# Monitoring Enzyme Catalysis with Mass Spectrometry\*

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Mass spectrometry is a rapid, sensitive, and accurate quantitative approach for the direct monitoring of enzyme-catalyzed reactions that does not require a chromophore or radiolabeling and thus provides a viable alternative to existing analytical techniques. In this study the proteolysis of intact viral capsid proteins, the  $\alpha$ -glucosidase-catalyzed hydrolysis of *p*-nitrophenyl- $\alpha$ glucopyranoside and the lipoprotein lipase-catalyzed ester hydrolysis of resorufin were examined. Matrixassisted laser desorption/ionization and electrospray ionization mass spectrometry were used to examine the proteolysis of viral protein capsids, providing information about capsid dynamics and the stabilizing force of viral protein/RNA interactions. In addition,  $k_{\text{cat}}$  and  $K_m$ values of enzyme-catalyzed hydrolysis were obtained (without the use of a chromophore). These results also demonstrate the effect an unnatural substrate can have on enzyme activity. Overall, mass spectrometry provides for efficient and quantitative analysis of enzymecatalyzed reactions, as well as the direct observation of reaction dynamics.

The development of efficient methods for monitoring enzymatic activity is important (1-15) especially for reactions that occur in vivo or are dynamic in nature. For example, the quantitative measurement of viral capsid proteolytic fragments offers a unique approach toward studying capsid dynamics (16, 17) (as of yet, no other spectroscopic methods exist to perform such mobility measurements). In addition, while spectroscopic probes exist for monitoring small molecule, enzyme-catalyzed reactions, these reactions typically require the release of a chromophore as the reaction proceeds. Unfortunately, the preparation of chromogenic substrates often requires substrate modification that can alter reaction kinetics or necessitate time consuming, multistep synthesis. Therefore, assay methodologies that allow for the rapid and quantitative measurement of enzyme activity, offer novel approaches toward monitoring protein interactions, and obviate the need for substrate modification (1, 13) would be very beneficial.

In recent years, mass spectrometry has emerged as a valuable tool in biochemistry offering unique insight into biological systems with respect to sensitivity and accuracy (2-14). More specifically, mass spectrometry permits protein sequencing (3, 4), the elucidation of protein folding pathways (5), for charac-

terizing post-translational modifications on peptides and proteins (6), and for detecting covalent and noncovalent proteinligand complexes (7-10). Moreover, the accuracy and reproducibility of the technique make it well suited for the quantitative study of enzyme-catalyzed reactions (11-14). Here the utility of electrospray ionization (ESI)<sup>1</sup> and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry is examined for the qualitative and quantitative characterization of viral capsid dynamics, as well as the hydrolysis of small molecules with  $\alpha$ -glucosidase and bovine lipoprotein lipase. The measurement of proteolytic fragments, as well as  $k_{cat}$  and  $K_m$ provides quantitative insight into viral capsid dynamics, verifies UV results, demonstrates the utility of an internal standard for accurate kinetic measurements, and extends its utility to the difficult analysis of lipase-catalyzed hydrolysis of an unmodified substrate.

## EXPERIMENTAL PROCEDURES

Chemicals—All organic solvents were purchased from Aldrich. Bovine lipoprotein lipase was purchased from Sigma. 1,2-O-Dilauryl-racglycero-3-glutaric acid resorufin ester was purchased from Roche Molecular Biochemicals. Resorufin and glutaric acid were purchased from Aldrich. 1,2-O-Dilauryl-rac-glycerol, 1,2-O-dilauryl-rac-glycero-3-glutaric acid, 1,2-O-dilauryl-rac-glycero-3-benzyl ether, 1,2-O-dilauryl-racglycero-3-glutaric acid hexyl ester, and 1,2-O-dilauryl-rac-glycero-3lauric acid ester were synthesized using the following method.

1,2-O-Glycerol acetonide was generated by refluxing a glycerol/acetone solution in the presence of p-toluene sulfonic acid. 1,2-O-Glycerol acetonide was then converted to the benzyl ether by deprotonation of the alcohol with sodium hydride in tetrahydrofuran followed by alkylation with benzyl bromide. The protected benzyl ether was subsequently deprotected with aqueous acid. The resulting glycerol-1-benzyl ether was deprotonated with 2.5 eq of sodium hydride and alkylated with 2.5 eq of lauryl bromide (1-bromododecane) at 0 °C. The reaction was allowed to stand for 24 h at 25 °C. After purification by flash chromatography, 1,2-O-dilauryl-rac-glycerol benzyl ether was obtained in 53% yield. The target compound, 1,2-O-dilauryl-rac-glycerol, was obtained by removal of the benzyl ether protection group by hydrogenation (H<sub>2</sub>/Pd/C, ethanol, 24 h). The resulting 1,2-O-dilauryl-rac-glycerol was obtained by flash chromatography in 95% yield.

1,2-O-Dilauryl-*rac*-glycerol was converted to 1,2-O-dilauryl-*rac*-glycero-3-glutaric acid by refluxing the diether with glutaric anhydride in the presence of 4-(dimethylamino)pyridine in pyridine solvent. After acidification and aqueous workup the target glutaric acid derivative was obtained in 72% yield.

1,2-O-Dilauryl-*rac*-glycero-3-glutaric acid hexyl ester was synthesized in 90% yield by the condensation of 1,2-O-dilauryl-*rac*-glycero-3glutaric acid and 1-hexanol at room temperature with 1,3-dicyclohexylcarbodiimide in methylene chloride and 4-pyrrolidinopyridine as a catalyst. Lauric acid,  $CH_3(CH_2)_{10}COOH$ , was first converted to the acid chloride by reaction with oxall chloride in benzene. The resulting acid chloride was added to a mixture containing 1,2-O-dilauryl-*rac*-glycerol, pyridine, and benzene at room temperature. 1,2-O-Dilauryl-*rac*-glycero-3-lauric acid ester was obtained in 96% yield.

Instrumentation-ESI experiments were performed using a Hewlett-

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<sup>&</sup>lt;sup>1</sup> The abbreviations used are: ESI, electrospray ionization; MALDI, matrix-assisted laser desorption/ionization; FHV, flock house virus; LC, liquid chromatography; MS, mass spectrometry.

Packard MSD, Perkin-Elmer SCIEX API 100 and API III mass spectrometers. LC/MS measurements were performed on a Hewlett-Packard 1090 MSD. MALDI-MS measurements were performed on a PerSeptive Voyager STR time-of-flight reflectron instrument. UV-visible measurements were performed on a Perkin-Elmer UV spectrophotometer attached to a constant temperature regulator. All experiments were conducted at 25  $^{\circ}$ C.

Viral Studies—20 µg (2.5 pmol) of wild type FHV and synthetic FHV (sFHV) were prepared to a final concentration of 0.5 mg/ml in 25 mM Tris buffer at pH 7.5. 0.5  $\mu$ l of the trypsin stock solution at 0.02  $\mu$ g/ $\mu$ l was added to FHV to start the digestion and the reactions were terminated by the addition of 10  $\mu$ l of acetic acid after 1.5 h. Limited trypsin digestion experiments were performed at room temperature at an E:Sratio of 1:2000 and the digestion was monitored as a function of time at different time points (5 min, 10 min, 15 min, 30 min, 1 h, and 1.5 h) using MALDI-MS. The LC/MS measurements were performed on the viruses after 1.5 h of digestion. Ethanol precipitation was used to remove viral RNA from tryptic peptides, which (if not removed) affected the quality of the LC separation. To perform ethanol precipitation, 0.5 µl of glycogen (2 mg/ml), 4.5 µl of 3 M NaOAc (pH 3.5), and 135 µl of ethanol was added to the digest. After centrifugation, the sample supernatants were transferred to separate Microfuge tubes, and dried under vacuum. The samples were then dissolved in H<sub>2</sub>O to a starting volume of 40 µl. Selected ion monitoring was used in LC/MS and a synthetic peptide was used as an internal standard. The synthetic peptide (VVTTTQTAPVPQQNVPR) was synthesized for its similarity to the sequence and ionization properties of the selected viral peptide (from  $\beta$ -protein 15–32: VVVTTTQTAPVPQQNVPR). A Supelcosil ABZ+ (3.3 cm  $\times$ 2.1 mm, 3 mm) column was used to achieve rapid and high resolution separation. 10 µl of digest samples were injected for every LC/MS analysis. Solvent A was 0.03% trifluoroacetic acid in  $H_2O$  and solvent B was 0.03% trifluoroacetic acid in ACN/H2O (95/5, v/v) and the chromatography (5-25% B gradient) was performed over 8 min. The LC/MS chromatography experiments were performed with an HP1090 liquid chromatograph and an HP1100 electrospray mass selective detector.

Kinetic Measurements-The kinetics of a-glucosidase (brewers' yeast) were determined by measuring the concentration of *p*-nitrophenvlate by ESI-MS and UV-visible spectroscopy in the presence of an internal standard. A calibration curve was generated starting with 2  $\mu$ M p-nitrophenyl- $\alpha$ -glucopyranoside, 2  $\mu$ M p-nitrophenol, and 2  $\mu$ M glucose, all in 25  $\mu{\rm M}$  sodium phosphate buffer (pH 7.5), and 2  $\mu{\rm M}$  8-anilino-1naphthylsulfonic acid in N,N-dimethylformamide, as internal standard, for experiments in negative mode. For end point kinetic studies, synthetic mixtures of the above reagents were prepared for calibration purposes by dilution. Substrate (1) concentration ranged from 0 to 120  $\mu$ M, and product (2 and 3) concentration ranged from 0 to 120  $\mu$ M, while the internal standard concentration constant was kept at 40  $\mu$ M. Reactions were initiated by adding 10  $\mu$ l of  $\alpha$ -glucosidase (34 nM in 25 mM sodium phosphate, pH 7.5) to 990  $\mu l$  of the reaction mixture, and the kinetics followed both by UV-visible at 452 nm and ESI-MS. For ESI-MS, reaction samples were stopped by taking 20-µl aliquots and diluting with 20  $\mu$ l of methanol. Samples were immediately injected into the ESI-MS. All  $k_{cat}$  and  $K_m$  experiments were done in negative mode using 2 μM 8-anilino-1-naphthylsulfonic acid in N,N-dimethylformamide, as internal standard.

The kinetics of bovine lipoprotein lipase were monitored with ESI-MS by determining the response of the sodium adducts of 1,2-Odilauryl-*rac*-glycerol **5**, and 1,2-O-dilauryl-*rac*-glycero-3-glutaric acid **6**, in the presence of 1,2-O-dilauryl-*rac*-glycero-3-benzyl ether, as the internal standard.  $2 \,\mu$ M Dioxane solutions of 1,2-O-dilauryl-*rac*-glycerol **5**, 1,2-O-dilauryl-*rac*-glycero-3-glutaric acid **6**, 1,2-O-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester **4**, and 1,2-O-dilauryl-*rac*-glycero-3-benzyl ether were used for calibration purposes.

Synthetic mixtures of the above reagents were used to prepare two separate calibration curves by dilution, so that in one case the concentration of substrate **4** was varied from 0 to 120  $\mu$ M, and the concentration of the alcohol product **5** was varied from 0 to 120  $\mu$ M, while keeping the concentration of the acid **6** and the internal standard constant at 40  $\mu$ M; in the other case, the concentration of substrate **4** was varied from 0 to 120  $\mu$ M, while keeping the to 120  $\mu$ M, and the concentration of the acid product **6** was varied from 0 to 120  $\mu$ M, while keeping the concentration of the alcohol **5** and the internal standard constant at 40  $\mu$ M. Reactions were initiated by adding 10  $\mu$ l of bovine lipoprotein lipase (0.2 nM in 25 mM sodium phosphate, pH 7.0) to 990  $\mu$ l of the reaction mixture, and the kinetics followed both by ESI-MS and UV-visible spectroscopy at 592 nm. For ESI-MS, reaction samples (20  $\mu$ l aliquots) were removed periodically and stopped by diluting with 200  $\mu$ l of dioxane. Samples were then immediately frozen until analyzed by ESI-MS in positive mode.



FIG. 1. MALDI-MS data obtained from tryptic peptides generated from viral capsid protein  $\alpha$  (*top*) free in solution and (*below*) intact virus over the same time period of digestion (the amount of protein in both experiments is the same). Viral capsid proteins from the intact virus are much less susceptible to proteolysis.

### RESULTS AND DISCUSSION

Proteolysis of Viral Capsid Proteins-The combined use of enzymology with mass spectrometry is providing new insight into the quantitative aspects of enzyme-catalyzed reactions such as glycosidation and ester hydrolysis, as well as in protein structure determination (15). In these experiments viral capsid proteins were examined using limited proteolysis combined with MALDI-MS (16-18), and ESI-MS was used as a means of generating quantitative information on protein capsid mobility. In previous experiments, enzyme digestion was used to map the surface proteins on the virus, leading to the fascinating evidence that the capsid proteins are mobile (16-18). Recently, trypsin digestion between active whole virus and isolated capsid proteins were compared using MALDI-MS, where the isolated capsid proteins were found to be significantly more susceptible to proteolysis than proteins in the intact capsid (Fig. 1). This result was consistent with the intact virus being highly protected both internally (by RNA) and externally (by other viral capsid proteins). In addition, the exact sites of proteolysis on the intact virus provided interesting insight into particle-structure dynamics.

Examination of the tryptic digestion fragments from the intact flock house virus (FHV, composed of 180 copies of the  $\beta$ -protein and  $\gamma$ -peptide, containing 363 and 44 amino acids, respectively) revealed that the first sites to be cleaved were, based on crystallographic data, located internal to the viral capsid. Based on the known sequence of the capsid proteins it was possible to map out the digestion course on the FHV capsid for the major  $\beta$ -protein. At low enzyme-to-substrate ratios, time-resolved digestion of the intact virus was observed to start at the N terminus of  $\beta$ -protein and moved inward. Sequentially, trypsin cleaved the  $\beta$ -protein at positions 10, 13, 31, and 34 in the first 15 min, reaching residue 57 after 3 h of incubation. Additional experiments performed with clostripain (Arg-C) also showed that digestion initially occurred on the N-terminal of the  $\beta$ -protein in the intact virus. Since the  $\beta$ -proteins N terminus is internal to the viral capsid (based on the crystal structure), the observed cleavage are believed to reflect transient exposure of these regions to the viral surface (16-18).

Trypsin proteolysis experiments performed on a mutant ver-



FIG. 2. LC ESI-MS data of a tryptic peptide released from a virus containing authentic RNA and a virus containing random cellular RNA. A dramatic 6-fold difference in the rate of digestion between particles packaging authentic *versus* cellular RNA is observed.

sion of FHV (deleted at N terminus residues 2–31 of the  $\beta$ -protein) also produced initial sites of cleavage via sequential digestion from the truncated N terminus albeit at a significantly slower rate than observed for wild type FHV. These results indicate that in both the wild type and mutant versions of FHV a significant portion of the N terminus is being transiently exposed on the surface of the intact virus. Further suggesting that, in solution, the intact virus undergoes significant conformational changes.

To further elucidate the relationship between viral dynamics and intermolecular interactions within the virus, a virus-like particle (sFHV) was examined with respect to the authentic wild type virus (FHV). The synthetic virus has the same capsid protein as wild type FHV, yet the particle contains random cellular RNA instead of authentic viral RNA. The difference between the two viral systems is that significantly less RNAcapsid interaction exists in sFHV than FHV (17), a difference that should be observed in viral capsid mobility.

Relative capsid dynamics were studied by trypsin digestion experiments performed in parallel (and reproduced in five separate experiments), where the rate of proteolysis was determined by LC/MS using a synthetic version of the capsid  $\beta$ -protein(16-32) as an internal standard. A representative separation is shown in Fig. 2 where relative ion intensities of the released peptide fragments were used to allow comparisons between reactions. Consistent with previous experiments (17), a dramatic increase in proteolysis was seen in sFHV, thereby supporting the idea that decreased intermolecular reactions decrease the stability of the virus. sFHV is more susceptible to trypsin digestion, which has a digestion rate six times higher than FHV, indicating a large stabilizing effect due to the authentic RNA. Because of the inherent accuracy of electrospray ionization measurements, especially when used in conjunction with an internal standard, these quantitative measurements present a solution-phase representation of the change in dynamics that correspond to the presence of the authentic viral RNA.

Glucosidase-catalyzed Hydrolysis—Reactions such as lipid ester hydrolysis and oligosaccharide glycosidation involve substrates and products that are readily analyzed and quantified by mass spectrometry (13), which is offering a new window into these enzyme-catalyzed reactions.  $\alpha$ -Glucosidase catalyzes the hydrolysis of linear oligosaccharides and releases one glucose residue at a time. This enzyme has been extensively characterized, and a variety of substrates are available that permit direct comparison of the mass spectrometry data with independent kinetic measurements. For this study, *p*-nitrophenyl- $\alpha$ -glucopyranoside (1) was chosen as a substrate which can be cleaved by  $\alpha$ -glucosidase, Scheme 1, to produce the *p*-nitrophenolate ion (2) and glucose (3). Selected ion monitoring was used



SCHEME 1. Hydrolysis of 1, catalyzed by  $\alpha$ -glucosidase in sodium phosphate buffer (pH 7.5) to produce *p*-nitrophenolate (2) and glucose (3).



FIG. 3. Hydrolysis of 1 catalyzed by  $\alpha$ -glucosidase at 25 °C in 25 mM sodium phosphate buffer with an initial substrate concentration of 120  $\mu$ M. The open circles correspond to the increase in *p*-nitrophenolate as monitored by UV-visible at 400 nm. The closed circles correspond to the increase in *p*-nitrophenolate ion as monitored by ESI-MS.

to generate a calibration curve for reaction products **2** and **3** (in the concentration range 0-1 mM) with either 8-anilino-1-naphthalenesulfonic acid (negative mode) or methyltriphenyl phosphonium ion (positive mode) as internal standards. The sodium ion adducts provided the best response and an excellent correlation between concentration and selected ion peak intensity were observed ( $r^2 = 0.998$  for glucose, and r = 0.994 for *p*-nitrophenolate).

The hydrolysis of 1 (10 µM to 1 mM) was performed at 25 °C in 25 mm sodium phosphate buffer (pH 7.5) containing either 8-anilino-1-naphthylsulfonic acid (40 µM) or methyltriphenylphosphonium bromide (2.5–500  $\mu$ M) in the presence of  $\alpha$ -glucosidase from brewers' yeast (Sigma). Aliquots were periodically removed from the reaction, diluted with methanol (1:2), and immediately analyzed by ESI-MS. Typical ESI-MS results are shown in Fig. 3. The change in intensity of the product ion relative to the internal standard was determined as a function of time. Product concentrations were then calculated directly from the calibration curve. Fig. 3 shows the reaction progress for the hydrolysis of 1, monitored by the increase of the pnitrophenolate ion concentration UV-visible at 400 nm and by ESI-MS. At a concentration of 120  $\mu$ M 1, the initial rates observed by UV-visible spectroscopy and ESI-MS were 4.82 and 4.92 µM/min, respectively. Similar guality data was obtained when the rate of formation of glucose was monitored by ESI-MS and compared with the rate of formation of *p*-nitrophenolate, as determined by UV-visible spectroscopy. Compound 1 at a concentration of 1.0 mM generated initial rates of 11.7 and 11.5  $\mu$ M/min, observed by UV-visible spectroscopy at 452 nm and ESI-MS, respectively. Both experiments illustrate the good quality of data that can be obtained over the time course of the reaction.

Fig. 4 shows a plot generated from the ESI-MS data where, in order to determine  $k_{cat}$  and  $K_m$ , the initial rates of hydrolysis



FIG. 4. Michaelis-Menten plot for the  $\alpha$ -glucosidase-catalyzed conversion of 1 to 2 plus 3. Initial rates were determined by monitoring the formation of 3 by ESI-MS as described in the legend to Fig. 3 and calculated from the first 15% of reaction; each point represents the average of two ((1)  $\geq$  0.8  $\mu$ M) or three ((1)  $\leq$  0.6  $\mu$ M) independent runs with standard errors indicated. The curve through the data is a best fit. The concentration of enzyme was 2.3 mg/liter.



SCHEME 2. Hydrolysis of resorufin (4) catalyzed by lipoprotein lipase, which predominantly proceeded along path *b*.

in Scheme 1 were obtained. The kinetic parameters calculated are  $k_{\rm cat} = 256 \pm 27 \text{ min}^{-1}$  and  $K_m = 0.535 \pm 0.192 \ \mu\text{M}$ . For comparison, the  $k_{\rm cat}$  and  $K_m$  values of  $266 \pm 30 \ \text{min}^{-1}$  and  $0.407 \pm 0.077 \ \mu\text{M}$  were determined by UV-visible spectroscopy, showing good agreement between the two methods. This approach was compared with the original work by Henion *et al.* (11, 12), whose investigations initiated the use of electrospray mass spectrometry for monitoring enzyme reactions. In addition, it was found (by direct comparison to UV-visible measurements) that addition of an internal standard further increased the accuracy of the ESI-MS results.

Lipase-catalyzed Hydrolysis-Performing kinetic measurements of lipases is difficult because of the limited solubility of the substrates can result in precipitation and aggregation (micelle formation). With this in mind, lipase-catalyzed hydrolysis of triacylglycerides and related substrates has been studied (19-21), where a variety of available substrates have been used to allow for a direct comparison of the mass spectrometry data with independent kinetic determinations. For example, 1,2-Odilauryl-rac-glycero-3-glutaric acid resorufin ester (4) has been reported to be an excellent substrate for lipases. Its hydrolysis has been described as a series of steps involving first the lipase-catalyzed formation of 1,2-O-dilaurylglycerol and glutaric acid-resorufin ester, followed by the spontaneous intramolecularly assisted hydrolysis of the latter product to release resorufin, as shown in Scheme 2, path a (22, 23, 26). This proposed mechanism is based on absorbance data and the fact that intramolecularly assisted chromophore release has been reported in other investigations (24, 25). Indeed, it was confirmed that the resorufin substrate is processed rapidly by bovine lipoprotein lipase. The time course of the reaction is monitored readily by the release of the resorufin chromophore



FIG. 5. Kinetic plot for the hydrolysis of 4 by lipoprotein lipase at 25 °C. The substrate concentration was 120  $\mu$ M in 25 mM sodium phosphate buffer (pH 7.0), containing 20% of a mixture of dioxane and acetonitrile. The enzyme concentration was 0.23 nM. The reaction mixture also contained 40  $\mu$ M 1,2-O-dilauryl-*rac*-glycero-3-benzyl ether as an internal standard. The mass selective detection of ESI-MS offers direct insight into biomodal reactions simultaneously providing the initial rate of formation of the alcohol 5 and the acid ester 6 at 3.19 and 33.6  $\mu$ M/min/mg, respectively.



SCHEME 3. Hydrolysis of non-activated dialkyl ester, 1,2-Odilauryl-rac-glycero-3-glutaric acid-hexyl ester (7), catalyzed by lipoprotein lipase which predominantly proceeded along path a.

at 572 nm. The initial rate was 39.7  $\mu$ M/min/mg of enzyme at 25 °C when the substrate concentration was 120  $\mu$ M in 25 mM sodium phosphate buffer (pH 7.0), containing 20% dioxane and acetonitrile.

To monitor the lipase reaction by ESI-MS (Fig. 5), selected ion monitoring was used first to generate a calibration curve for the alcohol product, 1,2-O-dilauryl-*rac*-glycerol (6), using 1,2-O-dilauryl-*rac*-glycero-3-benzyl ether as an internal standard. Aliquots of the lipase reaction were taken at various time intervals, diluted with dioxane, and then frozen until analyzed by ESI. Surprisingly, little of the expected product 5 was obtained. Instead of the glycerol ester of substrate 4 being cleaved, it was found that the resorufin ester was initially cleaved (Scheme 2, path b).

As shown in Scheme 2, initial formation of the acid ester product **6** correlates directly with the release of the resorufin chromophore. The initial rate of formation of the acid ester is 33.6  $\mu$ M/min/mg, in agreement with the value obtained spectrophotometrically. At longer times the concentrations of the acid ester **6**, as determined by ESI-MS, differ from those determined spectrophotometrically, possibly due to some precipitation of the product during the reaction and after the freezing procedure. The conversion of **6** to the alcohol **5** is very slow



SCHEME 4. Hydrolysis of non-activated dialkyl ester, 1,2-O-dilauryl-*rac*-glycero-3-lauric acid ester (8) catalyzed by lipoprotein lipase.

(0.186  $\mu$ M/min/mg), where the initial rate of formation of **5** from **4** is 3.19  $\mu$ M/min/mg. Contrary to expectations, the large aromatic resorufin-leaving group may orient the substrate within the active site in such a way as to favor cleavage of the activated ester over cleavage of the glycerol ester. The unique capabilities of ESI-MS thus provide direct insight into molecular changes that take place.

Because the activated diester 4 partitioned in a manner that was unexpected, the partitioning of a non-activated dialkyl ester, 1,2-O-dilauryl-rac-glycero-3-glutaric acid-hexyl ester 7, by lipoprotein lipase was examined to determine the extent to which the chromophore alters the course of the reaction (Scheme 3). This substrate lacks a chromophore, making its analysis difficult by other techniques. The formation of both alcohol 5 and acid ester 6 ions was monitored, and it was found that cleavage of the glycerol ester (path a, Scheme 3) is preferred over the terminal alkyl ester by a factor of 6.7:1. The initial rate of 5 is 46.1  $\mu$ M/min/mg, approximately the same as that of the resorufin substrate, which suggests that the resorufin chromophore greatly perturbs the normal processing of the glycerol esters. The hydrolysis of a more "natural" ester was also examined to place the above results in their proper context. The kinetics of 1,2-O-dilauryl-rac-glycero-3-lauric acid ester 8 showed a clean conversion of ester to product 5 and 9 in the presence of lipoprotein lipase (Scheme 4). The initial rate of cleavage increased to 109 µM/min/mg.

*Conclusion*—These experiments illustrate that mass spectrometry is qualitatively useful for examining reaction pathways and protein structure, and is also a viable approach for quantitative determination of kinetics. From macromolecular protein dynamics, kinetic measurements, and the effect of unnatural substrates on reaction dynamics, mass spectrometry offers excellent accuracy, reproducibility, and is especially well suited for assaying reactions that cannot be followed spectro-

photometrically. In cases where introduction of a chromophore drastically changes the fate of the reaction as a result of the structural features of the substrate (as illustrated by the resorufin-containing substrate), ESI-MS has been found to be especially valuable. The small sample size, minimal handling requirements, along with the potential for high throughput represent further significant advantages. Accurate mass measurements by ESI-MS also suggest the further utility of this technique for the kinetic analysis of reactions involving enzyme inhibitors (13).

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#### REFERENCES

- Matayoshi, E. D., Wang, G. T., Kraft, G. A., and Erickson, J. (1990) Science 247, 954–958
- 2. Siuzdak, G. (1996) Mass Spectrometry for Biotechnology, Academic Press, San Diego
- Hunt, D. F., Michel, H., Dickinson, T. A., Shabanowitz, J., Cox, A. L., Sakaguchi, K., Appella, E., Grey, H. M., and Sette, A. (1992) Science 256, 1817–1820
- 4. Chait, B. T., Wang, R., Beavis, R. C., and Kent, S. B. (1993) Science 262, 89
- Miranker, A., Robinson, C. V., Radford, S. E., and Dobson, C. M. (1996) *FASEB* J. 10, 93
- Kouach, M., Belaiche, D., Jaquinod, M., Couppez, M., Kmiecik, D., Ricart, G., Van Dorsselaer, A., Sautiere, P., and Briand, G. (1994) *Biol. Mass Spectrom.* 23, 283
- Krebs, J. F., Siuzdak, G. H., Dyson, J., Stewart, J. D., and Benkovic, S. J. (1995) Biochemistry 34, 720
- Siuzdak, G., Krebs, J. F., Benkovic, S. J., and Dyson, J. H. (1994) J. Am. Chem. Soc. 116, 7937
- 9. Ganem, B., Li, Y. T., and Henion, J. D. (1991) J. Am. Chem. Soc. 113, 6294
- 10. Katta, V., and Chait, B. T. (1991) J. Am. Chem. Soc. 113, 8534–8535
- 11. Lee, E. D., Mück, W., Henion, J. D., and Covey, T. R. (1989) J. Am. Chem. Soc. 111, 4600–4604
- Hsieh, F. Y. L., Tong, X., Wachs, T., Ganem, B., and Henion, J. (1995) Anal. Biochem. 229, 20–25
- Wu, J., Takayama, S., Wong, C. H., and Siuzdak, G., (1997) Chem. Biol. (Lond.) 4, 653–657
- Gerber, S. A., Scott, C. R., Turecek, F., and Gelb, M. H. (1999) J. Am. Chem. Soc. 121, 1102–1103
- Kriwacki, R., Wu, J., Tennant, L., Wright, P. E., and Siuzdak, G. (1997) J. Chromatogr. Sect. A 777, 23–30
- Bothner, B., Dong, X-F. A., Bibbs, L. D., Johnson, J. E., and Siuzdak, G. (1998) J. Biol. Chem. 273, 673–676
- Bothner, B., Schneemann, A., Marshall, D., Reddy, V., Johnson, J. E., and Siuzdak, G. (1999) Nat. Struct. Biol. 6, 114–116
- 18. Siuzdak, G. (1998) J. Mass Spectrom. 33, 203-211
- Wang, C., Hartsuck, J., Downs, D., and Bass, H. (1990) *Biochim. Biophys. Acta* 1043, 143
- Quinn, D., Shirai, K., Jackson, R., and Harmony, J. (1982) Biochemistry 21, 6872
- Jackson, R. (1983) The Enzymes, Vol. XVI, Chapter 5, Academic Press, New York
- 22. Chemnitius, G., Erdmann, H., and Schmid, R. (1992) Anal. Biochem. 202, 16
- 23. Neumann, U., Junius, M., and Batz, H. G. (1989) U. S. Patent 4847376
- 24. Washburn, W. N., and Dennis, E. A. (1990) J. Am. Chem. Soc. 112, 2040
- 25. Gong, B., Lesley, S. A., and Schultz, P. G. (1992) J. Am. Chem. Soc. 114, 1486
- 26. Neumann, U., Junius, M., and Batz, H. G. (1991) U. S. Patent 4988497