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Characterization of Morphine-Glucose-6-Phosphate Dehydrogenase Conjugates by Mass Spectrometry

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Abstract

A key characteristic of the analyte-reporter enzyme conjugate used in the enzyme-multiplied immunoassay technique (EMIT) is the inhibition of the conjugate enzyme upon anti-analyte antibody binding. Toward understanding the antibody-induced inhibition mechanism, characterization of morphine-glucose-6-phosphate dehydrogenase (G6PDH) conjugates as model EMIT analyte-reporter enzyme conjugates was pursued. Morphine-G6PDH conjugates were prepared by acylating predominantly the primary amines on G6PDH with morphine-3-glucuronide NHS-ester molecules. In this study, morphine-G6PDH conjugates were characterized using a combination of methods including tryptic digestion, immunoprecipitation, matrix-assisted laser desorption/ionization mass spectrometry, and electrospray ionization tandem mass spectrometry. Twenty-six conjugation sites were identified. The identified sites all were found to be primary amines. The degree of conjugation was determined to be less than the number of conjugation sites, suggesting heterogeneity within the morphine-G6PDH conjugate population. Two catalytically important residues in the active site (K22 and K183) were among the identified conjugation sites, explaining at least partially, the cause of activity loss due to the coupling reaction.

INTRODUCTION

The enzyme-multiplied immunoassay technique (EMIT) is a homogeneous assay technique widely used for small-molecule drug testing[1,2]. Similar to other enzyme immunoassays (EIA), EMIT relies on a reporter enzyme for signal generation. However, the reliance of EMIT on antibody-induced inhibition of the reporter enzyme distinguishes it from other EIA. Conceptually, EMIT is based on the reversible repression of reporter enzyme activity caused by anti-analyte antibody binding to an analyte-reporter enzyme conjugate[3]. When an antibody binds to an analyte or analyte-analog covalently coupled to the reporter enzyme, a physical blockage and/or conformational change of the enzyme active site occurs, thereby reducing its catalytic activity. When introduced, free analyte competes for antibody binding and at least partially prevents repression. Since the concentration of antibody binding sites

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available to inhibit the enzyme depends on the concentration of free analyte, the measurable reporter enzyme activity is related to the free analyte concentration.

Some advantages of EMIT include simple assay protocols, rapid assay time, and low detection limit. Perhaps most important, EMIT-based assays are conducted conveniently in homogeneous solution without the need for washing and separation steps (in contrast to ELISA, for instance). The assay time for commercial EMIT, at less than 1 minute[4], is much shorter than ELISA, and yet a low detection limit (< 1 nM) still can be achieved with EMIT[5]. These qualities have made EMIT attractive for lower molecular weight analytes where suitable reporter enzyme conjugates can be synthesized.

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) from *Leuconostoc mesenteroides* is certainly the most commonly used reporter enzyme for EMIT[4]. The bacterial G6PDH is a 109 kDa homodimer[6] that catalyzes oxidization of glucose-6-phospate (G6P) to 6-phosphogluconate with high specific activity using NAD^+ as the electron acceptor[7]. The rate of NADH production can be monitored either spectrophotometrically or fluorometrically. Analyte-G6PDH conjugates usually are prepared by acylating the primary amine ($–NH_2$) groups of lysines and the N-terminus with activated carboxyl (–COOH) groups of the analyte or analyte derivative. In a common coupling reaction, the hydroxyl (–OH) groups of tyrosines also can be acylated, but to a much lesser extent[8]. It has been established that analyte-G6PDH conjugates prepared in this fashion give significant repression of conjugate enzyme activity upon antibody binding[9,10], a key requirement for EMIT.

Although many EMIT assays have been constructed successfully with analyte-G6PDH conjugates made using the approach described, little is understood about the inhibition mechanism and conjugation sites. One of a few previously published reports showed that antibody-induced inhibition was caused by conformational change and non-cooperative antibody binding since anti-analyte Fab fragments can inhibit the analyte-G6PDH conjugate as effectively as the bivalent IgG[8]. In this report, the data regarding O^3-carboxymethylmorphine-G6PDH inhibition versus anti-morphine concentration was analyzed using a probability model. The modeling results suggested that most morphine was conjugated to G6PDH via a random subset of 12 readily available $–NH_2$ groups and 3 to 4 tyrosine residues. Less frequent conjugation to other $–NH_2$ groups was implied. The model also suggested that only 1 to 2 $–NH_2$ groups (on the homodimer) were associated with antibody-induced inhibition. However, among the 37 $–NH_2$ groups (lysines and the N-terminus) on each G6PDH monomer subunit, it still was not established which residues conjugated with morphine and/or were involved in the antibody-induced inhibition (Figure 1). Further, the conclusions drawn from the probability model were not substantiated with experimental data. Aside from this work, an unsuccessful attempt to identify antibody-induced inhibition sites by proteolytic hydrolysis methods was mentioned in a meeting abstract; however no experimental details or data were published[11]. Finally, a claim was made in a patent regarding genetically-modified G6PDH that suggested that some of the lysine residues (after conjugated with analyte) did not contribute to the antibody-induced inhibition[12]. However, the patent did not identify the lysine residues that are important to antibody-inducted inhibition. Further, only 8 of the lysine residues were discussed in the patent.

A more thorough understanding of the analyte-G6PDH conjugate population created by following a typical coupling reaction protocol can help improve EMIT assays. For example, by identifying sites that are important for antibody-induced inhibition, genetically modified G6PDH mutants can be created to enable more site-specific conjugations, thereby lowering the deactivation caused by general conjugations and allowing for more efficient coupling. In

fact, Dade Behring Inc. created a category of inhibitable, genetically modified G6PDH mutants that has a single conjugation per monomer subunit, thereby preventing deactivation caused by unnecessary conjugation[11]. The conjugation site is located within residues 45 to 60; it is created by an opportunistic cysteine substitution, because *L. mesenteroides* G6PDH includes no natural cysteine residues. However, the inhibition exhibited by the G6PDH conjugates based on the mutants follows a cooperative antibody binding mechanism, which differs from that with the native reporter enzyme. Further, little information about the G6PDH mutants and the EMIT systems employing the mutants has been disclosed; therefore, it is difficult to build upon this development.

In an effort to better understand the antibody-induced inhibition mechanism involving non-cooperative antibody binding, a model morphine conjugate based on native G6PDH was prepared and characterized in this study. A specific aim of this work was to identify the conjugation sites on the G6PDH reporter enzyme. Since the sites that are responsible for antibody-induced inhibition (AII sites) must be a subset of all conjugation sites, it was hoped that this study could narrow the field of candidate AII sites. A combination of methods including tryptic digestion, immunoprecipitation (IP), matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS)[13,14], and electrospray ionization tandem mass spectrometry (ESI-MS/MS)[15] was used to identify sites of morphine attachment on G6PDH. The conjugation site identity data also can be useful for developing strategies to improve EMIT assays. Further, the methods developed in this work could be applicable for characterizing and improving other analyte-reporter enzyme systems designed for EMIT.

MATERIALS AND METHODS

Materials

Morphine-3-β-D-glucuronide (M3G), glucose-6-phosphate dehydrogenase (G6PDH) from *L. mesenteroides* (lyophilized powder), *N*,*N*-dimethylformamide (anhydrous, 99.8 %) (DMF), D-glucose-6-phosphate sodium salt (G6P), β-nicotinamide adenine dinucleotide hydrate (NAD^+), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), gelatin from porcine skin (type A), sodium phosphate monobasic salt, sodium phosphate dibasic salt, Trizma Pre-set crystals (pH 8.0), sinapinic acid (SA), 2, 5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (recrystalized prior use) and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO). Sheep anti-morphine antibody (whole serum) was obtained from AbD Serotec (Raleigh, NC). 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), Protein-G Spin Columns, and Luminol Enhancer Solutions were purchased from Thermo Fisher Scientific (Rockford, IL). Amersham Biosciences PD-10 desalting columns and nitrocellulose membranes were obtained from GE Healthcare (Waukesha, WI). Microcon YM-30 centrifugal filters were obtained from Millipore (Billerica, MA). Methanol, acetonitrile (ACN), trifluoroacetic acid (TFA), lanolin, phosphoric acid, and Tween 20 were obtained from Fisher Scientific (Pittsburgh, PA). NuPAGE Novex 4–12 % Bis-Tris gel, MOPS SDS Running Buffer (20×), NuPAGE LDS Sample Buffer (4×), and NuPAGE Transfer Buffer (20×) were purchased from Invitrogen (Carlsbad, CA). Rabbit anti-sheep antibody-HRP was obtained from Bio-Rad (Hercules, CA). Modified trypsin was obtained from Promega (Madison, WI). GELoader tips were purchased from Eppendorf (Westbury, NY). PolySULFOETHYL aspartamide strong cation exchange material (4 μm) was obtained from PolyLC, Inc. (Columbia, SC). Jupiter Proteo reversed phase media (4 μm, C12) was purchased from Phenomenex (Torrance, CA). POROS R1 and POROS R2 reversed phase material were purchased from Applied Biosystems (Framingham, MA). Finally, Empore C8 extraction discs were obtained from 3M Bioanalytical Technologies (St. Paul, MN).

Morphine-G6PDH Conjugation

The –COOH groups of M3G in 10 mM solution were first activated in 20 mM EDC and 40 mM NHS in DMF for 1 to 2 hours. The activated M3G (M3G-NHS-ester) solution was added to G6PDH in 100 mM sodium phosphate buffer (SPB), pH 7.3, to give a final concentration of 5.4 μM (500 U/mL) G6PDH and 20 % DMF. G6PDH concentrations ranging from 10 U/mL to 500 U/mL, in combination with different M3G concentrations (0.1 – 1.6 mM), were used to prepare different conjugate samples. The mixtures were allowed to react for 4 hours to form the morphine-G6PDH conjugate. At the end of the 4-hour period, the excess crosslinkers and M3G were removed by gel filtration with PD-10 columns and three rounds of centrifugal filtration with Microcon YM-30 centrifuge filters. The conjugation reaction and purification steps were conducted under ambient conditions.

Western Blot

Western Blots were performed according to standard procedures[16]. Briefly, protein samples were denatured in NuPAGE LDS Sample Buffer at 70 °C for 10 minutes. The denatured samples were loaded onto a Bis-Tris gel. Electrophoresis was conducted at 200 V for 55 minutes in MOPS SDS running buffer. The proteins then were transferred to a nitrocellulose membrane in NuPAGE transfer buffer by applying 30 V for 90 minutes. Subsequently, the membrane was incubated in blocking solution consisting of 50 % Qiagen blocking reagent and 2.5 % milk in Tris-buffered saline (200 mM Tris-buffer and 150 mM NaCl) with 0.5 % Tween (TBST) overnight at 4°C, then in sheep anti-morphine Ab solution (500× diluted in TBST) for 2 hours at room temperature, and finally, in a rabbit anti-sheep IgG-HRP solution (2000× diluted in TBST) for 1 hour at room temperature. Between each incubation step, the membrane was washed 3 times in TBST. The HRP signal was amplified using Luminol Enhancer Solutions.

Repression of Morphine-G6PDH Conjugate Activity upon Antibody Binding

In a 1-cm-pathlength cuvette, 3 μL of calibrator solution was added to 136 μL of Syva Reagent 1, which consisted of 4.2 μg/mL sheep polyclonal antibodies to morphine, 10 mM G6P and 6 mM NAD^+. Purified water and 1 mM M3G were used as the calibrator solutions for the repressed and unrepressed state measurements, respectively. The mixture was allowed to equilibrate at 37 °C for 6 minutes. Ten μL of the 2.5 nM morphine-G6PDH solution (in 50 mM Tris-HCl at pH 8.0, 0.1M NaCl and 0.1 % gelatin) was added to the mixture and mixed by repeated micropipetting. The optical density at 340 nm (OD_{340}) was recorded at 37 °C, and the initial change in OD_{340} per minute (ΔOD_{340}/min) was determined. From the ΔOD_{340}, the enzyme activity in units-per-milliliter (U/mL) was calculated using Beer's Law where 6.22 mM^{-1} cm^{-1} was used as the NADH extinction coefficient.

MALDI MS of Intact Morphine-G6PDH Conjugates

The MALDI MS spectra were obtained using an Applied Biosystems Voyager DE-STR MALDI-TOF mass spectrometer. The protein sample was desalted using a Microcon YM-30 centrifugal filter to obtain a final salt concentration < 1 mM, and a final protein concentration of ~5 μM. Sinapinic acid dissolved in 70/30 ACN/water (*v/v*), 0.1 % TFA at a concentration of 20 mg/mL was used as the MALDI matrix. The sample (1.0 μL) and the matrix (1.2 μL) were spotted on the MALDI stainless steel target using the sandwich spotting method[17]. Spectra were recorded in linear mode at 25 kV. The resulting data were processed and analyzed using Voyager Data Explorer (Applied Biosystems, Foster City, CA). The average molecular weight of a conjugate was calculated from the average centroid mass of 3 to 8 mass spectra.

Trypsin Digestion

In-solution trypsin digestion was performed according to the protocol described in Ytterberg et al.[18]. Briefly, proteins were precipitated with greater than 90 % acetone at −20 °C overnight. The precipitates were collected by centrifugation, were resuspended and subsequently were incubated in 80 % acetone, 10 % methanol, and 0.2 % acetic acid at −20 °C for 30 min. The resuspended precipitates then were centrifuged, the supernatant was removed, and residual acetone was evaporated. Next, the pellets were dissolved in 20 μL DMSO and subsequently diluted to 50 mM NH_4HCO_3 and 30 % DMSO. Trypsin was added to a final protease:protein ratio of 1:20, and proteins were digested overnight at 37 °C. The peptide mixtures were dried completely and then resuspended in 5 % formic acid (FA). For in-gel digestion, proteins were separated by SDS-PAGE and stained with Coomassie blue. Stained protein spots were manually excised, washed and digested with trypsin as described in Rosenfeld et al.[19].

Immunoprecipitation of Tryptic Digest

Ten μL of the tryptic digest sample was dried under vacuum and subsequently resuspended in 20 μL of binding buffer (100 mM SPB, pH 7.3). To the resuspended tryptic digest, 20 μL of sheep anti-morphine antibody (whole serum) was added. The resulting mixture was incubated at 4 °C overnight to allow for binding of antibody to M3G tryptic peptides. After this incubation, the mixture was added to 40 μL of protein-G resin (binding capacity: 11–15 mg human IgG/mL), which had been equilibrated with the binding buffer. The sample was incubated at room temperature with mixing for 30 to 60 minutes. Subsequently, the sample was centrifuged at 5,000 × *g* for 2 min, and the supernatant was collected for analysis. The pellet was washed 3 times with 400 μL water and was resuspended in 60 μL of 5 % FA to elute the antibody and antibody-bound tryptic peptide from the protein-G resins. The preparation then was centrifuged, and the resulting eluate (supernatant) was collected and desalted using a POROS R2 packed microcolumn[20].

MALDI MS of Tryptic Digest

The tryptic digest was dried and resuspended in 5 % FA. One half μL of the sample was spotted on the target; subsequently, 0.5 μL of the matrix was mixed into the sample spot. The sample and matrix were allowed to dry and co-crystallize. DHB in 25 % ACN, 15 % isopropanol, 0.5 % phosphoric acid at a concentration of 10 mg/mL or CHCA in 70/30 ACN/water, 0.1 % TFA at a concentration of 7 mg/mL was used as the matrix. MALDI mass spectra were recorded in reflector mode (Voyager DE-STR MALDI-TOF) at 20 kV.

Fractionation of Modified Peptides and ESI-MS/MS Analysis

In-gel digested G6PDH and morphine-G6PDH were analyzed by LC-MS/MS (Synapt HDMS, Waters Corportation, Manchester, UK) using a 40-minute HPLC gradient between 10–95 % ACN/0.1 % FA (*v/v*). The data were processed by searching against an in-house version of the SwissProt sequence databases using Mascot (Matrix Science, London, UK). The mass tolerances for precursor and fragment ions were set to 0.3 Da. A maximum of two missed cleavages in the tryptic digestion were allowed. Variable modifications used in the search included: oxidation of methionine; addition of morphine-3-glucuronide (+443.1580 Da, neutral loss 285.1365 Da) to tyrosine, lysines and the N-terminus; pyroglutamate formation from the N-terminal glutamine and glutamate. All MS/MS spectra were verified manually.

To improve the sequence coverage of modified peptides, in-solution digested morphine-G6PDH was fractionated manually using strong cation exchange (SCX) chromatography. In brief, a plug of 3M Empore C8 extraction discs was put into a P10 pipette tip, and

Polysulfoethyl aspartamide stationary phase was added to a height of 4 mm. After equilibration, the sample was loaded onto the column in 20 % ACN and 1 % FA. The peptides were eluted with elution steps of increasing concentration of ammonium formate, followed by evaporation. The peptides were then resuspended in 5 % FA, loaded onto a microcolumn packed with POROS R2 resins and eluted directly into the glass capillaries (Proxeon Biosystems, Odense, Denmark) to be analyzed manually by ESI-MS/MS (Synapt HDMS). One of the SCX fractions contained several of the potentially modified peptides and was therefore fractionated further. Similar to the fractionation using SCX chromatography, C12 reversed-phase material was packed into P10 tips to a height of 4 mm. Fractions were eluted stepwise using increasing ACN concentration in 1 % FA. After evaporation and resuspension in 5 % FA, the peptides were loaded onto microcolumns and eluted directly into glass capillaries to be analyzed by ESI-MS/MS.

Hydroxylamine Treatment

An equal volume of 2 M hydroxylamine solution, pH 8.5 (prepared fresh daily) was added to the intact morphine-G6PDH and G6PDH (27 μM) samples. The mixture was incubated for 1–5 hours at room temperature. After the incubation, the hydroxylamine was removed using a POROS R1 microcolumn. For tryptic digest samples, 10 μL of tryptic digest was dried, and subsequently resuspended in 10 μL of purified water with vortex treatment. Next, 10 μL of 2 M hydroxylamine, pH 8.5 (prepared fresh daily) was added to the tryptic digest and incubated for 1 to 5 hours. After the incubation, hydroxylamine was removed using a POROS R2 microcolumn.

RESULTS AND DISCUSSION

Morphine-G6PDH Conjugation

Morphine was conjugated to G6PDH using an NHS-enhanced carbodiimide-mediated coupling method[21]. The –COOH group of M3G, a morphine derivative, was first activated by EDC and NHS, forming an NHS-ester. The NHS-ester was then reacted with an $–NH_2$ group from lysines or the N-terminus of G6PDH to form an amide bond. Although coupling could also occur between the NHS-ester and the –OH group from tyrosine, the majority of the labeling is expected to occur at the $–NH_2$ groups because $–NH_2$ is substantially more nucleophilic than –OH at pH 7.3 at which conjugation took place.

To confirm morphine-G6PDH conjugation using this approach, Western blot analysis was employed. The anti-morphine antibody showed affinity for the morphine-G6PDH conjugate samples as indicated by the band that appeared above the molecular weight of a single 54 kDa G6PDH monomer (Figure 2). The absence of signals corresponding to the G6PDH bands also indicated that the binding of the anti-morphine antibody was specific to the M3G molecules. This result verified that the G6PDH monomers were linked covalently to M3G molecules.

Characterization of Intact Morphine-G6PDH

The degree of conjugation and the repression level upon antibody binding are important characteristics of an analyte-G6PDH conjugate used in EMIT-assays, and they often are related. To determine these characteristics and to study their relationship, MALDI MS and activity assay analyses were performed on morphine-G6PDH conjugate samples prepared using coupling reaction mixtures containing M3G and G6PDH at molar ratios of approximately 75:1 (MG1), 150:1 (MG2), 225:1 (MG3) and 300:1 (MG4). MALDI MS was employed to determine the average mass increase of the modified protein from the covalent attachment of M3G to G6PDH, thereby providing an estimate of the degree of conjugation. Figure 3 shows representative MALDI MS spectra of unmodified G6PDH, as well as the

MG1, MG2, MG3, and MG4 preparations. The G6PDH sample gave a peak at *m/z* 54,420 ± 30, which corresponds to the expected molecular weight of a G6PDH monomer. For the conjugate samples, the attachment of each M3G molecule is expected to give a mass increase of 443 Da. The M3G molecular weight is 461 Da, but the coupling reaction results in the release of one water molecule (18 Da). The average molecular masses of the MG1, MG2, MG3, and MG4 preparations were found to be of *m/z* 54,860 ± 60, 55,200 ± 40, 55,600 ± 100, and 55,950 ± 60, which indicated that there was an average of 1.0 ± 0.1, 1.8 ± 0.1, 2.6 ± 0.2, and 3.4 ± 0.2 M3G molecules attached to each G6PDH monomer, respectively. With increased degree of conjugation, an increase in the deactivation of conjugate activity also was observed. The full width at half maximum (FWHM) of the peaks from all four morphine-G6PDH conjugate populations were larger than that of the unmodified G6PDH peak, suggesting there was a distribution in the number of M3G attachments. The FWHM also increased with degree of conjugation, implying a broader distribution as expected.

A high level of antibody-induced reporter enzyme repression is an important EMIT-assay characteristic because it contributes directly to higher assay sensitivity through a lower background signal. To determine the achievable repression level, the enzyme activities measured in the presence and absence of excess anti-morphine antibody were compared. The repression levels were found to be 13 ± 2 %, 27 ± 2 %, 38 ± 2 %, and 45 ± 1 % for MG1, MG2, MG3, and MG4, respectively, as summarized in Figure 3. The unmodified G6PDH showed a negligible level of repression (3 ± 2 %), indicating that the specific binding of antibody to the conjugated M3G caused the observed repression of reporter enzyme activity. The increase in reporter enzyme activity repression with increased degree of conjugation is expected, since the probability of M3G attachment on enzyme sites that cause repression upon antibody binding increases with degree of conjugation.

Studies on morphine-G6PDH conjugates prepared at varying G6PDH concentrations in the coupling reaction mixture revealed that the G6PDH concentration did not affect the degree of conjugation. Using 1.6 mM M3G, morphine-G6PDH conjugates were prepared with G6PDH concentrations ranging from 0.1 to 5.4 μM. The resulting conjugates displayed the same levels of repression, implying the same degree of conjugation. Further, the MALDI MS analysis showed that the degree of conjugation of morphine-G6PDH prepared with 1.1 μM was also ~3.4. These observations, indicating that the degree of conjugation depended on the M3G concentration but not the G6PDH concentration, suggest that spontaneous M3G-NHS-ester hydrolysis limited conjugation reaction progress.

ESI-MS/MS Analysis of MG4 Tryptic Digests

Proteolytic digestion in combination with MALDI MS and MS/MS methods is widely used in protein characterization[22]. In this work, morphine-G6PDH conjugates and unmodified G6PDH were digested by trypsin which cleaves specifically at the carboxyl side of lysine and arginine with the exception of the cases where there is a succeeding proline[23,24] or a M3G-modified lysine. Subsequently, the tryptic digest was analyzed by ESI MS/MS and MALDI MS methods to locate conjugation sites.

MS/MS spectra were obtained from peptides in the MG4 tryptic digest samples. Fifteen M3G tryptic peptides (peptides with bound M3G) were identified. Figure 4a shows a typical deconvoluted ESI-MS/MS spectrum of a M3G tryptic peptide. The deconvoluted ESI-MS/MS spectrum was transformed from the triply charged ion of *m/z* 697.01, which corresponds closely to the *m/z* of expected tryptic peptide IYDEAEVNKYFVR with a single M3G attachment (*m/z* 696.99 or 2087.98 Da). An abundant product ion of *m/z* 286.14 signal also was observed in the spectrum that is consistent for the loss of morphine from M3G. Further, a series of expected b- and y-product ions resulting from cleavage of the peptide backbone

amide bonds confirmed the peptide identity and led to the assignment of the M3G conjugation site, which was determined to be the lysine residue of the tryptic peptide, or K283 of the G6PDH monomer.

The morphine fragment ion (*m/z* 286.14) also was used as a reporter ion, which aided the screening of other M3G tryptic peptides. Figure 4b shows the fragmentation pattern of the 15 M3G tryptic peptides identified using ESI-MS/MS. Left facing bars indicate b-ions and right facing bars indicate y-ions. In 10 out of 15 peptides, the modification sites could be assigned unambiguously, while in four cases the sites were assigned assuming only lysine and tyrosine residues could be modified. In addition, another conjugation site was determined to be at either the N-terminus or K6 based on the fragmentation pattern of the N-terminal peptide (2572.3 Da). As expected, missed trypsin cleavage was observed at all the M3G-modified lysine residues[24].

MALDI MS Analysis of Tryptic Digests

MALDI MS analyses of tryptic digests and anti-morphine immunoprecipitated tryptic digests (IP-digest) also were utilized to identify M3G tryptic peptides. Although the MALDI MS method does not provide amino acid content information, the amino acid sequence of the measured masses can be inferred from the G6PDH sequence. Since the mass and corresponding amino acid sequence of each G6PDH tryptic peptide can be predicted, it is straightforward to deduce the amino acid sequence of the measured masses.

Figure 5 shows representative MALDI MS spectra of the G6PDH tryptic digest (G6PDH-TD), the MG4 tryptic digest (MG4-TD), the G6PDH IP-digest (G6PDH-IPTD) and the MG4 IP-digest (MG4-IPTD) samples. In addition to G6PDH, another control sample, G6PDH reacted with EDC and NHS (G6PDH-EN), was prepared and subsequently subjected to tryptic digestion. As shown in Figure 5, the MALDI MS spectrum of G6PDH-EN showed no detectable difference from that of the G6PDH. Therefore, only the G6PDH control was considered in further analyses.

The overall pattern of MALDI MS spectra (*m/z* 800–5000) for G6PDH-TD and MG4-TD were similar, but the *m/z* 2000–2500 region revealed some minor peaks present only in the MG4-TD spectrum. This observation was anticipated, since peaks representing M3G tryptic peptides were expected to appear only in the MG4-TD spectrum. By identifying peaks that were present in the morphine-conjugated G6PDH mass spectra and absent in the G6PDH mass spectra, masses of M3G tryptic peptides were determined. These measured masses were further matched against the masses of expected M3G tryptic peptides, thereby obtaining the corresponding amino acid sequences. Based on this MALDI-tryptic digest (MALDI/TD) analysis, 45 M3G tryptic peptides were identified.

Anti-morphine immunoprecipitation (IP) also was employed to search for additional M3G tryptic peptides and to further substantiate the previously identified peptides. Since the IP eluate should contain only peptides with affinity for the anti-morphine antibody, the peaks on the MG4-IPTD mass spectra were expected to represent the masses of M3G tryptic peptides. Although background signals were present from non-specific binding, significantly lower signals were measured for the G6PDH-IPTD, suggesting non-specific binding was minimal. From the MALDI/IPTD analysis, 46 M3G tryptic peptides were found, 32 of which were identified in the MALDI/TD study.

Although the MALDI MS method did not provide direct sequence information on M3G conjugation, the results obtained from the MALDI/TD and MALDI/IPTD analyses nonetheless are convincing for several reasons. M3G tryptic peptides were present in the MG4/TD and/or MG4-IPTD samples while absent in the control samples. Moreover, the

MALDI MS results were consistent with the ESI-MS/MS data. Over 90 % (14/15) of the peptides identified by MS/MS also were identified by the MALDI/TD and/or MALDI/IPTD analysis. Finally, the results from these two analyses corroborated one another to a large extent as illustrated by Figure 5 (*m/z* 2000 to 2500). MG4-TD and MG4-IPTD spectra displayed similar peaks that were absent in the control spectra. In fact, over 70% (32/45) of the M3G tryptic peptides identified by the MALDI/TD analysis showed affinity for anti-morphine antibody. It was expected that some of the M3G tryptic peptides (14 in this case) could only be identified by the MALDI/IPTD analysis and not the MALDI/TD analysis because IP generally amplifies signals enabling the identification of modified peptides present at lower concentration. There were 13 peptides that appeared only in the MG4-TD but not the MG4-IPTD spectra, and this observation could be caused by material loss during the wash of the IP experiment. The MALDI/TD and MALDI/IPTD results both confirm and complement one another.

The exact conjugation sites of many M3G tryptic peptides identified by MALDI MS were unknown, because many of the peptides have more than a single lysine or tyrosine residue. In order to narrow down the possible conjugation sites, several MG4 samples were subjected to hydroxylamine treatment to remove any conjugation that occurred via the –OH groups of tyrosine. This process was useful in determining whether the M3G molecule was conjugated through an $–NH_2$ or –OH group for a given tryptic peptide. Three MG4 samples from different batches were treated with hydroxylamine prior to tryptic digestion. The intact mass and repression level of the hydroxylamine-treated MG4 preparation was statistically the same as that of the untreated MG4, suggesting that conjugation occurring through –OH groups was absent or insignificant. However, MALDI/TD and MALDI/IPTD analyses of the hydroxylamine-treated MG4 samples revealed the loss of four tryptic peptide peaks, suggesting that M3G was conjugated to –OH groups on these peptides. However, different results were obtained when the same analyses were performed on two MG4 samples that underwent hydroxylamine treatment after the tryptic digestion. Three out of four peptides reappeared that were lost after hydroxylamine pretreatment; moreover, two different M3G tryptic peptide peaks disappeared. Since MG4 samples treated with hydroxylamine before and after tryptic digestion produced different results, no peptide was confirmed to have M3G conjugation through an –OH group. Nevertheless, the M3G tryptic peptides that remained after hydroxylamine treatment (whether before or after digestion) helped in determining $–NH_2$ conjugation sites on M3G peptides identified by MALDI.

Conjugation Site Determination of M3G Peptides identified by MALDI MS

A given peptide sequence could have more than one possible conjugation site (e.g., it could contain more than a single lysine or tyrosine), and thus the exact amino acid residues engaged in coupling to M3G may not be determined unambiguously without additional information. The task was simplified in several cases by making the following commonly accepted assumptions: (1) lysine, the N-terminus and tyrosine are the only possible conjugation sites, (2) modification of lysines causes missed cleavage, and (3) hydroxylamine removes all M3G groups coupled to tyrosine. These assumptions are well established in the literature[8,25,26], and they also are consistent with the MS/MS results obtained with this particular system. The MS/MS data directly support the first two assumptions, as shown by the fact that all the conjugation sites identified by MS/MS are amines and missed cleavages are observed on all the M3G-modified lysines. The MS/MS results also indirectly support the third assumption. Since all conjugation sites found by the MS/MS method were $–NH_2$ groups, it is consistent with hydroxylamine results, which also suggest that most, if not all, conjugation sites were $–NH_2$ groups. Based on these assumptions, 12 additional lysine residues were identified as conjugation sites, yet the exact conjugation sites for a number of remaining peptides still could not be determined.

Conjugation Site Categorization

Conjugation sites were categorized into three tiers based on the certainty of their identities. The M3G tryptic peptides identified by ESI-MS/MS based on their masses and sequences were highly certain and they were categorized into Tier 1. Peptides that were identified by both MALDI/TD and MALDI/IPTD showed that the peptides had affinity for anti-morphine antibody; these peptides were assigned to Tier 2. Tier 3 M3G-peptides were identified by only MALDI/TD or MALDI/IPTD. After assigning M3G-peptides into different tiers, conjugation sites were then categorized accordingly. The conjugation sites were placed into the same tier as the peptide(s) from which they were deduced. If a conjugation site was identified by multiple peptides, the conjugation site was placed in the highest tier. A total of 26 conjugation sites were confirmed by the data, 14 of which were placed in Tier 1, 6 in Tier 2 and 6 in Tier 3 (Table 1a).

All 26 identified sites were found to be lysine residues, and an additional conjugation site was determined (by MS/MS) to be either the N-terminus or K6. Three other lysine residues, K97, K149 and K209, were potential conjugation sites as suggested by peptides observed at *m/z*, 4119.87, 4147.90, and 2633.32 respectively (Table 1b). However, these sites could not be further pinpointed because of the existence of other possible M3G attachment sites on the peptides. This study demonstrated the primary conjugation sites to be $–NH_2$ groups. It also demonstrated that, among the 37 $–NH_2$ groups on each G6PDH monomer, at least 27 of the groups can be acylated by activated –COOH compounds (in this case M3G-NHS-ester), with perhaps a small amount of modified tyrosines. It also may be worth noting that 4 of the 6 remaining $–NH_2$ sites (K253, K260, K312 and K442), if modified, would generate M3G tryptic peptides with masses over 4400 Da, which could be difficult to detect with the MALDI MS method. Based on the results, all $–NH_2$ groups except for K353 and K377 were found to be at least potential conjugation sites. Therefore, K353 and K377 can be excluded as AII (antibody-induced inhibition) sites.

Although the number of conjugation sites was determined to be at least 27 for MG4, a degree of conjugation of 3.4 suggests that there is a heterogeneous population of morphine-G6PDH conjugates, i.e., different G6PDH monomers have different M3G conjugation sites. Based solely on the MALDI/TD data, 17 conjugation sites were found in the MG1 samples. Since the repression level for MG4 (46 %) was higher than that of MG1 (11 %), the conjugation sites present only in MG4 may be particularly important for antibody-induced inhibition. However, the MALDI MS signal of many of the modified peptides also decreases with decreasing degree of conjugation, which could cause a decrease in repression level. No information on identifying AII sites could be obtained from this result.

The results of this study are mostly consistent with a previous study conducted by Ullman et al.[8], which focused on the characterization of a similar conjugate, O^3-carboxymethylmorphine (O^3-CMM)-G6PDH, fitting inhibition versus anti-morphine concentration data to a probability model. Both studies showed that the level of inhibition increased with degree of conjugation, and lysines were the primary conjugation sites for activated COOH-analyte. A heterogeneous population of analyte-G6PDH was suggested by both studies. There were, however, a few different conclusions between the studies. First, the O^3-CMM-G6PDH study assigned a higher fraction of conjugation sites to tyrosine residues. This inconsistency perhaps could be explained by the different conjugation reaction pH used in the two studies. The O^3-CMM-G6PDH conjugate was prepared at pH 8, while the morphine-G6PDH conjugate in this work was prepared at pH 7.3. A higher pH could increase the nucleophilicity of –OH thereby making the –OH groups more likely to be acylated. Some more minute details, such as the number of conjugation sites and the repression level, also were different between the two systems. Despite a few minor

differences, the majority of the conclusions between the two studies were generally consistent.

Implications for EMIT

An analyte-G6PDH conjugate is a major component in an EMIT system. Therefore, the characterization of analyte-G6PDH conjugates is important for understanding and improving EMIT systems. Although a previous study focused on characterization of O^3-CMM-G6PDH conjugates, this work describes a different approach for characterizing morphine-G6PDH using a combination of several methods including enzyme activity assay, proteolytic digestion, immunoprecipitation and mass spectrometry. Unlike the previous study, the current approach uses MALDI MS to determine the degree of conjugation, obviating the need to handle radioisotopes. More importantly, this study identified conjugation sites via tryptic digestion and MS analysis, information not provided in the previous study.

A useful finding of the current study was that two catalytically important residues in the active sites, K22 and K183[12,27,28], were conjugated with M3G molecules. This explains at least a partial cause of enzyme activity loss due to the conjugation reaction. Within the heterogeneous morphine-G6PDH population, the deactivated morphine-G6PDH molecules are useless for the EMIT assay, because antibody binding to a deactivated enzyme would not generate any change in activity. Further, the M3G molecules on the deactivated enzyme also compete for antibody binding, and as a result, the detection limit would increase. The ability to prevent conjugation on the catalytically important residues could improve EMIT assay performance. Yet, it would be even more useful to be able to perform site-specific conjugation. An existing patent described two mutation schemes to create genetically-modified G6PDH toward site-specific conjugation, but scarce results on the first scheme and no results on the second scheme were reported[12]. The first scheme involved mutation of specific lysine residues to arginine. Since arginine is not reactive to activated –COOH compounds, conjugation on undesired amino acid positions could be prevented. The second proposed scheme involved genetically modifying residues that are important for antibody-induced inhibition to cysteines, and subsequently conjugating the analyte to the cysteine residues. Since the current study revealed that K22 and K183 were conjugation sites for M3G, the lysine to arginine mutation approach would be inadequate. Although it is still unknown which conjugation sites are important for antibody-induced inhibition, the second scheme is a better approach, because it could prevent alteration of catalytically important residues either through genetic modification or covalent conjugation. Perhaps creating a series of G6PDH mutants such that each mutant has a different lysine substituted with cysteine could be a part of the development strategy.

To estimate the number of important sites for antibody-induced inhibition, the inhibition and degree of conjugation data obtained in this study were utilized. Based on probability, the total number of combinations (T) of N conjugation sites at x degree of conjugation is

$$T=\frac{N!}{x!(N-x)!} \qquad \text{Equation 1}$$

If there are d deactivation sites, which cause complete deactivation if modified with M3G, the number of combinations that are still active (not deactivated) is T_{nd}

$$T_{nd}=\frac{N-d!}{x!(N-d-x)!} \qquad \text{Equation 2}$$

Now, if in addition to *d* deactivation sites, there are also *r* sites which if bound by anti-morphine antibody will cause complete inhibition, then the number of combinations that are still active is $T_{nd,nr}$

$$T_{nd,nr}=\frac{N-d-r!}{x!(N-d-r-x)!} \qquad \text{Equation 3}$$

And the % repression measured will be

$$\%\ repression=1-\frac{T_{nd,nr}}{T_{nd}}\times 100\,\% \qquad \text{Equation 4}$$

In order to estimate *r*, the values for $N - d$, *x*, and *% repression* are required. The degree of conjugation (*x*) and the corresponding *% repression* have been measured in this study. The value of $N - d$ is not clear, since *N* and *d* are unknown. However, $N - d$ has an upper bound of 68. This is because K22, K149, K183 have been shown to be catalytically important[27,28,29], meaning that there are at least 6 catalytically important lysine residues per G6PDH homodimer, $d \geq 6$. Since *N* cannot exceed 74 (the total number of $–NH_2$), $N - d \leq 68$. By fitting the *% repression* vs. *x* to Equations 2–4 with the constraint that $N - d \leq 68$, the result shows that *r* cannot exceed 6. Because *r* represents the number of repression sites on each G6PDH homodimer, this suggests at most 3 unique amino acid residues that are important for antibody-induced inhibition. This is consistent with Ullman's study, which estimated 1 important unique amino acid residue.

CONCLUSIONS

In this work, morphine-G6PDH conjugates have been characterized using a combination of methods including an enzyme activity assay, tryptic digestion, immunoprecipitation, and mass spectrometry. This study utilized MALDI MS to determine the degree of conjugation, obviating the need to handle radioisotopes while providing results consistent with a previous O^3-carboxymethylmorphine-G6PDH characterization study. Further, tryptic digestion and MS analyses allowed for conjugation site identification, information not provided in the previous studies. The 26 unambiguously identified conjugation sites all were found to be lysine residues, demonstrating that lysines are the primary conjugation sites and that a large portion of the 36 lysines on the G6PDH are accessible to and can be modified by activated –COOH compounds. Further, the catalytically important lysine residues were among the 26 identified conjugation sites. The insights gained in this study partially explain the cause of enzyme deactivation resulting from the conjugation reaction and are useful in devising strategies for creating new G6PDH mutants to improve EMIT.

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Figure 1.

The amino acid sequence of *L. mesenteroides* G6PDH used in this study as available from the Swiss-Prot protein sequence database (accession number P11411) with the 36 lysines (black) and 21 tyrosines (gray) highlighted. This sequence differs from that in the RCSB Protein Data Bank (PDB ID: 1DPG) by the addition of a methionine at the N-terminus and the substitution of a serine for cysteine at position 62 (both in italics).

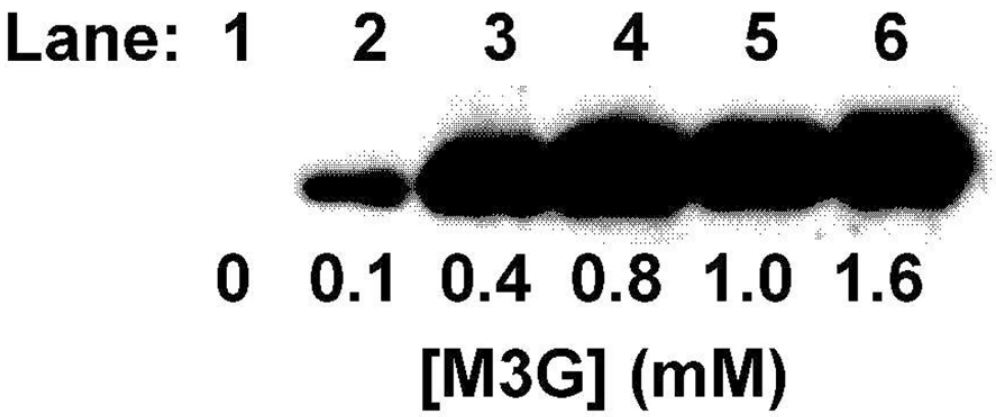


Figure 2.
Western blot (using anti-morphine antibody) of morphine-G6PDH conjugates prepared with the NHS-enhanced carbodiimide-mediated coupling method. Morphine-G6PDH conjugates prepared with 0.1 μM G6PDH and varying M3G concentrations: 0.1, 0.4, 0.8, 1.0 and 1.6 mM (lane 2 – 6). The absence of signals corresponding to unmodified G6PDH (lane 1) indicated that the binding of the anti-morphine antibody was specific to the M3G molecules.

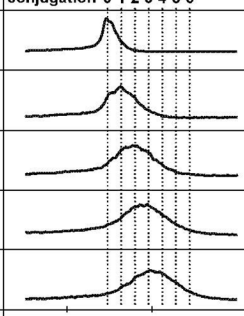
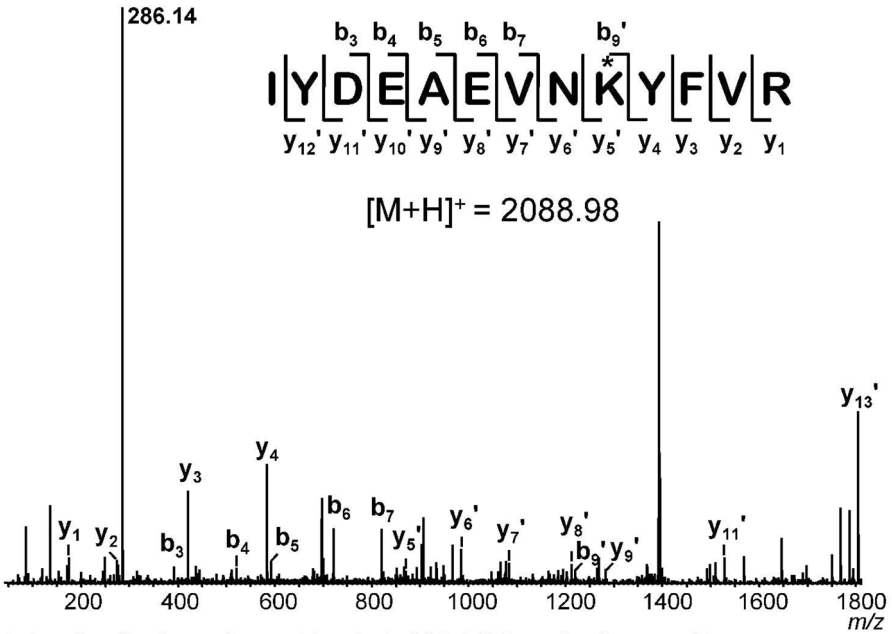
Sample	Representative MALDI Spectra	[M3G] in conjugation (mM)	*m/z*	FWHM (Da)	Degree of conjugation (M3G/ monomer)	Repression
G6PDH		0	54,420 ± 30	660 ± 30	0	3 ± 2 %
MG1		0.4	54,860 ± 60	1380 ± 50	1.0 ± 0.1	13 ± 2 %
MG2		0.8	55,200 ± 40	1780 ± 90	1.8 ± 0.1	27 ± 2 %
MG3		1.2	55,600 ± 100	2050 ± 60	2.6 ± 0.2	38 ± 2 %
MG4		1.6	55,950 ± 60	2180 ± 60	3.4 ± 0.2	45 ± 1 %

Figure 3.

MALDI mass spectra and data (*m/z* and FWHM), the degree of conjugation and the repression levels of intact G6PDH and morphine-G6PDH conjugates (MG1, MG2, MG3 and MG4). The $[M+H]^+$ (centroid mass at 50 % of peak height) and FWHM values are average values of 3 to 6 independent samples; the ± values represent standard errors. The degree of conjugation was calculated based on the difference in $[M+H]^+$ between the conjugate sample and unmodified G6PDH divided by the mass increase of each M3G modification (443 Da). The ± values on % repression data represent standard errors (n = 3).



' *denotes the loss of morphine (m/z 285.14) from the fragment ions*

[M+H]+ *(calc.)*	Fragmentation pattern	Conjugation site(s)	Mascot score
1561.79	AAK*NAAFNALK	K266	22
1576.72	K*NTPEPYER	K410	8
1814.93	K*LYPSVFNLYK	K22	10
1907.90	LNAK*SVEDAFNTR	K387	27
2004.02	GYLQK*HFAIVGTAR	K38	38
2018.97	QALNDDEFK*QLVR	K56	14
2026.02	TLVTFFGGTGDLAK*R	K20	23
2049.95	TIDLGWTVSDEDK*K	K409	18
2088.98	IYDEAEVNK*YFVR	K283	37
2143.02	YLK*SEGLLADTGYNR	K132	58
2235.99	EAIEEAADK*FDIDGNR	K106	17
2430.16	IDHYLGK*EM°VQNIAALR	K183	57
2573.28	°M°VSEIK*TLVTFFGGTGDLAK	N-term. or K6	34
2645.29	FVDAISAVYTADK*APLETYK	K455	32
3066.45	AAK*NAAFNALK*IYDEAEVNK	K266 & K274	

Figure 4.

(a) ESI-MS/MS spectrum (deconvoluted with MaxENT3 software) of tryptic peptide IYDEAEVNKYFVR with a single M3G attachment, $[M+H]^+$ = 2088.98. The $[M+H]^+$ = 286.14 signal corresponds to the mass of the morphine moiety from M3G, indicating the loss of morphine from the backbone fragment. A series of expected b- and y-ions accounted for the morphine loss (b′ and y′ denote ions with the morphine loss), confirmed the peptide identity and allowed for the assignment of the M3G conjugation site, which is the ninth residue of the tryptic peptide or K283 of the G6PDH monomer.

(b) Fragmentation patterns of the M3G tryptic peptides obtained from ESI-MS/MS spectra (* denotes the conjugation sites, and ° denotes oxidized residues). Spectra were deconvoluted with either the MaxENT3 or Mascot Distiller software. While most spectra identified the modified peptide with the highest score when searching the Mascot database, others had to be interpreted manually. The fragmentation pattern of peptide $[M+H]^+$ = 2573.28 suggested either the N-terminus or K6 to be a conjugation site.

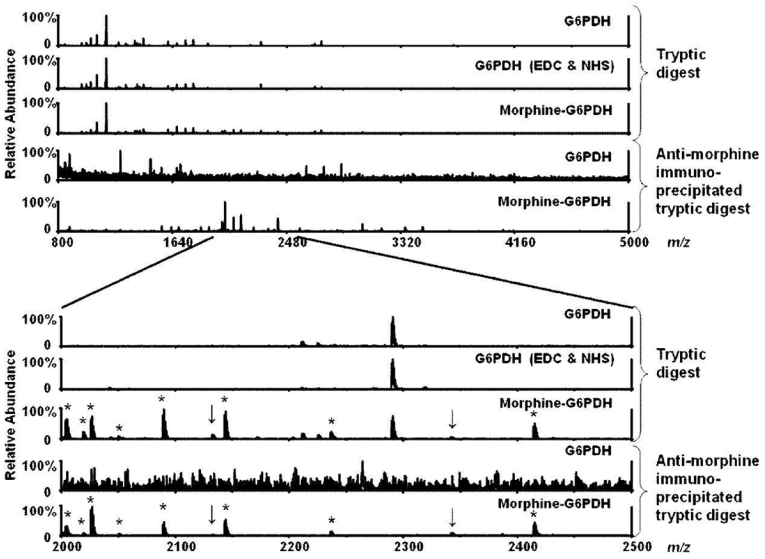


Figure 5.

MALDI MS spectra (top: *m/z* 800–5000 Da, bottom: *m/z* 2000–2500 Da) of G6PDH tryptic digest, G6PDH-EN tryptic digest, MG4 tryptic digest, G6PDH IP-digest, MG4 IP-digest. The y-axes were scaled relative to the peak with the highest intensity in the selected range. * and ↓denote peaks that represent M3G tryptic peptides; * marks M3G tryptic peptides that also were identified by the MS/MS method.

Table 1

M3G conjugation sites (a) and potential M3G conjugation sites (b) categorized into 3 tiers. Tier 1: conjugation sites derived from MS/MS data. Tier 2: conjugation sites derived from M3G tryptic peptides identified by both MALDI-TD and MALDI-IPTD analyses. Tier 3: conjugation sites derived from M3G tryptic peptides identified by either MALDI-TD or MALDI-IPTD analysis.

	M3G conjugation site	**Peptide *m/z***	**Peptide Sequence**[a]
Tier 1	K20	2025.02	TLVTFFGGTGDLA**K**R
	K22	1813.93	**K**LYPSVFNLYK
	K38	2003.02	GYLQ**K**HFAIVGTAR
	K56	2017.97	QALNDDEF**K**QLVR
	K106	2234.99	EAIEEAAD**K**FDIDGNR
	K132	2142.02	YL**K**SEGLLADTGYNR
	K183	2413.21	IDHYLG**K**EMVQNIAALR
	K266	1560.79	AA**K**NAAFNALK
	K274	3065.45	AAKNAAFNAL**K**IYDEAEVNK
	K283	2087.98	IYDEAEVN**K**YFVR
	K387	1906.90	LNA**K**SVEDAFNTR
	K409	2048.95	TIDLGWTVSDED**K**K
	K410	1575.72	**K**NTPEPYER
	K455	2644.29	FVDAISAVYTAD**K**APLETYK
Tier 2	K64	3175.40	DSI**K**DFTDDQAQAEAFIEHFSYR
	K129	1629.84	FFGTIA**K**YLK
	K205	2341.10	FGNPIFDAAWN**K**DYIK
	K299	3128.41	AQYGAGDSADF**K**PYLEELDVPADSK
	K462	2310.03	APLETY**K**SGSMGPEASDK
	K485	1803.88	LLAANGDAWVF**K**G
Tier 3	K32	2846.41	LYPSVFNLY**KK**GYLQK
	K33	2846.41	LYPSVFNLY**KK**GYLQK
	K339	889.43	SG**K**R
	K344	1229.64	LAA**K**QTR
	K383	1498.80	GAIEL**K**LNAK
	K473	2793.29	SGSMGPEASD**K**LLAANGDAWVFK

	Potential M3G conjugation site	**Peptide *m/z***	**Peptide Sequence**[a,b]
Tier 1	N-terminus	2572.28	**NH_2**-MVSEIKTLVTFFGGTGDLAK
	K6	2572.28	NH_2-MVSEI**K**TLVTFFGGTGDLAK
Tier 2	K209	2633.32	DYI**K**NVQVTLSEVLGVEER
Tier 3	K97	4119.87	AHDVTDAASYAVL**K**EAIEEAADKFDIDGNR
	K149	4147.90	LMIE**K**PFGTSYDTAAELQNDLENAFDDNQLFR

[a] Residues in bold denote conjugation sites and potential conjugation sites indicated in the second column.

[b]The underlined residues indicate other possible conjugation sites suggested by the M3G tryptic peptide.