Investigation of Glucose-dependent Insulinotropic Polypeptide-(1–42) and Glucagon-like Peptide-1-(7–36) Degradation in Vitro by Dipeptidyl Peptidase IV Using Matrix-assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry

A NOVEL KINETIC APPROACH*

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The incretins glucose-dependent insulinotropic polypeptide (GIP1–42) and glucagon-like peptide-1-(7–36)-amide (GLP-17–36-amide), hormones that potentiate glucose-induced insulin secretion from the endocrine pancreas, are substrates of the circulating exopeptidase dipeptidyl peptidase IV and are rendered biologically inactive upon cleavage of their N-terminal dipeptides. This study was designed to determine if matrix-assisted laser desorption/ionization-time of flight mass spectrometry is a useful analytical tool to study the hydrolysis of these hormones by dipeptidyl peptidase IV, including kinetic analysis. Spectra indicated that serum-incubated peptides were cleaved by this enzyme with only minor secondary degradation due to other serum protease activity. Quantification of the mass spectrometric signals allowed kinetic constants for both porcine kidney- and human serum dipeptidyl peptidase IV-catalyzed incretin hydrolysis to be calculated. The binding constants ($K_m$) of these incretins to purified porcine kidney-derived enzyme were $1.8 \pm 0.3$ and $3.8 \pm 0.3 \mu M$, whereas the binding constants observed in human serum were $39 \pm 29$ and $13 \pm 9 \mu M$ for glucose-dependent-insulinotropic polypeptide and glucagon-like peptide-1-(7–36)-amide respectively. The large range of $K_m$ values found in human serum suggests a heterogeneous pool of enzyme. The close correlation between the reported kinetic constants and those previously described validates this novel approach to kinetic analysis.

Incretins are hormones of the enteroinsular axis, which potentiate the actions of glucose on the endocrine pancreas (1). The most potent known incretins are glucose-dependent inhibitory polypeptide; GLP-1, glucagon-like peptide-1 (GLP-1,36-amide and GLP-1,37); both are members of the glucagon family of hormones sharing considerable N-terminal sequence homology (2, 3). Both hormones are released from the gut in response to ingested nutrients and were recently shown to be substrates of the circulating exopeptidase dipeptidyl peptidase IV (DP IV, EC 3.4.14.5) (4, 5). This enzyme is a highly specific protease, preferentially hydrolyzing peptides with N-terminal Xaa-Pro and Xaa-Ala motifs (6). Hydrolysis of GIP1–42 and GLP1–36 by DP IV yields GIP3–42 and GLP1–36 and the dipeptides Tyr-Ala and His-Ala, respectively. Activation or inactivation of biologically active peptides is frequently associated with DP IV catalysis. Work by ourselves and others (7, 8) has demonstrated that GIP3–42 and GLP1–36 are biologically inactive, and it has been hypothesized that serum degradation of GIP1–42 and GLP1–36 by DP IV is the primary step in the metabolism of these hormones in the circulation (4, 5, 9).

In 1993 Mentlein and co-workers (4) reported on the kinetics of enzymatic degradation of GIP1–42 and GLP1–36 by purified human placental DP IV, as determined by high performance liquid chromatography (HPLC), and suggested that this may be a physiologically important pathway for the degradation of these hormones. This proposal was supported by Kieffer et al. (5) who administered physiological concentrations of intravenous $^{125I}$-GIP1–42 and $^{125I}$-GLP1–36 into anesthetized rats and monitored the fate of the injected label. HPLC analysis of purified plasma revealed that over 50% of both incretins were hydrolyzed into DP IV reaction products in less than 2 min (5).

The present study describes further investigations on serum degradation of GIP1–42 and GLP1–36 and clarifies the role of DP IV in the breakdown of these hormones. Currently used methods for studying the degradation of peptides rely on radioimmunoassay and/or measurement of radioligand metabolites by HPLC, or capillary electrophoresis, all of which are labor-intensive and require the extensive use of controls. Since these approaches offer only limited information on incretin metabolites, this study was designed to use matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) to investigate incretin degradation in human serum, and to study the kinetics of GIP1–42 and GLP1–36 hydrolysis by human serum and purified porcine kidney DP IV.

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1 The abbreviations used are: GIP, glucose-dependent insulinotropic polypeptide or gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1-amide; DP IV, dipeptidyl peptidase IV; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight; MS, mass spectrometry; HPLC, high performance liquid chromatography; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Since MALDI-TOF MS is tolerant of heterogeneous samples (buffers, salts, and contaminants), this technology is ideally suited for analysis of biological fluids such as serum and is able to accurately resolve all analyte metabolites on the basis of their m/z, thereby overcoming a significant limitation of other approaches.

**EXPERIMENTAL PROCEDURES**

**Instrumentation and General Procedures**

Matrix-assisted laser desorption/ionization mass spectrometry was carried out using a Hewlett-Packard G2025 mass spectrometer with a linear time of flight analyzer. The instrument was equipped with a 337-nm nitrogen laser, a high-potential acceleration source (5 kV), and a 1.0-m flight tube. Detector operation was in the positive-ion mode, and signals were recorded and filtered using a LeCroy 9350 M digital storage oscilloscope linked to a personal computer. The spectrometer was externally calibrated using the low molecular weight standard Hewlett-Packard (G2051A).

The DP IV used in this study was purified from porcine kidney according to a previously described method (10) (this enzyme was kindly prepared by C. Krüger, Hans-Knoell-Institute of Natural Product Research Jena, Halle-Saale, Germany). The specific activity measured using H-Gly-Pro-4-nitroanilide as a chromogenic substrate was 45 unit/mg.

To obtain mass spectra of GIP1–42 (Peninsula) and GLP-17–36 (Bachem), in the presence or absence of DP IV, substrate was incubated at 30°C with 0.1 mM Tricine buffer, pH 7.6, and either enzyme or water in a 2:2:1 ratio. Samples (4 µl) of the incubation mixture were removed at various time intervals and mixed with equal volumes of 2:3-dihydroxyacetophenone as matrix solution (Aldrich). A small volume (<1 µl) of this mixture was transferred to a probe tip and immediately evaporated in a vacuum chamber (Hewlett-Packard G2024A sample prep accessory) to ensure rapid and homogeneous sample crystallization. All spectra were obtained by accumulating data generated by 250 single shots with laser power between 1.5 and 4.5 µJ.

**Dependence of MALDI-TOF MS Signal on the Concentration of GIP1–42 and GLP-17–36**

Various concentrations of synthetic porcine GIP1–42 (Peninsula) and synthetic human GLP-17–36 (Bachem) were mixed with buffer and water as described above, and 1-µl samples ranging from 0.5 to 6 pmol/sample of GIP1–42, and 3.75 to 10 pmol/sample GLP-17–36 were analyzed by MS in order to determine the relationship between concentration of hormone versus MS signal intensity. Spectra for each peptide concentration were generated in triplicate. Quantification of GIP1–42 and GLP-17–36 signals was accomplished by dividing the peak intensity by the base-line intensity resulting in a signal intensity normalized to spectra base lines.

**Monitoring in Vitro Degradation of GIP1–42 and GLP-17–36 by DP IV Using MALDI-TOF MS**

Incubation of Peptide with Purified Kidney DP IV—To study the hydrolysis of GIP1–42 (5 µM) and GLP-17–36 (15 µM) by DP IV, peptides were incubated in buffer and enzyme (0.58 nM for GIP1–42 incubations and 2.9 nM for GLP-17–36 incubations) under the aforementioned standard conditions. Samples of GIP1–42 (2.5 pmol) and GLP-17–36 (7.5 pmol) were removed from the incubation mixture at 4, 9, and 16 min and prepared for MS analysis as described above.

Incubation in Human Serum—In order to study proteolytic degradation of GIP1–42 (30 µM) and GLP-17–36 (30 µM) in serum, peptides were incubated in buffer containing 20% human serum under standard conditions. Serum was pooled from three individuals and obtained from the Medical Science Division (courtesy of Dr. S. Heins, Department of Child Diseases), Martin-Luther University, Halle-Wittenberg, Germany. Samples of the respective peptides (15 pmol) were removed from the incubation mixture at hourly intervals for 15 h and analyzed using the MS.

**Kinetic Analysis of DP IV-mediated GIP1–42 and GLP-17–36 Hydrolysis Using MALDI-TOF MS**

Hydrolysis with Varying Concentrations of DP IV—In order to determine the feasibility of studying the time dependence of an enzymatic reaction using MALDI-TOF MS and to establish a convenient DP IV concentration for subsequent kinetic analysis, GIP1–42 (5 µM) and GLP-17–36 (15 µM) were incubated under standard conditions with varying concentrations of purified DP IV (ranging from 0.29 to 5.8 nM for GIP1–42 incubations and from 1.5 to 12 nM for GLP-17–36). Samples were removed at various time intervals after the start of the reaction and analyzed by MS. The relative amounts of GIP1–42 and GLP-17–36 were calculated from net substrate peak intensity divided by the sum of the net substrate and net product peak intensities and plotted versus time. Net peak height was defined as peak intensity minus base-line intensity. Before transferring to the probe for MS analysis, these samples were diluted so that the final amount of peptide on the probe tip was 2.5 pmol for GIP1–42 metabolites and 7.5 pmol for GLP-17–36 metabolites. The linearity between rate of hydrolysis and enzymatic concentration was determined from a plot of the initial slopes of substrate turnover (µmol/min) versus enzyme concentration.

**Determination of Kinetic Constants**—The kinetic constants of DP IV-catalyzed GIP1–42 and GLP-17–36 hydrolysis were determined by introducing a specific and kinetically characterized DP IV inhibitor into the incubation mixture and observing the relative reaction rates of inhibited and uninhibited substrate hydrolysis as described by Crawford et al. (11). GIP1–42 (20 µM) and GLP-17–36 (30 µM) were incubated with DP IV (0.59 and 2.9 nM, respectively) under standard conditions, in the presence or absence of either Ala-thiazolidide (20 µM – K of 3.4 µM) or Ile-thiazolidide (20 µM – K of 0.126 µM). Both are specific, competitive inhibitors of DP IV synthesized in our laboratory (12, 13). Simiarly, GIP1–42 (30 µM) and GLP-17–36 (30 µM) were incubated with 20% human serum in the presence or absence of inhibitors. Samples were appropriately diluted and assayed by MS. Quantification of relative amounts of substrate after various time intervals was calculated as described in the previous section. The initial slopes of peptide turnover with purified DP IV or human serum DP IV activity in the presence and absence of inhibitors were used to calculate reaction velocities. To validate this approach, two different competitive inhibitors, exhibiting fairly different K values (more than one order of magnitude different), were used. The K of DP IV-catalyzed peptide hydrolysis was calculated according to Equation 1:

\[ K_m = \frac{(v_i - v_o)}{1 + (K_i)} + (v_o/v_i) \]  

(Eq. 1)

where v and v are the uninhibited and inhibited relative reaction rates, respectively; S is the substrate concentration; I is the inhibitor...
To estimate these kinetic constants for serum DP IV activity, it was necessary to determine the concentration of purified DP IV equivalent to human serum DP IV activity. A standard curve of DP IV activity was necessary to determine the concentration of purified DP IV equivalent to the molar mass of DP IV.

Values for $k_{cat}$ were calculated using $M = 110$ kDa per catalytically active subunit as the molar mass of DP IV.

Confirmation of MS-derived $K_m$ Values Using a Spectrophotometric Competition Assay

To confirm the kinetic constants determined using MALDI-TOF MS, the inhibition constant of GIP$_{1–42}$ as a competitive substrate of the DP IV-catalyzed hydrolysis of a chromogenic substrate was determined spectrophotometrically. Three concentrations of H-Gly-Pro-4-nitroanilide (50, 100, and 200 μM) were incubated in 0.04 M HEPES buffer, pH 7.6, at 30°C and monitoring the rate of H-Gly-Pro-4-nitroanilide hydrolysis. Data acquisition was carried out using a Kontron 930 Uvicon uv-visible spectrophotometer as outlined above. Data were analyzed using nonlinear regression (Graphit 3.01) yielding an inhibition binding constant ($K_i$) for GIP$_{1–42}$. Since GIP$_{1–42}$ is simultaneously an inhibitor to DP IV-catalyzed H-Gly-Pro-4-nitroanilide hydrolysis, as well as a substrate of DP IV, this inhibition binding constant should be an approximation to the $K_m$ for DP IV-catalyzed GIP$_{1–42}$ hydrolysis.

RESULTS

Concentration Dependence of GIP$_{1–42}$ and GLP-1$_{7–36}$ on MS Signal Intensities—Polypeptide concentration was plotted versus the ratio of GIP$_{1–42}$ and GLP-1$_{7–36}$ signal intensities normalized to spectral base lines (Fig. 1). This simple approach resulted in graphs indicating the concentration range of polypeptide during which signal intensity increased with increasing concentration of substance without the necessity of internal standards. By knowing this unique concentration window, bound by the limit of detection and the highest normalized signal intensity, the optimum analyte to matrix ratio for subsequent sample dilution was chosen. The molar GIP$_{1–42}$/matrix ratio was optimum at 2.5 × 10$^{-5}$, whereas the GLP-1$_{7–36}$/matrix optimum was 7.5 × 10$^{-5}$.

In Vitro Degradation of GIP$_{1–42}$ and GLP-1$_{7–36}$ by DP IV—Fig. 2 shows the MS spectra of GIP$_{1–42}$ and GLP-1$_{7–36}$ and their DP IV reaction products at various time intervals during incubation with purified DP IV. The relative heights of the substrate signal (GIP$_{1–42}$ and GLP-1$_{7–36}$) decreased as the relative heights of the peaks corresponding to the DP IV hydrolysis products (GIP$_{3–42}$ and GLP-1$_{9–36}$) increased. The average m/z mass ratios of GIP$_{1–42}$ and GIP$_{3–42}$ were 4980.1 and 4745.2 representing an error of 0.09 and 0.10% relative to [M + H]$^+$, respectively. The error between [M + H]$^+$ is less than 0.10% for GLP-1$_{7–36}$ and 0.05 and 0.06% for GIP$_{3–42}$.

In order to gain insight into the identity of the major metab-
olites found in the circulation, GIP$_{1-42}$ and GLP-1$_{7-36}$ were incubated in human serum. The MS spectra generated at the stated time intervals are shown in Fig. 3. Metabolites were identified on the basis of their $m/z$ ratio. Table I summarizes the $[M + H]^{+}$ versus $[M + H]_{\text{calc.}}$ of possible metabolite sequences. Indistinct minor peaks were not considered for analysis nor were sequences where the error between $[M + H]_{\text{exp.}}$ and $[M + H]_{\text{calc.}}$ were $>0.20\%$.

Over a 15-h period, serum-incubated GIP$_{1-42}$ showed a consistently gradual decrease in the relative peak height of the intact peptide with a complementary increase in the relative peak height of a degradation product having $m/z$ corresponding to GIP$_{3-42}$. Only after approximately 3 h, by which time more than half of the GIP$_{1-42}$ was already converted to GIP$_{3-42}$, were minor peaks due to secondary stepwise degradation by other serum proteases observed. These results support the hypothesis that DP IV is the primary serum protease acting on GIP$_{1-42}$.

Similarly, serum-incubated GLP$_{1-36}$ was degraded by serum DP IV activity to GLP$_{1-36}$-ethyl ester. The serum degradation spectra for GLP$_{1-36}$ at different times are illustrated in Fig. 3B and show doublet peaks for both GLP$_{1-36}$ and GLP$_{1-36}$-ethyl ester. The $m/z$ difference between these doublets was consistently 29, a mass corresponding to an ethyl group most likely attached as a protecting group to a glutamate residue during peptide synthesis of the commercial product. As the incubation time increased, the heights of the mass peaks corresponding to $[M + H]^{+}$ of a metabolite of GLP$_{1-36}$-ethyl ester decreased relative to the height of the mass peak of the unesterified GLP$_{1-36}$ metabolite. This suggests that nonspecific serum esterases remove the ethyl group over time. Parallel studies of GLP$_{1-36}$ using the same commercially available substance, with purified DP IV did not result in doublet peaks but only the mass peak of GLP$_{1-36}$-ethyl ester (Fig. 2.). Presumably this occurs because the purified enzyme preparation is free of contaminating nonspecific esterases.

**Kinetic Analysis Using MALDI-TOF MS**—Under normal circumstances increasing the concentration of an enzyme while maintaining a constant substrate concentration results in an increased rate of product formation. Fig. 4 illustrates that MALDI-TOF MS analysis of DP IV-catalyzed GIP$_{1-42}$ and GLP$_{1-36}$ hydrolysis can be used to demonstrate this relationship. Peptide turnover varies linearly with increasing concentrations of DP IV (Fig. 4, inset; $R^2 = 0.9986$ and 0.9849 for GIP$_{1-42}$ and GLP$_{1-36}$ hydrolysis, respectively). MALDI-TOF MS was used to demonstrate that GIP$_{1-42}$ and GLP$_{1-36}$ turnover was attenuated by Ala-thiazolidide and Ile-thiazolidide inhibition of purified DP IV and serum DP IV as predicted by the inhibitor binding constants ($K_i$) (Fig. 5.). These results lend more credibility to MALDI-TOF MS as a feasible method for quantitative kinetic analysis, as well as allowing the $K_m$ values for purified porcine kidney-catalyzed GIP$_{1-42}$ and GLP$_{1-36}$ hydrolysis to be calculated. These results are summarized in Table II and, where appropriate, are
expressed as a range derived from the two inhibitors.

Serum DP IV was determined to have the equivalent activity of 1.3 × 10⁻⁵ mgml⁻¹ of purified porcine kidney, as measured by the rate of H-Gly-Pro-4-nitroanilide hydrolysis using the standard curve in Fig. 6. The kinetic constants (k_cat) for GIP-42 and GLP-17-36 hydrolysis by serum DP IV activity were calculated and compared in Table I. The binding constant of GIP-1–42 derived from the competitive inhibition of porcine kidney DP IV-catalyzed hydrolysis of H-Gly-Pro-4-nitroanilide was found to be 54 ± 8 μM (mean ± standard error).

**DISCUSSION**

With the introduction of electrospray ionization (14, 15) and MALDI (16) as soft ionization methods that greatly decrease the fragmentation of fragile biomolecules, mass spectrometry has become an important tool in biological research. Subsequent to the development of these techniques, mass spectrometry has been used to analyze a wide range of substances including polypeptides, proteins, oligonucleotides, polysaccharides, and other bio-organic compounds. An important feature of mass spectrometry is the high sensitivity and excellent resolution, allowing detection of picomole to femtomole amounts of substance up to molecular masses of 300 kDa with an accuracy of 0.1 to 0.01% (17). In the present study, MALDI-TOF MS, a particularly versatile and easily used method of mass spectrometry, was used to monitor the *in vitro* degradation of GIP-1–42 and GLP-17–36 in human serum, as well as to investigate the kinetics of DP IV catalysis of these peptides.

It was observed by ourselves in this study and others (17) that absolute quantification of MALDI signals is extremely difficult due to inconsistent shot to shot and sample to sample reproducibility. Although several studies have tried to address this issue (18–20), laser beam heterogeneity and irradiance, as well as inconsistent sample preparation and crystallization, are still cited as the most significant problems in obtaining consistent results. Fig. 1 illustrates the MALDI-TOF MS signal profile over a range of GIP-1–42 and GLP-17–36 concentrations. As previously observed, signal intensity does not continue to increase but rather plateaus or decreases as the relative amount of analyte increases with respect to matrix (21). The observation that diluting analyte results in a more intense signal is not uncommon. One explanation is that decreasing the amount of analyte relative to matrix results in a more optimum analyte:matrix ratio (22). Tang and colleagues (21) suggest this nonlinearity is likely due to changes in the number of analyte layers that the laser can penetrate in order to produce intact ions that ultimately can reach the detector. This conclusion was based on their finding that increasing the number of analyte molecules while maintaining a constant analyte:matrix

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**TABLE I**

| GIP-1–42 degradation (10 h) | GLP-17–36 degradation (10 h) |
|-----------------------------|-----------------------------|
| m/z | Sequence | [M + H]calc. | Difference | m/z | Sequence | [M + H]calc. | Difference |
| 4975.3 | 1–42 | 4975.5 | 0.00 | 3323.7 | (7–36) + CH₂CH₃ | 3267.7 | -0.09 |
| 4872.4 | (1–41) + Na | 4869.4 | 0.06 | 3296.1 | 7–36 | 3297.7 | -0.05 |
| 4809.8 | 2–42 | 4811.4 | -0.03 | 3115.2 | (9–36) + CH₂CH₃ | 3118.6 | -0.11 |
| 4740.9 | 1–40 | 4745.4 | -0.09 | 3087.9 | 9–36 | 3089.6 | -0.06 |
| 4327.2 | 3–42 | 4318.3 | 0.01 | 2584.3 | 7–34 | 2587.4 | 0.17 |
| 4462.8 | 2–39 | 4469.2 | -0.14 | 2555.7 | 7–32 | 2557.4 | -0.06 |
| 4192.4 | 8–42 | 4193.1 | -0.02 | 2771.0 | (7–31) + CH₂CH₃ | 2773.3 | -0.08 |
| 4149.7 | 3–37 | 4147.1 | 0.06 | 2771.0 | (10–34) + CH₂CH₃ | 2776.4 | -0.19 |
| 3995.5 | 4–38 | 4001.0 | -0.13 | 2762.8 | (11–35) + CH₂CH₃ | 2768.4 | -0.19 |
| 3502.9 | 11–42 | 3508.0 | -0.05 | 2743.8 | 7–31 | 2744.0 | -0.06 |
| 3740.6 | 1–32 | 3736.9 | 0.10 | 2562.3 | 7–30 | 2562.3 | 0.00 |
| 3878.8 | 5–37 | 3876.9 | 0.10 | 2534.9 | 9–31 | 2536.2 | -0.05 |
| 3629.6 | 13–35 | 3627.9 | 0.05 | 2487.2 | 7–29 | 2487.3 | 0.00 |
| 3560.2 | 14–42 | 3565.9 | 0.00 | 2375.0 | 7–30 | 2374.1 | 0.04 |
| 3509.2 | 3–32 | 3508.0 | 0.00 | 2371.9 | (8–29) + CH₂CH₃ | 2371.9 | -0.17 |
| 3375.2 | 4–33 | 3371.0 | 0.03 | 2357.8 | (11–31) + CH₂CH₃ | 2357.1 | -0.17 |
| 3421.2 | 13–41 | 3498.5 | 0.09 | 2353.4 | 8–29 | 2350.1 | 0.14 |
| 3629.0 | 19–42 | 3627.9 | 0.05 | 2297.5 | 9–30 | 2297.0 | 0.00 |
| 3373.2 | 3–31 | 3374.7 | -0.04 | 2296.1 | 7–27 | 2297.0 | -0.04 |
| 3748.2 | 18–42 | 3748.0 | 0.06 | 2166.3 | 9–28 | 2166.0 | 0.01 |
| 2896.1 | 6–29 | 2901.5 | -0.19 | 2164.0 | 11–30 | 2164.0 | 0.11 |
| 2827.5 | 8–31 | 2827.4 | 0.04 | 2168.2 | 18–36 | 2168.2 | 0.00 |
| 2827.5 | 12–35 | 2832.5 | 0.18 | 2019.2 | 9–27 | 2018.9 | 0.01 |
| 2657.2 | 15–37 | 2657.1 | -0.14 | 1971.0 | 7–29 | 1969.9 | 0.06 |
| 2544.4 | 20–41 | 2564.6 | 0.03 | 1828.5 | 7–23 | 1826.8 | 0.09 |
| 2544.4 | 21–42 | 2564.6 | 0.03 | 1828.5 | 7–23 | 1826.8 | 0.09 |
| 2544.4 | 22–42 | 2441.1 | 0.12 | 1825.0 | 18–33 | 1825.0 | 0.19 |
does not improve the linearity of analyte concentration versus signal intensity. For GIP$_{1-42}$ and GLP$_{17-36}$, the concentrations yielding signals of greatest intensity were 5 and 15 m$m^\text{M}$, respectively, and subsequent incubations using higher peptide concentrations were diluted to these concentrations prior to MS analysis. MALDI-TOF MS proved a highly sensitive technique to confirm the removal of N-terminal dipeptides from GIP$_{1-42}$ and GLP$_{17-36}$ due to DP IV catalysis, on the basis of the mass difference between substrate and product. Equally significant was the observation that monitoring the time course of peptide hydrolysis was an appropriate application of this analytical tool (Fig. 2). However, methodologically, the great advantage of MALDI-TOF MS over other approaches, and even over other types of mass spectrometry, is the tolerance of impurities in the analyte solution. An objective of this study was to analyze the metabolism of GIP$_{1-42}$ and GLP$_{17-36}$ in serum, without prior purification, to test the hypothesis that DP IV is the principal protease responsible for serum inactivation of these hormones. This study clearly affirms that more than 50% of GIP$_{1-42}$ and GLP$_{17-36}$ was converted to GIP$_{3-42}$ and GLP$_{19-36}$, respectively, before significant secondary degradation was observed (Fig. 3). Addition of specific DP IV inhibitors (Fig. 5) reduced this conversion as predicted by inhibitor binding constants ($K_i$), suggesting that the serum protease responsible for the initial hydrolysis was DP IV. The data presented in Table I suggest that secondary degradation of GIP$_{1-42}$ may include stepwise N-terminal removal of amino acids due to serum amino peptidases, resulting in $m/z$ corresponding to GIP$_{8-42}$, GIP$_{11-42}$, GIP$_{12-42}$, GIP$_{13-42}$, GIP$_{15-42}$, GIP$_{18-42}$, GIP$_{21-42}$, and GIP$_{22-42}$. This does not preclude the possibility that GIP metabolites are susceptible to hydrolysis by other serum proteases resulting in other sequences listed in Table I. Regardless of interpretation, however, serum DP IV is unquestionably the primary enzyme responsible for GLP$_{17-36}$ metabolism in serum as well. Although mass spectrometry has been used extensively for analyzing protein degradation products, Hsieh and colleagues (23) combined mass spectrometric analysis of enzymatic degradation with quantitative mass spectrometry to demonstrate the feasibility of studying enzyme kinetics in real time using HPLC-coupled electrospray mass spectrometry. Classical
Fig. 5. Quantification of the DP IV-catalyzed GIP1–42 and GLP-17–36 hydrolysis in the presence of specific DP IV inhibitors using MALDI-TOF MS. A and B, GIP1–42 (20 μM) and GLP-17–36 (30 μM) were incubated with purified porcine kidney DP IV (0.59 and 2.9 nM respectively) in 0.1 mM Tricine buffer, pH 7.6, at 30 °C in the presence of absence of alanine-thiazolidide (20 μM) and isoleucine-thiazolidide (20 μM), two specific, reversible inhibitors of DP IV. C and D, GIP1–42 and GLP-17–36 (30 μM for both) were also incubated in 20% human serum under the same experimental conditions. Samples of analyte (15 pmol) were removed from the incubation mixture for MS analysis. Spectrum peaks were quantitatively analyzed as outlined under “Experimental Procedures.” Squares represent substrate turnover in the absence of inhibitor, whereas triangles and circles represent turnover in the presence of alanine-thiazolidide and isoleucine-thiazolidide, respectively.

Table II

Kinetic constants for the degradation of GIP1–42 and GLP-17–36 by DP IV as determined by quantitative MALDI-TOF MS

| Peptide   | DP IV source                      | K_m (μM) | V_max (μmol min⁻¹ mg⁻¹) | k_cat (s⁻¹) | k_cat/K_m (μmol min⁻¹ mg⁻¹) |
|-----------|-----------------------------------|----------|------------------------|-------------|-----------------------------|
| GIP1–42   | Porcine kidney                    | 1.8 ± 0.3a | 13.6 ± 0.2             | 23          | 13.10a                     |
| GIP1–42   | Human serum                       | 39 ± 20a  | 27 ± 12                | 20          | 0.5610a                    |
| GLP1–36   | Porcine kidney                    | 34 ± 3    | 3.8 ± 0.2              | 7.6         | 0.2210a                    |
| GLP1–36   | Human serum                       | 3.8 ± 0.3a | 5.45 ± 0.05           | 9           | 2.310a                     |
| GLP1–36   | Human serum                       | 13 ± 9a   | 11 ± 2                 | 14          | 1.110a                     |
| GLP1–36   | Plasma                            | 4.5 ± 0.6 | 0.97 ± 0.05           | 1.9         | 0.4310a                    |

a K_m values were calculated from data of experiments using two DP IV inhibitors, Ala-thiazolidide and Ile-thiazolidide.

FIG. 6. Standard curve for matching human serum DP IV activity with purified porcine kidney DP IV activity. The rate of hydrolysis of H-Gly-Pro-4-nitroanilide (0.4 μM), a standard chromogenic substrate of DP IV, versus serially diluted purified porcine kidney DP IV was measured spectrophotometrically in 0.04 M HEPES buffer, pH 7.6, at 30 °C (r = 0.004376; r² = 0.9979).

methods for investigating enzyme kinetics such as refractive index monitoring, radioimmunoassay, HPLC, or capillary zone electrophoresis are time consuming, use large amounts of substrate, and are often insensitive. In the case of colorimetric assays, chromogenic substrates must often be synthesized, and many of these do not adequately parallel the kinetics of the substrate they were designed to mimic. Mass spectrometry offers a rapid, accurate and easy approach to study enzyme kinetics. This is especially relevant for analyzing large biomolecules such as proteins. In order to study the kinetics of DP IV-catalyzed GIP1–42 and GLP-17–36 hydrolysis, protocols were developed for the quantification of MS signals. This typically involves the incorporation of an internal standard to the sample mixture, allowing an unknown quantity of analyte to be normalized relative to the standard (21, 24–26). When measuring the activity of protein kinase and phosphatase, however, Craig et al. (27) avoided the use of an internal standard by quantifying substrate and product peaks relative to each other. Essentially, these peaks served as their own internal standards.

The approach of relative quantification was used in the present study. The feasibility of this method is demonstrated in Fig. 4 which shows the relationship between the rate of DP IV-catalyzed peptide hydrolysis and enzyme concentration. As expected, the initial reaction rates increased linearly as a function of DP IV concentration providing convincing evidence that our approach to MS quantification was valid.

Incubation of GIP1–42 and GLP-17–36 with purified porcine kidney DP IV or human serum in the presence and absence of two known specific DP IV inhibitors (Fig. 5) allowed the kinetic constants for peptide hydrolysis to be calculated. The K_m values calculated for purified DP IV correspond well to those previously reported for GIP1–42 and GLP-17–36 hydrolysis by purified human placental DP IV (Table II) (4). The error in the MS-derived constants for GIP1–42 and GLP-17–36 as determined using only single trials of two DP IV inhibitors, was 17 and 7.9% respectively. This compared with errors of 8.8 and 13% as determined by seven HPLC-analyzed trials (4). Although MS- and HPLC-generated kinetic analysis result in
comparablevariability, this study demonstrates that MS offers some considerable advantages. Significantly fewer trials mean that MS is less time consuming and labor-intensive, and since MALDI-TOF MS can detect picomole amounts of analyte, complete kinetic analysis can occur with only minimal amounts of substance, making this approach much less expensive.

The fact that MALDI-TOF MS is tolerant of sample impurities also makes it an ideal tool to study the kinetics of serum proteases without prior purification. The rate specificity constants \( (k_{cat}/K_m) \) for GIP\(_{1-42} \) and GLP-17–36 hydrolysis by human serum DP IV were between 10\(^5\) and 10\(^7\), suggesting that DP IV-mediated peptide hydrolysis is significant at physiological concentrations of these hormones. The large variability in the \( K_m \) values of peptide hydrolysis by human serum DP IV is likely due to the presence of a distinct DP IV isoenzyme in serum. In this regard, a novel 175-kDa soluble form of DP IV was recently identified and purified from human serum (28). Inhibitor binding constants \( (K_i) \) of Ala-thiazolidide and Ile-thiazolidide, the DP IV inhibitors used to estimate the kinetic constants of peptide hydrolysis in human serum, were evaluated using purified 105–110 kDa membrane-derived porcine kidney DP IV. Presumably, inhibitor interaction with the serum DP IV is not identical to that with the membrane-associated enzyme, resulting in the disparate \( K_m \) values of GIP\(_{1-42} \) and GLP-17–36 hydrolysis. Thus, these experiments support the findings of Duke-Cohan and colleagues (28, 29) that human serum DP IV is a unique protease having similar, yet distinct kinetic properties as compared with the insoluble form.

The close correlation between MALDI-TOF MS-derived kinetic constants and those previously reported, or determined using a spectrophotometric competition assay, validate MS as a reliable method for kinetic analysis.

The serum degradation experiments combined with the serum kinetic experiments provide considerable evidence that DP IV plays a significant role in GIP\(_{1-42} \) and GLP-17–36 hydrolysis. Although this \textit{in vitro} study may model the physiology of DP IV degradation of incretins \textit{in vivo}, one important limitation must be considered. Membrane-associated DP IV is found on the surface of T-lymphocytes, endothelial cells of the vasculature, epithelial cells of the intestine, brush border membranes, as well as in most other tissues (30). Thus, it is likely that the inactivation of circulating biologically active peptides is much more rapid than predicted by the kinetics of serum DP IV alone. This underscores the importance of DP IV plays in incretin physiology.

DP IV catalysis of GIP\(_{1-42} \) and GLP-17–36 to GIP\(_{3-42} \) and GLP-1\(_{9-36} \) renders these hormones biologically inactive. Subsequent evidence has suggested that this hydrolysis represents the first step in hormone metabolism. MALDI-TOF MS was used in the present study to test this hypothesis and investigate the kinetics of DP IV-catalyzed incretin hydrolysis, thereby introducing a novel application of MALDI-TOF MS: kinetic analysis. \textit{In vitro} experiments indicate that DP IV acts rapidly on GIP\(_{1-42} \) and GLP-17–36, converting more than 50% of either peptide before significant secondary degradation was observed. On the basis of this and previous studies, \textit{in vivo} inhibition of DP IV is predicted to have a profound effect on the enteroinsular axis. An exaggerated incretin response would be expected in response to an increased half-life of endogenously released GIP\(_{1-42} \) and GLP-17–36. This is currently under investigation.

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