of N-dansyl-Tyr-Val-Gly to N-dansyl-Tyr-Val-NH_{2} by the described RP-HPLC assay [111]. Conversion of the N-dansyl-Tyr-Val-Gly substrate to the products N-dansyl-Tyr-Val-NH_{2} and glyoxylate, was analyzed by a RP-HPLC PAM activity assay (Chapter 2), to ensure 100 % conversion of the substrate in order to verify the reaction products at [N-dansyl-Tyr-Val-NH_{2}] = [glyoxylate] = 20μM. Following the PAM reaction, and product analysis, an aliquot of 2U/mL of ascorbate oxidase was added
to all PAM reactions at 40mM MES pH 6.3 to oxidize the remaining ascorbate. It was necessary to oxidize all remaining ascorbate in the PAM reaction, as the reductive properties of ascorbate suppress the oxidation of luminol, necessary for light production. The resultant solution was utilized as a standard PAM produced glyoxylate stock, for use in the glycolate oxidase assay for PAM produced glyoxylate.

Aliquots of the PAM reaction corresponding to variable concentrations of glyoxylate (7nM, 20nM, 80nM, 200nM) were taken for analysis by the chemi-luminescent glycolate oxidase assay. Aliquots were added to a solution containing 100mM sodium phosphate buffer pH 7.8, reactions were initiated by the addition of 0.48U/mL glycolate oxidase, 0.2mM FMN and reacted for one hour at 37°C at a final volume of 600μL. The RLU response per [glyoxylate] sample was analyzed as described (chapter 3).
Results and Discussion

Resorufin Fluorescent Standard Curve

A solution of resorufin standard was prepared from a purified solid sample. A stock concentration of 15μM was utilized to obtain the slope of 33.5RFUμM⁻¹ (RFU = relative fluorescent units) was obtained (Fig. 30). This value acts as an empirical standard for the concentration dependent fluorescence of Resorufin at λ_{ex} = 560nm and λ_{em} = 584nm.

Fluorescent Response of Resorufin;  
\( \lambda_{ex} = 560\text{nm}, \lambda_{em} = 584\text{nm} \)

\[ y = 33.46x + 5.1914 \]
\[ R^2 = 0.9665 \]

Figure 30. Fluorescent response of resorufin. An initial standard curve for the detection limit of Resorufin was generated as a reference standard.
Standardization of H$_2$O$_2$ Produced Fluorescence

An increase in fluorescence from the H$_2$O$_2$ driven HRP dependent oxidation of Amplex Red was linear with H$_2$O$_2$ concentration and yielded a slope of 40.5RFU$\mu$M$^{-1}$ at pH 6.0, and a slope of 42.5RFU$\mu$M$^{-1}$ at pH 7.8 (Figs. 31 & 32). Hydrogen peroxide detection provided an value for which all enzymatic based developed assays could be compared, and was within 17 % error of the resorufin standard.

**Resorufin Standard Curve for Detection of H$_2$O$_2$ at pH 6.0**

![Resorufin Standard Curve for Detection of H$_2$O$_2$ at pH 6.0](image)

$y = 40.53x - 2.8473$

$R^2 = 0.9964$

**Figure 31.** Resorufin standard curve for detection of H$_2$O$_2$ at pH 6.0. An initial standard curve for the detection limit of hydrogen peroxide produced resorufin was generated as a reference standard at pH 6.0.
Resorufin Standard Curve for Detection of H$_2$O$_2$ at pH 7.8

Figure 32. Resorufin Standard Curve for Detection of H$_2$O$_2$ at pH 7.8. Initial standard curve developed to define the detection limit of hydrogen peroxide produced resorufin at pH 7.8.
Glyoxal Oxidase (GLOX) Fluorescent Assay for α-Amidated Peptides

Glyoxal oxidase oxidizes the glyoxylate gem diol in the presence of O$_2$ to produce a stoichiometric quantity of H$_2$O$_2$ and oxalate (Fig. 22). Glyoxylate oxidation drives the H$_2$O$_2$, HRP dependent oxidation of Amplex Red to produce an increase in fluorescence proportional to [glyoxylate] (Fig. 31 & 32). The extinction coefficients derived from these data are, $\varepsilon = 25.8\text{RFU}\mu\text{M}^{-1}$ (methyl glyoxal) and $\varepsilon = 12.8\text{RFU}\mu\text{M}^{-1}$ (glyoxylate). The methylglyoxal slope is within 36% error of the $\varepsilon$ value for H$_2$O$_2$, and within 23% error of the value obtained for standard resorufin. The $\varepsilon$ value of 12.8 obtained for glyoxylate is 3 fold reduced from the standard values. The cause for this market decrease in sensitivity is a ramification of the significantly reduced $k_{cat}/K_M$ value for methyl glyoxal as compared to glyoxylate. The efficiency of glyoxal oxidase is reduced approximately 4-fold under glyoxylate oxidation conditions (Fig. 33). This decrease in efficiency results in the reduced sensitivity on account of two main factors. First, the incubation time for glyoxylate oxidation is 50% longer as compared to methyl glyoxal, resulting in the formation of a higher background fluorescence for the zero glyoxylate control. Secondly, as mentioned in the introduction to chapter three, low signal to noise is the foremost cause of sensitivity loss in fluorescent assays. Therefore, on account of the reduced enzyme efficiency the detection limit of this assay suffers. The ultimate detection limit for glyoxylate with this assay was 0.3 nanomoles.
Glyoxal Oxidase Activity Standard Curve

Figure 33. Glyoxal Oxidase Activity Standard Curve. Glyoxal oxidase activity for methyl glyoxal as compared to glyoxylate was analyzed to determine the stoichiometric response for glyoxal oxidase glyoxylate oxidation. The error bars represent the standard deviation of triplicate samples.

Glyoxal oxidase activity is severely inhibited in the presence of a reductant. Kurek and Kersten [104] define catechol as a severe inhibitor of glyoxal oxidase activity, therefore catechol could not replace ascorbate in the PAM reaction for the glyoxal oxidase dependent glyoxylate fluorescent assay. Furthermore, a treatment of the catechol supported PAM reaction with catechol oxidase was tried as an avenue for the removal of catechol. Unfortunately, the oxidized dione product is intensely dark which inhibits all resorufin fluorescence. Several other known PAM reductants were analyzed, however their presence also markedly hindered the ability of the glyoxal oxidase assay to detect PAM produced glyoxylate. Recall from chapter three that glyoxal oxidase must be in the oxidized form to support catalysis, on account of its copper radical mechanism, therefore presence of such reductants inactivates glyoxal oxidase. For these reasons, it was
decided that glyoxal oxidase was not the enzyme of choice for the detection of PAM produced glyoxylate.
**Glycolate Oxidase Fluorescent Assay for α-Amidated Peptides**

Glycolate oxidase oxidizes glycolate in the presence of O₂ to glyoxylate and H₂O₂ (chapter 2, Fig 12). The gem diol of glyoxylate an alternate substrate for glycolate oxidase is oxidatively (stoichiometric O₂ required) converted to H₂O₂ and oxalate (Chapter 2, Fig. 14). Glyoxylate / glycolate oxidation drives the H₂O₂, HRP dependent oxidation of Amplex Red to produce an increase in fluorescence proportional to [glyoxylate] / [glycolate] consumed (Fig. 34). The extinction coefficients derived from standard curve analysis are \( \varepsilon = 5.83\text{RFU}\mu\text{M}^{-1} \) (glycolate) and \( \varepsilon = 3.78\text{RFU}\mu\text{M}^{-1} \) (glyoxylate).

![Standard Curve of the Glycolate Oxidase Dependent Fluorescent Assay for Glycolate vs. Glyoxylate](image)

**Figure 34.** Standard curve of the glycolate oxidase dependent fluorescent assay for glycolate vs. glyoxylate. Glycolate oxidase activity for glycolate as compared to glyoxylate was analyzed to determine the stoichiometric response for glyoxal oxidase glyoxylate oxidation.
The glycolate and glyoxylate slopes are within 36% error of the each other and approximately 10-fold reduced for the value obtained by H₂O₂ Amplex Red oxidation. Glycolate oxidase is a flavin dependent enzyme, therefore requires a minimal amount (0.05mM) flavin prosthetic for activity. The FAD supported catalysis for the data presented in chapter three shows a ten fold decrease in sensitivity as a result of the presence of FAD. The fluorescence spectra of FAD has a $\lambda_{\text{ex}} = 450\text{nm}$ and a $\lambda_{\text{em}} = 530\text{nm}$, which overlays with the fluorescent spectra of FAD (Fig. 35). The result of fluorescence overlap is decreased sensitivity since the observed fluorescent emission in this case is not a ‘dark’ process. The excitation/emission overlap greatly hinders the sensitivity of this particular fluorescent assay. The two values obtained for glycolate ($\varepsilon = 5.83\text{RFU}\mu\text{M}^{-1}$) and glyoxylate ($\varepsilon = 3.67\text{RFU}\mu\text{M}^{-1}$) are within 36% error, the difference owing to decreased enzyme efficiency for glyoxylate.

**FAD / Resorufin Fluorescent Spectra**

![FAD / Resorufin Fluorescent Spectra](image)

**Figure 35. FAD / Resorufin fluorescent spectra.** An equi-molar overlay of the fluorescent spectra of FAD and resorufin at a $\lambda_{\text{ex}} = 530\text{nm}$.
Data obtained for the detection of PAM produced glyoxylate was linear with respect to glyoxylate, and well within error of the reported ε value for glyoxylate (Fig.36). Comparatively, the PAM produced glyoxylate standardization was within 14%, of that obtained from a standard glyoxylate solution. This value relates to the effectiveness of the proposed assay to stoichiometricly detect α-amidated/glycine-extended peptides. In conclusion, the fluorescent glycolate oxidase assay was able to detect α-amidated peptide production at a sensitivity of 0.3 nanomoles (0.5μM in 300μL), whereas the glyoxal oxidase assay had considerable difficulties with detection of PAM-produced glyoxylate.

**Standard Curve for the Glycolate Oxidase Dependent Fluorescent Enzyme Assay for PAM Produced Glyoxylate**

![Graph](image_url)

Figure 36. **Standard Curve for the glycolate oxidase dependent fluorescent enzyme assay for PAM produced glyoxylate.** Aliquots of independently quantified glyoxylate were subjected to analysis by the glycolate oxidase assay for glyoxylate analysis.
The H$_2$O$_2$ dependent HRP catalyzed luminol chemi-luminescent RLU response (RLU = relative chemi-luminescence) was linear over a range from 10 to 80nM with respect to [H$_2$O$_2$] concentration and yielded a slope of 618.2RLU/nM$^{-1}$ (Figs. 37 & 38). This was within 7.0 % of the value obtained by the glycolate oxidase dependent H$_2$O$_2$ production from [glyoxylate] / [d-YVG] of 574.7RLU/nM$^{-1}$ (Fig. 35). Chemi-luminescent standard curve analysis of PAM produced glyoxylate was provided results comparable to exogenous H$_2$O$_2$. The RLU value deviates from linearity approaching 100nM (30 picomoles). The assay is sensitive within error to 5nM hydrogen peroxide and 15nM [glyoxylate]. The chemi-luminescent assay has sensitivity well within the nM range (~5-15nM).

The microplate formatted assay has a detection limit of approximately 1.5 x 10$^{-12}$ moles peroxide, 4.5 X 10$^{-12}$ moles glyoxylate at a final volume of 300μL. The luminescent assay displays a marked increase in sensitivity as a result of the large difference in light emission of luminol as compared to resorufin. Recall, that one major contributing factor to the detection of peroxide in the fluorescence assay was the overlap of FAD and resorufin. The red emission at 584nm of Amplex red in the fluorescent assay is affected by the orange emission of FAD at 525nm. Luminol light emission is within the range of $\lambda_{em}$ 440-480nm. The drastic blue shift in emission markedly decreases the background
emission of flavin allowing for greater sensitivity. Additionally, intrinsic protein (enzyme) fluorescence is not an additive factor for a low signal / noise ratio chemi-luminescent assays, as proteins do not intrinsically chemi-luminescence.
Figure 37. Chemi-luminescent standard curve for hydrogen peroxide. Data was obtained by taking the double integration with limits of RLU signal per time and RLU signal per well.

Figure 38. Glycolate oxidase chemi-luminescent standard curve for PAM produced glyoxylate. The tri-peptide dansyl-Tyr-Val-Gly was collected from a RP-HPLC separation, lyophilized, and treated with PAM.
The glyoxal oxidase assay (data not shown) provided the least sensitive determination of glyoxylate dependent production of peroxide. In addition to the decreased sensitivity the enzyme is not readily available and would require the growth of wood rot fungus (*Phanaerochaete chrysosporium*, [99]), as a source for enzyme purification. Therefore, the glycolate oxidase assay was chosen as the premiere assay for peptide discovery.

In conclusion, the chemi-luminescent assay proved most sensitive of all developed assays. Fluorescent assays are often very sensitive into the nM range (Fig. 30) of analyte. However the flavin interference, in addition to long incubation times increasing background fluorescence, presented a definite loss in fluorescent sensitivity. The luminescent assay was chosen as the premiere assay for $\alpha$-amidated peptide discovery. Luminol light emission is generally within the range of 440nm to 480nm, thus there is no flavin interference to adversely affect the signal. Albeit, the development of three novel
assays for glyoxylate, not all demonstrated usefulness towards the outlined platform technology.
Chapter Four

Platform Technology Process

Introduction

Application of the Glyoxylate Assays to the Discovery of α-Amidated Hormones

In order to establish the feasibility of the proposed platform technology to identify novel α-amidated peptides from cell culture, it must first be demonstrated as working prototype for the discovery of a known α-amidated peptide accumulated from cell culture. α-Amidated peptide secreting cells when grown in the presence of a PAM inhibitor, accumulate the glycine-extended precursors (PAM substrates). Recall, from chapters 2 and 3 that each developed glyoxylate assay was applied to the detection of PAM produced glyoxylate (from a peptide substrate) as an initial feasibility study. To further this work, glyoxylate detection was applied to the discovery of an α-amidated/glycine-extended peptide accumulated in cell culture. The detection of a known glycine-extended peptide accumulated in cell culture, served as an empirical trial to establish the performance of the glyoxylate assay(s) as a general tool for the detection of α-amidated hormones.
Utilization of the Mouse Pituitary Cell Line in the Platform Technology

A cell line known to express PAM, and an α-amidated hormone in appreciable quantities was chosen for the cell culture empirical trial. For this purpose a mouse neuro-intermediate pituitary, corticotropic tumor cell line (AtT-20) known to express high levels of PAM and mouse joining peptide (mJP-Gly; an α-amidated peptide) was chosen as a pragmatic trial. mJP-Gly, in addition to α-MSH-Gly are the proteolytic cleavage products of the parent peptide pro-adrenocorticotropic (ACTH/endorphin) (Fig. 37). Following dibasic proteolytic cleavage (Lys-Arg, mJP; Lys-Lys, α-MSH) the corresponding glycine-extended prohormones are produced [33]. Both mJP-Gly and α-MSH-Gly are substrates for PAM. Mouse joining peptide (mJP) was chosen as the peptide of interest for this study based previous data which established mJP-Gly as the peptide in majority (Fig. 40 & 41).

Proteolytic Processing of the Mouse pro-ACTH / Endorphin Homologue

![Figure 40](image)

Figure 40. Proteolytic processing of the mouse pro-ACTH / endorphin homologue. Processing and the respective nomenclature of each resulting peptide [113].
Mains et al [4] report an eighty percent inhibition of mJP-Gly amidation in At-T20 cells when grown in the presence of 0.5 μM – 2.0 μM disulfiram (1,1'-dithiobis(N,N-diethylthioformamide), a copper chelating PAM inhibitor. Disulfiram was chosen based on previous work which demonstrated its use as a PAM inhibitor in AtT-20 cells, and the resultant accumulation of mJP-Gly. Moreover, disulfiram is not extremely lethal to cell growth, and is a reversible inhibitor of PAM, as the disulfiram is metabolized over time, PAM activity is restored.

| Ala-Glu-Glu-Glu-Ala-Val-Trp-Gly-Asp-Gly-Ser-Pro-Glu-Pro-Ser-Pro-Arg-Glu-Gly |
| Ala-Glu-Glu-Glu-Ala-Val-Trp-Gly-Asp-Gly-Ser-Pro-Glu-Pro-Ser-Pro-Arg-Glu-NH₂ |

**Figure 41. mJP Sequence.** Sequences of both the glycine-extended form of mJP-Gly (19-mer) and the PAM amidated product (18-NH₂) [105].
Utilization of the Rat Neuro-intermediate Cell Line in the Platform Technology

Rat CA-77 cells which produce glycine-extended calcitonin gene-related peptide (CGRP-Gly, Fig. 42) were cultured by Unigene Laboratories, Inc. As a blind analysis Unigene Labs sent samples to USF for the verification of the presence of CGRP-Gly from a cellular extract.

**Figure 42. CGRP Peptide Sequence.** Sequence of the glycine-extended and amidated forms of the rat thyroid CGRP peptide [114].

```
Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-
Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-
Glu-Ala-Phe-Gly

Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-
Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-
Glu-Ala-Phe-NH₂
```
Materials and Methods

Materials

Mouse pituitary AtT-20 cells, Ham’s F-12K Medium, horse serum, and bovine serum albumin were purchased from the American Type Cell Culture Collection (www.atcc.org). Benzamide, MES buffer, ascorbate oxidase, and all HPLC grade solvents were purchased from Sigma-Aldrich. All cell culture flasks (T-125, 100mm Petri dishes) were purchased from Gibco-BRL. Disulfiram was purchased from Fluka. Sep-Pak® Plus C\textsubscript{18} cartridges, and an Atlantis® dC\textsubscript{18}, 4.6 x 250 mm, 5\textmu m column was purchased from Waters Corp. The mouse joining peptide-Gly (mJP-Gly) and mouse joining peptide-NH\textsubscript{2} (mJP-NH\textsubscript{2}) were synthesized in-house at the USF core peptide synthesis and Mass Spec facility run by Dr. Ted Gauthier. CGRP-Gly was purchased from American Peptide, Inc. PAM was a generous gift from Unigene Labs, Inc. All other reagents and solvents were of the highest quality available.
Methods

*At-T20 Cell Growth Conditions*

Mouse pituitary cells were grown in Hams F-12K® culture medium supplemented with 15% (v/v) horse serum, 2.5% (v/v) fetal bovine serum, containing 1% (v/v) of the antimicrobial Pen-Strep (10,000 units Penicillin (Base)/mL and 10,000 μg Streptomycin (Base)/mL in 0.85% NaCl (liquid). Cells were rapidly thawed from liquid N₂, added to fresh media and incubated in the presence of 5% CO₂ at 37°C. The AtT-20 cells were non-adherent, therefore cells were passed at a ratio of 1:3 into fresh media after 40% confluency was reached in each culture flask. A sterile laminar flow hood in combination with sterile technique was utilized for all cell culture work. Cells were grown in a 37°C incubator equipped with a constant flow of 5% CO₂ to maintain cell growth.
At-T20 Cell Growth for the mJP-Gly Accumulation Methodology

A 2.5mM disulfiram stock solution was prepared in 70% EtOH for addition to cell culture suspensions. The AtT-20 cells were grown in T-75 cell culture flasks (~20mL of media+cells/flask), prior to cell harvesting for accumulation studies. A total of 5, T-75 flask cell suspensions (Vt of cells & media ≈ 100mL) were collected for disulfiram dependent PAM inhibition. The cell suspension was collected in 15mL centrifuge tubes, centrifuged (2,000 x g) for 3 min., and the supernatant spent media removed by aspiration. The centrifugation and aspiration step was repeated until all the entire suspension was collected from the 8, T-75 culture flasks. A total of 7 centrifuge tubes were utilized to collect all AtT-20 cells.

Seven, 100mm cell culture flasks were prepared for disulfiram inhibition by the addition of 7mL of media containing 5μM disulfiram (16μL of 2.5mM stock). The cells were collected from the 7 centrifuge tubes by resuspension in 1mL media, and added to the 100mm culture flasks. The final volume of each flask was 8mL media containing 5μM disulfiram, flasks were incubated at 37°C, in a 5% CO₂ atmosphere, for 20 hours. The entire procedure was repeated for the non-disulfiram treated negative control cells, with substitution of simply the 70% EtOH carrier for disulfiram.
**Extraction of mJP-Gly from At-T20 Cells**

Cells were collected by centrifugation, the supernatant spent media was decanted and acidified to 0.1% (v/v) TFA with a 6% TFA stock (1.6 mL 6% TFA added to 100 mL). Whole cells were homogenized at 4°C in a ground glass homogenizer with an acid extract containing 0.1M HCl, 5% (v/v) formic acid, 1% (w/v) NaCl, 1% (v/v) TFA [4]. The homogenate was centrifuged, the supernatant collected and added to the acidified media. The remaining cell pellet was again homogenized, centrifuged, and the supernatant added to the acidified media. The solution was approximately 125mL of 0.1% TFA spent media including the homogenized extract. The same procedure was followed for the non-treated cells.
Purification of Cellular Extract on a Sep-Pak® Plus Cartridge

The acid extract was initially purified by solid phase extraction on a Waters Sep-Pak® Plus cartridge prior to HPLC separation. This initial step served to both de-salt and concentrate the spent media/cellular extract containing the accumulated glycine-extended peptides. A 5mL syringe was used to load the Sep-Pak® Plus cartridge with 5mL of a column pre-wetting solution composed of 0.1%(v/v) TFA/80% Acetonitrile at a flow rate $\leq 2.0$mL/min. The cartridge was then rinsed with an aqueous solution of 0.1% (v/v) TFA at a flow rate of 5.0mL/min. The entire spent media/cell extract ($V_t = 125$mL) was loaded onto the cartridge at a rate of 3.0mL/min, followed by a wash of 20mL of 0.1% TFA (v/v). Elution of the desired peptide mixture was accomplished by the addition of 3mL of 0.1% (v/v) TFA/80% at a flow rate $\leq 0.5$mL/min. The 3mL eluent was collected and lyophilized on a Savant SpeedVac® concentrator equipped with a Savant VLP 120 Vacuum pump, then resuspended in 250μL of 0.1% TFA (v/v)/0.001 % Triton-X for injection on the HPLC.
**Preparation of the Cellular Extract for HPLC Purification**

The entire 250μL portion of the resuspended cellular extract was divided into four separate samples for further analysis. Two of the four samples were purely experimental for the analysis of mJP-Gly accumulation by PAM inhibition in cell culture, by both MALDI-TOF and glyoxylate analysis (samples A and B). The remaining two were prepared as spiked standards and 2.5 nmoles of the standard mJP-Gly was added to the 60ul aliquot prior to HPLC separation (samples C and D). For experimental purposes samples were denoted as A-D which corresponded to the sample injected onto the HPLC and 1-70 to indicate fraction number. To exclude any cross contamination or analyte carry-over, the non-spiked cell extract samples A and B were injected and separated prior to the injection of spiked samples C and D.
**RP-HPLC Separation of the Accumulated mJP-Gly**

A quaternary solvent delivery HP-1100 LC unit equipped with a autosampler / autoinjector, a heated column compartment, and a ChemStation software package was utilized to perform a binary linear gradient separation of 100% 0.1 % TFA to 52 % acetonitrile over the duration of sixty-five minutes. A flow rate of 1.0mL/min at a detection $\lambda$ of 278nm at 37°C was utilized to define the standard retention time and signal of pure mJP-Gly. An initial RP-HPLC standard curve was performed on an mJP-Gly standard as a control for the confirmation of analyte retention time and signal. Additionally, RP-HPLC of an equimolar mixture of mJP-Gly and mJP-NH$_2$, verified that both the amidated and glycine extended forms co-elute as stated by Mains et. al. [4].

A 60$\mu$L sample injection volume ($V_t = 65\mu$L for internal standard samples C and D) of the crude AtT-20 peptide extracts was loaded onto a Atlantis® dC$_{18}$, 4.6 x 250mm, 5$\mu$m, reverse phase analytical HPLC column. A Bio-Rad 1200 fraction collector was set in-line to collect HPLC fractions at the time interval of 1.0mL / min and was operator controlled. To avoid any HPLC sample carry over spiked fractions containing 2.5nanomoles mJP-Gly internal standard were analyzed after accumulated samples. Following HPLC fraction collection all fractions were lyophilized to complete dryness on a Savant Speed Vac® concentrator equipped with a Savant VLP 120 vacuum pump and stored at -80°C. It must be noted that no true qualitative and/or quantitative analysis of
mJP-Gly accumulation can be made based solely on the results of the RP-HPLC separation data, and therefore RP-HPLC was solely a preparative method.
**Definition of Collected Sample Content for mJP-Gly Characterization**

For the initial characterization of mJP-Gly accumulation in cell culture samples, a duplicate set of samples were prepared and analyzed by two independent methods. As previously described samples were designated A through D to inform of the contents, for example non-spiked (A, B) and spiked (C, D). For notation purposes a subscript number following the letter informs of the trial number, and a regular font sized number records the fraction number. For example, A\textsubscript{2}-35 defines the sample as non-spiked, trial 2, fraction number thirty-five. Fraction sample sets were split, each set to be designated as glyoxylate analysis (odd numbers A\textsubscript{1,3,5} through D\textsubscript{1,3,5}) or MALDI-TOF analysis (even numbers A\textsubscript{2,4,6} through D\textsubscript{2,4,6}) (see Table 5.). A total of samples A\textsubscript{1-6} through D\textsubscript{1-6} were analyzed by both methodologies, for a total of three sets of glyoxylate vs. mJP-Gly analysis. Initially, samples A\textsubscript{1-D1} were characterized by the methods of the newly developed platform technology for the verification of mJP-Gly via glyoxylate detection. Thus, the duplicate sample sets A\textsubscript{2} - D\textsubscript{2} demonstrated inhibition of PAM in cell culture via MALDI-TOF analysis for the presence of the mJP-Gly. After detection of glyoxylate in samples A\textsubscript{1} - D\textsubscript{1}, and mJP-Gly in samples A\textsubscript{2} - D\textsubscript{2}, duplicate sets were then analyzed.
### Definition of Platform Technology
#### Sample Notation

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Analyte Detected</th>
<th>Method of Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1; 1 - 70</td>
<td>Accumulated mJP-Gly/glyoxylate</td>
<td>Chemi-luminescence</td>
</tr>
<tr>
<td>B1; 1 - 70</td>
<td>Accumulated mJP-Gly/glyoxylate</td>
<td>Chemi-luminescence</td>
</tr>
<tr>
<td>C1; 1 - 70</td>
<td>Spiked mJP-Gly/glyoxylate</td>
<td>Chemi-luminescence</td>
</tr>
<tr>
<td>D1; 1 - 70</td>
<td>Spiked mJP-Gly/glyoxylate</td>
<td>Chemi-luminescence</td>
</tr>
<tr>
<td>A2; 1 - 70</td>
<td>Accumulated mJP-Gly</td>
<td>MALDI-TOF Mass Spec</td>
</tr>
<tr>
<td>B2; 1 - 70</td>
<td>Accumulated mJP-Gly</td>
<td>MALDI-TOF Mass Spec</td>
</tr>
<tr>
<td>C2; 1 - 70</td>
<td>Spiked mJP-Gly</td>
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</tr>
<tr>
<td>D2; 1 - 70</td>
<td>Spiked mJP-Gly</td>
<td>MALDI-TOF Mass Spec</td>
</tr>
</tbody>
</table>

**Table 5.**

*Definition of platform technology sample notation.* Samples were defined by analyte, detection method, and fraction number. A designated notation was necessary to keep the large sample numbers organized.
Treatment of RP-HPLC Fractions with PAM; Production of mJP-NH₂/Glyoxylate

Fractions 26 through 35 from samples A₁;26-35 through D₁; 26-35, were treated with PAM. Lyophilized samples were resuspended in 100µL of a PAM reaction mixture containing 40 mM MES buffer pH 6.3, 1.0mM sodium ascorbate, 0.5µM CuSO₄, 10U/mL HRP, and 0.1 mg PAM, and reacted in a 37°C water bath overnight. An aliquot of 2U/mL ascorbate oxidase was added, and the reaction incubated for 1 hour at 37°C.

Analysis of mJP-Gly Dependent Glyoxylate Production by Chemi-luminescence

Fractions 26 – 35 of sample set A₁ through D₁ were brought up to 600µL in glycolate oxidase reaction conditions. The pH was verified as pH 7.8, and samples were tested for glyoxylate utilizing the newly developed chemi-luminescent glyoxylate assay (chapter 3).

Fractions 26 – 35 of sample set A2 through D2 were removed from storage at -80°C and resuspended in 20μL of 0.01% (v/v) TFA for Mass Spec analysis. A matrix of α-cyano-4-hydroxy-cinnamic acid was spotted in a 1:1 ratio of matrix : analyte on a 384 carbon plate and evaporated to dryness to allow for complete sample : matrix co-crystallization. A MALDI-TOF equipped with an N2 laser of 337nm was operated in the positive ion analysis mode, utilizing an acceleration velocity of 19.00kV at the initial ion source, a 130nanosecond time delay, and a second ion source at the extraction plate of 16.35kV. All MALDI-TOF data was collected from the reflector detector and calibrations were performed externally with a standard peptide mixture.
Demonstration of the Platform Technology by a Blind Experiment; Analysis of Peptide-Gly Samples Sent by Unigene Laboratories, Inc.

Unigene Cell Growth Conditions

Rat CA-77 cells which produce CGRP-Gly were grown in T75 flasks to 60 – 90% confluency in DMEM:F10 media (supplemented with insulin, transferrin and selenium) and 10% fetal bovine serum (FBS). Cells were collected and washed twice with PBS to remove residual serum/media. The cells were then grown in serum free media (DMEM:F10) supplemented with transferrin and selenium for either 24 or 48 hours under accumulation conditions. 0.1μM of the dexamethasone secretagogue and, 100μM of diethylidithiocarbamic acid a PAM inhibitor were added to the media for the accumulation of CGRP-Gly. Insulin, present at high concentration in the medium, was omitted from the accumulation growth medium to avoid potential interference during purification. Conditioned medium was harvested and cellular debris was removed by centrifugation.
Approximately 270mL the conditioned medium was loaded onto a Bio-Cad Sprint Perfusion LC System equipped with an Amberchrom CG300M column (1.1cm x 14.5cm) equilibrated with 0.1% TFA (v/v) / 2% acetonitrile. After sample loading the column was washed with 1% (v/v) TFA / 10% acetonitrile to remove cell culture by-products. The absorbance was monitored at 220nm, with a flow rate of 27Ml / min. Peptides were eluted with a mobile phase containing 0.1% TFA (v/v) / 50% acetonitrile and the resultant peak, which had previously been shown to contain the majority of the peptides in the conditioned medium, was collected. The peptide fraction was concentrated approximately 10-fold by lyophilization, and acetonitrile was added to a final concentration to $\approx 5\%$. All particulate matter following the acetonitrile addition was removed by centrifugation.
Unigene Purification of Rat CGRP-Gly by RP-HPLC

The concentrated peptide fraction was loaded onto a Rainin HPLX, HPLC equipped with a Hypersil BDS C_{18} column (4.6mm x 250mm) equilibrated with 0.1% (v/v) TFA / 20% acetonitrile, and set in-line to an LKB Bromma Model 2112 Redifrac fraction collector. The column was initially washed with 0.1% TFA (v/v) / 20% acetonitrile for 18 minutes. Peptides were eluted with a linear gradient from 0.1% TFA (v/v) / 20% acetonitrile to 0.1% TFA (v/v) / 52% MeCN over a duration of 60 minutes. The column was operated at 1.2mL / min, and the effluent monitored at an absorbance of 220nm by an Applied Biosystems model 683 programmable spectrophotometric detector. Approximately 1.5mL fractions were collected over 60 minutes. Fractions 15 through 41 (fraction set # 1) were collected and concentrated to dryness by lyophilization, and sent to the University of South Florida for CGRP-Gly analysis. The retention time of CGRP-Gly was determined by the injection of a pure CGRP-Gly standard. Duplicate fraction sets # 1- 3 were prepared by the described methodology.
Analysis of Unigene Fractions for CGRP-Gly by the Developed Platform Technology

Unigene fractions 20-29 from fraction set # 1 were selected to undergo the described platform technology. The fractions were resuspended in 100μl of PAM reaction conditions, 40mM MES pH 6.3, 1.0mM sodium ascorbate, 0.5mM CuSO₄, 10U/mL HRP, and 0.015U/mL PAM. The PAM reaction conditions for CGRP-Gly amidation were specified by Unigene Laboratories, Inc. The reaction proceeded for 1 hour at 37°C, and was followed by a 1 hour incubation with 2U/mL of ascorbate oxidase. The samples were brought to 600μL in 100mM Phosphate Buffer pH 7.8, and analyzed by the developed chemi-luminescent assay for glyoxylate. The platform technology was repeated on fractions 18 – 34 from fraction set #1, and fractions 27 – 32 of fraction set # 3.
**Matrix Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry Analysis for CGRP-Gly Accumulation**

Fractions 20 – 31 of fraction set # 2 were sent from Unigene Laboratories, Inc. for Mass Spectral Analysis of CGRP-Gly. A standard CGRP-Gly (American Peptide, Inc.) was initially analyzed to establish the CGRP-Gly parent ion (m/z), by the described methods (chapter 4). Fractions 27-32 were resuspended in 10μL of 0.1% (v/v) TFA, and spotted in a 1 : 1 ratio (matrix : analyte) on a 384 carbon plate and evaporated to dryness. The MALDI-TOF procedure for fraction analysis was performed as described (chapter 4).
Results and Discussion

Demonstration of the Platform Technology to Identify mJP-Gly Accumulation by the Inhibition of PAM in Cell Culture

RP-HPLC of the mJP-Gly Standard

Injection of standard mJP-Gly resulted in an average $R_t$ of $31.42 \pm 0.0564$. The average $R_t$ and standard deviation was calculated over a total of 21 injections (Fig. 43).

![Standard Curve for the Absorbance at 278nm of mJP-Gly as Analyzed by RP-HPLC; mJP-Gly $R_t = 31.417 \pm 0.0564$](image)

$y = 171.36x + 173.8$

$R^2 = 0.9903$

Figure 43. Standard curve for the absorbance at 278nm of mJP-Gly as analyzed by RP-HPLC. Standard curve analysis of mJP-Gly resulted in the determination of the average $R_t$ and RP-HPLC sensitivity of the standard peptide.
RP-HPLC Separation of the Accumulated and Spiked mJP-Gly from At-T20 Cell Culture

The mJP-Gly and mJP-NH₂ standards, confirmed that both the mJP-Gly and mJP-NH₂ co-elute in the described separation. Thus, both the mJP-Gly $M_w = 1999\text{g/mol}$, and mJP-NH₂ $= 1941\text{g/mol}$, appear in the same HPLC fraction by Mass Spectral analysis (Fig. 50). Moreover, RP-HPLC coupled to spectrophotometric detection is not a technique sensitive enough to detect and quantify the accumulated mJP-Gly peptide within the linear detection range of mJP-Gly peptide (Fig. 43).
Analysis of mJP-Gly Dependent Glyoxylate Production by Chemi-luminescence

Fractions 29-32 of A_{1,3,5} and B_{1,3,5} show a positive luminescent signal for fractions 30 and 31. Although results were not stoichiometrically quantifiable, the signal of mJP-Gly accumulation is relative to (Figs. 44, 46 & 45, 47). The chemi-luminescent signals define fractions 30 and 31 positive for glyoxylate: mJP-Gly in both accumulated and spiked samples. These fraction numbers are within standard deviation of the RP-HPLC Rt of mJP-Gly as determined by standard analysis, the non-disulfiram treated cell extract displayed no positive luminescent signal for fractions 29 -32.

**Figure 44.** Chemi-luminescent analysis of accumulated mJP-Gly from At-T20 cells. Fractions 29 – 32 were analyzed by the described method for glyoxylate dependent peroxide production. A blank solution containing all necessary enzymes and cofactors was analyzed between fractions to establish the luminescent baseline signal.
Figure 45. Chemi-luminescent Analysis of Spiked mJP-Gly from At-T20 Cells. Fractions 29 – 32 were analyzed by the described method for glyoxylate dependent peroxide production. A blank solution containing all necessary enzymes and cofactors was analyzed between fractions to establish the luminescent baseline signal.
Chemiluminescent Analysis of Accumulated mJP-Gly from At-T20 Cells

Figure 46. Chemiluminescent Analysis of Accumulated mJP-Gly from At-T20 Cells. Fractions were analyzed by the described method for glyoxylate dependent peroxide production. Values represent RLU minus the average blank value obtained between samples.

Chemiluminescent Analysis of Spiked mJP-Gly from At-T20 Cells

Figure 47. Chemiluminescent analysis of spiked mJP-Gly from At-T20 cells. Fractions were analyzed by the described method for glyoxylate dependent peroxide production. Values represent RLU minus the average blank value obtained between samples.
Summary of Spiked vs. Non-Spiked Cellular Extract for mJP-Gly

<table>
<thead>
<tr>
<th>Fraction #</th>
<th>Spiked</th>
<th>Non-spiked</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>0</td>
<td>20811</td>
</tr>
<tr>
<td>30</td>
<td>767759</td>
<td>177711</td>
</tr>
<tr>
<td>31</td>
<td>68905</td>
<td>72778</td>
</tr>
<tr>
<td>32</td>
<td>74788</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6. Summary of spiked vs. non-spiked cellular extract for mJP-Gly. Values are obtained from the total RLU gained minus the average blank value from each fraction analyzed for glyoxylate:H₂O₂ content.

The luminescent data demonstrates an approximate 4-fold increase in mJP-Gly in the spiked extract. Thus, the approximate amount of mJP-Gly accumulated is 0.625 nanomoles as compared to the 2.5 nanomole spike.
**MALDI-TOF Analysis of Standard mJP-Gly and mJP-NH₂**

Mass Spectral analysis of the purified standards defined the absolute \( m/z \) values for mJP-Gly and mJP-NH₂. Amidation of mJP-Gly results in a total mass loss of 59 \([C₂H₃O₂]\) from the glycine extended peptide. The amidated product gains one proton on the terminal \( \alpha \)-amine during catalysis, resulting in a true difference of 58 mass units between the glycine-extended and amidated forms of mJP-Gly. Respectively, the \( m/z \) value for mJP-Gly is 1999 and 1941 for \( \alpha \)-amidated mJP-Gly (Figs. 48, 49).

![Mouse joining peptide precursor](image)

**Figure 48. Mouse joining peptide precursor.** MALDI-TOF of a standard mJP-Gly sample, depending on calibration standards the parent ion is found 1998 \( \leq m/z \leq 2001 \).
Figure 49. **Mouse joining peptide.** MALDI-TOF of a standard mJP-NH₂ sample, depending on calibration standards the parent ion is found $1940 \geq m/z \leq 1942$. 
**MALDI-TOF Analysis of Accumulated and Spiked mJP-Gly**

Based on the HPLC co-elution of mJP-Gly/mJP-NH$_2$ and incomplete PAM inhibition both parent ions (mJP-Gly, mJP-NH$_2$) are present by MALDI-TOF within the same fraction (Figs. 50-52). MALDI-TOF is not a quantitative methodology based on several variables which affect the signal of a given analyte. These variables include, matrix composition, matrix : analyte co-crystallization, and sample ionization. Other parent ions evident within the analyzed fractions are result of incomplete purity of cellular fractions and most likely are remnants of the cell culture media / serum. Although the identity of these other molecules is not known, their existence is not harmful as it is evident that they do not interfere with the platform technology. Likewise, the existence of other peaks is expected, and will vary among cell lines, and culture mediums used for cell growth. MALDI-TOF analysis demonstrates the disulfiram dependent inhibition of PAM in cell culture and thereby further validates the developed platform technology.
Figure 50. MALDI-TOF of accumulated mJP-Gly. MJP-Gly accumulated in sample B2-30, accumulated mJP-Gly was found in samples B2 30 – 31.
Figure 51. MALDI-TOF of spiked mJP-Gly. MJP-Gly from spiked sample D2-30, samples D2 30 - 32 were positive for mJP-Gly.
MALDI-TOF of mJP-Gly Accumulated Vs. Spiked mJP-Gly

Figure 52. MALDI-TOF of accumulated vs. spiked mJP-Gly. (A) An example of MALDI-TOF data from accumulated sample set A4 - 31, and (B) spiked sample D4 - 31 displaying the isotopic resolution of mJP-Gly within the spiked sample.
Demonstration of the Platform Technology to Identify CGRP-Gly Accumulation by the Inhibition of PAM in Cell Culture

Analysis of Unigene Fractions for CGRP-Gly by Chemi-luminescence

Fractions 20-29 of Unigene sample set #1 were analyzed by the described chemi-luminescent assay for glyoxylate and displayed a positive signal for rat CGRP-Gly in fraction 29 (Fig. 53). Repeat analysis on sample set #1 of fractions 18-34, also defined fraction 29 as positive for glyoxylate (data not shown).

Chemi-luminescent Analysis of Unigene Fraction Set # 1 for CGRP-Gly

![Chemi-luminescent Analysis of Unigene Fraction Set # 1 for CGRP-Gly](graf.png)

Figure 53. Chemi-luminescent analysis of Unigene fraction set # 1 for CGRP-Gly. Luminescent analysis defines sample 29 as positive for glyoxylate.
Analysis of Unigene CGRP-Gly Standard by the MALDI-TOF

A rat CGRP-Gly standard was supplied by Unigene for analysis of the CGRP-Gly parent ion. The parent ion at \( m/z \) of 3860 corresponds to the standard molecular weight of rat CGRP- (Fig. 54).

MALDI-TOF of Standard CGRP-Gly

Figure 54. MALDI-TOF of standard CGRP-Gly. The CGRP-Gly standard supplied by Unigene had a \( m/z \) of 3860, this value corresponds to the calculated molecular weight.
Analysis of Unigene Fractions for CGRP-Gly by the MALDI-TOF

Fractions 21-32 of Unigene fraction set #2 analyzed by MALDI-TOF displayed the parent ion of 3860 \( m/z \) (Fig. 55).

Figure 55. Fraction 29. Fraction 29 from the Unigene fraction set #2. Analysis of fractions 21 -32 displays the presence of CGRP-Gly \( (m/z \ 3860) \) accumulated from cell culture.
Conclusion

The cellular accumulation of the glycine-extended peptides is the premise on which this newly designed platform resides, without the accumulation of the glycine-extended peptides detection of PAM produced glyoxylate is futile. The production of glyoxylate upon treatment of cellular fractions with PAM is compelling for the accumulation of the glycine-extended peptides but not definitive. Mass spectral analysis of glyoxylate positive fractions for the presence of the glycine-extended peptides provides not only verification of the glyoxylate signal, but also it defines the framework for platform technology. The utilization of PAM presents a novel method for the discovery of α-amidated peptides. This method relies on the ability to detect glyoxylate, a stoichiometric product of the PAM dependent amidation of glycine-extended peptides. This platform technology has been thoroughly optimized towards the design of both specific and sensitive assays for glyoxylate.

This body of work has described the development of seven novel spectroscopic enzyme based assays for glyoxylate. Detection of glyoxylate is not only useful as a PAM assay, but is also useful in a clinical setting. The presence of glyoxylate in urine is used a marker for monitoring kidney stone formation. To date, many of the prior glyoxylate assays have been developed for this use, or for detection of ureidoglycolate activity (chapter 1).
Each novel glyoxylate assay can also be used for the detection of PAM-produced glyoxylate, moreover the chemi-luminescent assay has proven itself most useful for the identification of glycine-extended peptides. The development of this new platform technology has proven useful as a ‘proof of concept’, as this technology has been successful at identification of model glycine-extended peptides accumulated from cell culture. The platform technology is now poised to be efficacious for the identification of novel glycine-extended peptides accumulated from cell culture. Although work remains to be done as different cell lines may require alternate PAM inhibitors, and identification of a novel peptides will undoubtedly be difficult, an efficient and robust platform technology for glycine-extended peptide discovery has been developed.
Future Work for Novel Peptide Identification and Characterization

The platform technology is now poised for novel peptide discovery from a variety of sources. Future work relies on the choice of source material, namely cell line choice. Classically, hormones are chemical messengers which carry messages from one cell to another. By definition, this does not imply that both PAM and an $\alpha$-amidated hormone are both synthesized in the same tissue, (cell source) which ultimately means that the cell lines used in the platform technology do not have to express PAM. However, as mentioned in chapter two, PAM has been linked to both exocrine and autocrine growth loops. This feature imparts a unique decision making option to cell line choice for the platform technology; cells which do express PAM and cells which do not express PAM may both be logical choices.

One definite aspect of future work lies in the process for discovery of a novel peptide. The putative peptide must initially be purified to near homogeneity by a variety of chromatographic techniques including reverse-phase and ion-exchange chromatography, coupled to analytical HPLC for purity assessment. It is possible that the platform technology may find a glycine-extended protein fragment, although this is not likely as there are no known endoproteases which cleave at the C-terminal side of glycine. A variety of techniques when employed in tandem will easily dissolve any issues concerning the identity of all novel peptides discovered by this platform technology.
Initially, it is most important to determine the peptide sequence, a goal easily achieved by a variety of techniques, including N-terminal sequence analysis (Edman degradation) or LC-MS-MS. Alternatively, if Edman degradation fails sequences data can be obtained by enzymatic C-terminal sequencing in conjunction with amino acid analysis. Sequence data will provide the initial information to discover if in fact peptides are novel, screening peptide databases with the putative novel peptide sequence will discover if this peptide is simply a fragment of a larger known peptide and/or protein, and if this peptide is an already documented α-amidated peptide. Often, peptides are classed into families because they exist together in one reading frame (e.g. mJP-Gly of the mouse pro-ACTH homologue fig. 40). It is likely that a putative peptide may be contained within an open reading frame known to contain other peptide hormones. One key feature of all the α-amidated peptides is the proteolytic pathway to their formation. Recall, from chapter 1 that all peptides which ultimately become glycine-extended undergo a series of sequence specific proteolytic events. The signature sequence for all peptides to become glycine-extended is a pair of dibasic amino acids flanking the internal glycine. This hallmark of α-amidation will most definitely provide useful information as to if the discovered peptide hormones; are indeed truly novel α-amidated hormones. In conclusion, there are three main factors which must be defined for identification of newly discovered peptides; if the sequence has the correct proteolytic processing sites, is entirely contained within one open reading frame, and contains a C-terminal glycine.

Defining the identity newly discovered α–amidated hormones may prove trivial as compared to in vitro characterization of the newly discovered peptide. It will most likely
require a great deal of metabolic and genomic studies to define physiological the role of any newly discovered peptide. The peptide will need to be produced in large enough quantities for bio-characterization, a goal accomplished by recombinant overexpression of the peptide/peptides in *E. coli*. Parameters such as tissue distribution, disease relevance, and bio-characterization will require time, and will most likely differ among newly discovered peptides. Northern blot analysis can provide valuable information as to the population of peptide transcripts within a specific tissue, and aid in gathering information as to the population in various disease states. Gene knockout and or siRNA can also provide information as to the phenotypical and/or metabolic role of the novel peptide in cell culture or animal studies. Several tools for the *in vitro* characterization of the newly discovered peptide exist, and many can be develop as information of the peptides role unfolds during initial studies. The use of this assay in time may lead to the discovery of many novel peptides essential to the study of α-amidated hormones and their physiological role. Moreover, peptides are the chemical messengers that keep living systems in a homeostasis and abnormalities arising from their over/under production leads to the onset of hormone induced diseases (Table 2). A platform technology for the discovery of novel α-amidated peptides is currently in place, and the ramifications of this technique will provide the scientific community with the basic the knowledge to pioneer the biochemistry of novel α-amidated hormones.
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Appendix
Appendix A: Abbreviations

CGRP-Gly – calcitonin gene related peptide
Dansyl -  5-dimethylamino-naphthalene-1-sulfonyl chloride
DMAB - 3-(dimethylamino)benzoic acid
GLOX – glyoxal oxidase
GO – glycolate oxidase
GR – glyoxylate reductase
HRP – horse radish peroxidase
Luminol - 3-aminophthalhydrazide
MALDI-TOF MS – matrix assisted laser deionization – time of flight mass spectrometry
MBTH - 3-methyl-2-benzothiazolinone hydrazone
mJP-Gly – mouse joining peptide
MOPS - 3-(N-morpholino)-propanesulfonic acid
MS - malate synthase
MD - malate dehydrogenase
MS / MD Assay – malate synthase / malate dehydrogenase assay
MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetraolium, inner salt
NAD+/NADH - nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide reduced form
PAM – peptiylglycine α-amidating monooxygenase
PMS - phenazine methosulfate
RFU – relative fluorescent units
RLU – relative chemi-luminescent units
RP-HPLC – reverse phase high performance liquid chromatography
TEA - triethanolamine
TFA - trifluoroacetic acid
About the Author

Sarah Elizabeth Carpenter received a Bachelor of Arts Degree in Biology from Franklin Pierce College (1997). During her college career, Sarah held a summer position at the Baltimore Medical Examiners, where she assisted pathologists in autopsies to determine cause of death. Sarah also received a Bachelor of Science in Biochemistry and Molecular Biology from the University of Massachusetts, Amherst (2000). As an undergraduate at UMASS she performed independent research in the laboratory of Dr. Jennifer Normally, where she was awarded two Howard Hughes Medical Scholarships, and a National Science Foundation Summer Support Grant to continue her research over the summer semester. She also performed research in the laboratory of Dr. John P. Burand in the Department of Microbiology and Entomology at UMASS. Prior to beginning her Ph.D. work at The University of South Florida, she also worked as a substitute high school teacher in Agawam, Mass. As an undergraduate she was also hired by Spectrum Analytical, Inc., as an analyst in the Organic Extraction Department. Sarah was also hired as an adjunct Natural Sciences faculty member at Manatee Community College while finishing her Ph.D.

Sarah has also participated and performed research on many other projects in Dr. Merkler’s laboratory in the Department of Chemistry. Sarah has studied classical ballet, pointe, jazz, and modern dance at the Firethorn School of Dance, and was a member of the competitive dance team. Her performance repertory at the Tampa Bay Performing
Arts Center includes; Paquita, Don Quiote, and Giselle variations. She was also selected to perform in the Moscow Ballet’s Great Russian Nutcracker, in the Arabian variation.