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Mechanistic Studies on Enzymatic Reactions by Electrospray Ionization MS Using a Capillary Mixer with Adjustable Reaction Chamber Volume for Time-Resolved Measurements

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Mass spectrometry (MS)-based techniques have enormous potential for kinetic studies on enzyme-catalyzed processes. In particular, the use of electrospray ionization (ESI) MS for steady-state measurements is well established. However, there are very few reports of MS-based studies in the pre-steady-state regime, because it is difficult to achieve the time resolution required for this type of experiment. We have recently developed a capillary mixer with adjustable reaction chamber volume for kinetic studies by ESI-MS with millisecond time resolution (Wilson, D. J.; Konermann, L. ***Anal. Chem.*** **2003,** ***75,*** **6408−6414). Data can be acquired in kinetic mode, where the concentrations of selected reactive species are monitored as a function of time, or in spectral mode, where entire mass spectra are obtained for selected reaction times. Here, we describe the application of this technique to study the kinetics of enzyme reactions. The hydrolysis of *p*-nitrophenyl acetate by chymotrypsin was chosen as a simple chromophoric model system. On-line addition of a "makeup solvent" immediately prior to ionization allowed the pre-steady-state accumulation of acetylated chymotrypsin to be monitored. The rate constant for acetylation, as well as the dissociation constant of the enzyme−substrate complex obtained from these data, is in excellent agreement with results obtained by conventional stopped-flow methods. Bradykinin was chosen to illustrate the performance of the ESI-MS-based method with a nonchromophoric substrate. In this case, the unfavorable rate constant ratio for acylation and deacylation of the enzyme precluded measurements in the pre-steady-state regime. Steady-state experiments were carried out to determine the turnover number and the Michaelis constant for bradykinin. The methodologies used in this work open a wide range of possibilities for future ESI-MS-based kinetic assays in enzymology.**

Enzyme catalysis is a vital component of all biological systems. Enzyme mechanisms range from simple two-step processes to complex multistep reactions.[1] Kinetic experiments are among the most important tools for elucidating these reaction mechanisms. Immediately after the initiation of an enzymatic reaction, there is a short period of time (milliseconds to seconds, depending on the rate constants involved) during which reaction intermediates become successively populated. It is during this "pre-steady-state" period that the rate constants of individual steps can be measured. It is often possible to directly deduce reaction mechanisms based on pre-steady-state studies, whereas this is usually not the case for the more commonly employed steady-state measurements.[2−4]

With very few exceptions,[5] pre-steady-state kinetic studies require a time resolution in the millisecond range, which can only be achieved by using automated rapid mixing techniques. Stopped-flow rapid mixing involves quickly flushing reactants through a mixer and into an observation cell. The flow is then stopped, and the reaction is monitored in real time by optical methods.[6] Quench−flow experiments also involve rapid mixing, but the reaction is quenched after predetermined delay times through addition of a suitable agent (e.g., acid, base, or organic solvent). Subsequently, the mixture is analyzed off-line, e.g., by chromatography-based methods.[7] For continuous-flow studies, reactants are continuously passed through a mixer and into a reaction capillary. The reaction time at any point along this capillary depends on the tube dimensions and on the flow rate used. Continuous-flow methods can have a time resolution in the submillisecond range.[8]

Typically, the kinetics in these different types of rapid mixing experiments are monitored optically, e.g., by UV−visible absorption or by fluorescence spectroscopy. However, most reactions of enzymes with their "natural" substrates cannot be studied in this way because there are no associated chromophoric changes. For this reason, kineticists often use artificial substrate analogues that undergo a color change upon turnover. Obviously, this approach is problematic because the kinetics observed with these

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analogues are often different from those that would be observed with the natural substrate(s).[9] In some cases, the use of radioactively labeled substrates provides an alternative approach.[10,11] However, radiochemical methods are somewhat cumbersome, and problems can arise due to nonspecific entrapment of the label.

In recent years, mass spectrometry (MS)-based techniques have shown great promise in the area of chemical and biochemical kinetics.[12−21] The most significant advantage offered by MS-based studies is that they do not require chromophoric substrates or radioactive labeling. Consequently, there is great interest in the application MS for kinetic studies on enzyme-catalyzed processes.[9,22−27] Electrospray ionization mass spectrometry (ESI-MS), in particular, has enormous potential as an alternative to the traditional methods for monitoring enzyme kinetics, because the reaction mixture can often be injected directly into the ion source for on-line analysis, while the reaction occurs in solution. This approach allows the identification of reactive species based on their mass-to-charge ratio, their MS/MS characteristics, or both, while an analysis of the measured intensity−time profiles can provide reaction rates.[28] Our group has recently developed a capillary mixer with adjustable reaction chamber volume for on-line kinetic studies by ESI-MS.[29] This system allows the monitoring of processes having rate constants in the range from ∼1 s^{-1} up to at least 100 s^{-1}. When used in conjunction with a quadrupole mass spectrometer, reaction kinetics can be monitored either by measuring entire mass spectra for selected reaction times ("spectral mode") or by recording detailed intensity−time profiles for selected ions ("kinetic mode"). This unique combination of features should make this device a powerful tool for on-line studies on enzyme-catalyzed processes, particularly in the pre-steady-state regime.

Chymotrypsin is a member of the serine protease family.[30−32] Ser_{195} represents the reactive nucleophile in the active site of this enzyme. Although the physiological role of chymotrypsin is to serve as an endopeptidase, it also catalyzes the hydrolysis of esters, including numerous synthetic substrate analogues. Chymotrypsin shows a moderate degree of specificity for aromatic or bulky aliphatic substrates; hydrolytic cleavage occurs preferentially at the C-terminal side of phenylalanine, tyrosine, tryptophan, or leucine.[33] The generally accepted reaction mechanism for chymotrypsin-catalyzed hydrolysis is depicted in Scheme 1.[1−3]

Scheme 1

$$E + S \underset{}{\overset{K_d}{\rightleftharpoons}} ES \xrightarrow{k_2} EP_2 \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$\downarrow P_1$$

In the first step of this reaction sequence, the enzyme E and the substrate S form a noncovalent enzyme−substrate complex, ES, that is characterized by the dissociation constant K_d. Subsequently, Ser_{195} forms a covalent bond with the carbonyl carbon of the substrate, thus releasing the first hydrolysis product P_1. The rate constant of this acylation step is denoted as k_2. The subsequent deacylation has a rate constant of k_3, and it leads to regeneration of the free enzyme by hydrolysis of the Ser_{195}−ester bond, through release of the second hydrolysis product P_2. For conditions where S is present in large excess, it can be shown that the concentration of P_1 as a function of time t in the pre-steady-state regime is given by[2,3]

$$[P_1](t) = C_1(1 - \exp(-k_{obs}t)) + C_2t \qquad (2)$$

and the concentration−time profile of the covalent EP_2 complex can be expressed as

$$[EP_2](t) = C_3(1 - \exp(-k_{obs}t)) \qquad (3)$$

Consequently, the sum of the concentrations of free enzyme and ES complex are given by

$$([E_{free}] + [ES])(t) = C_4 \exp(-k_{obs}t) + C_5 \qquad (4)$$

C_1, ..., C_5 in these expressions are constants and k_{obs} is given by

$$k_{obs} = k_3 + \frac{k_2[S]}{K_d + [S]} \qquad (5)$$

where [S] is the substrate concentration. Measurements of k_{obs} as a function of substrate concentration allow the determination of the parameters k_2, k_3, and K_d in Scheme 1.

For $t \gg 1/k_{obs}$, the exponential terms in eqs 2−4 become negligible, thus marking the transition from the pre-steady-state to the steady-state regime. Under steady-state conditions, $[EP_2]$,

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$[E_{free}]$, and [ES] remain constant, whereas $[P_1]$ and $[P_2]$ increase linearly with time. The rate of reaction under these steady-state conditions is given by the Michealis−Menten expression[1]

$$\frac{d[P_1]}{dt} = \frac{d[P_2]}{dt} = \frac{k_{cat}[E]_0[S]}{K_M + [S]} \quad (6)$$

where $[E]_0$ is the total enzyme concentration. Measurements of the reaction rate as a function of [S], therefore, provide the turnover number k_{cat} and the Michaelis constant K_M.[4,34,35]

This work explores the application of our recently developed capillary mixer[29] for kinetic studies on enzymatic reactions by ESI-MS. Using chymotrypsin as a model system, we will initially describe results obtained with the chromophoric substrate *p*-nitrophenyl acetate (*p*-NPA). The hydrolysis kinetics measured for this compound by ESI-MS are compared to optical data obtained by standard optical stopped-flow spectroscopy. Subsequently, the ESI-MS-based approach is used for studies on the hydrolysis of the peptide bradykinin, which represents a non-chromophoric substrate. It will be seen that the method employed here can provide detailed information on the kinetics and mechanisms of enzyme-catalyzed processes.

EXPERIMENTAL SECTION

Chemicals. Chymotrypsin (nominally in its α form, see below) and p-NPA were obtained from Sigma (St. Louis, MO). Distilled grade methanol and hydrochloric acid were supplied by Caledon (Georgetown, ON, Canada), glacial acetic acid was supplied by BDH (Toronto, ON, Canada), and ammonium hydroxide was supplied by Fisher (Nepean, ON, Canada). These chemicals were used without further purification. Bradykinin, supplied by Bachem (Torrence, CA), was extensively dialyzed against distilled water using a 100 MWCO Float-A-Lyzer (Spectrum Laboratories, Rancho Dominguez, CA) prior to use.

On-Line Kinetic ESI-MS Experiments. ESI-MS-based kinetic experiments were carried out on a custom-built continuous-flow mixing apparatus described previously,[29] which has been coupled to a triple quadrupole mass spectrometer (model Sciex API 365, Concord, ON, Canada). Briefly, this flow setup consists of two concentric capillaries that are connected to sample injection syringes. Reactions are initiated by mixing of two solutions at the outlet of the inner capillary. The reaction time is determined by the solution flow rate and by the distance between the mixing point and the end of the outer capillary. For experiments in kinetic mode, the inner capillary is steadily withdrawn from the end of the outer capillary, while the mass spectrometer is set to monitor selected m/z values, corresponding to specific solution-phase species, as a function of time. In spectral mode, the inner capillary is set at specific distances from the end of the outer capillary, such that entire mass spectra can be obtained for selected reaction times.

For the experiments described here, both reactant solutions were introduced into the apparatus at 20 μL/min using syringe pumps (Harvard Apparatus, Saint Laurent, PQ Canada) for a total flow rate of 40 μL/min after the mixer. One important modification compared to our earlier setup is the addition of a mixing "tee" at the end of the outer capillary, which allows the addition of an "ESI-friendly" makeup solvent to the reaction mixture, immediately prior to ionization. The makeup solvent was infused at a flow rate of 40 μL/min, for a total flow rate of 80 μL/min at the ESI source. The second mixer is made from Flexon HP tubing (Alltech, Deerfield, IL); its two inlets accommodate the end of the outer capillary and a fused-silica capillary (100 ± 1.5 μm i.d., 167 ± 3 μm o.d., Polymicro Technologies, Phoenix, AZ) for addition of the makeup solvent. The mixer outlet is connected to a 1-cm fused-silica capillary (75 ± 1.2 μm i.d., 150 ± 2 μm o.d. Polymicro Technologies) that ends at the ESI source. Ionization takes place by pneumatically assisted ESI in the positive ion mode at a sprayer voltage of 6 kV. All measurements were carried out on a triple quadrupole mass spectrometer (PE Sciex, API 365, Concord, ON, Canada). It is noted that the makeup solvents used (see below) also act as chemical quenchers of the enzymatic reactions studied here. Therefore, the residence time of the solution in the flow system downstream of the second mixer (∼30 ms) does not contribute to the total dead time of the kinetic measurements, which is also estimated to be ∼30 ms. Analysis of the kinetic data obtained was carried out based on a framework described previously that takes into account laminar flow effects in the reaction capillary.[29] Safety considerations pertinent to the operation of the ESI-MS-coupled capillary mixer have also been discussed in that previous work. Protein mass spectra were deconvoluted using the Biomultiview analysis package provided by the instrument manufacturer.

Optical Stopped-Flow Experiments. Optical control experiments were carried out on an SFM-4 instrument (Bio-Logic, Claix, France) operated in absorbance mode, using an observation wavelength of 405 nm to monitor the release of *p*-nitrophenol (*p*-NP).[36] The two stepper motor-driven syringes were advanced at 3.5 mL s^{-1} each, for an instrument dead time of 3.3 ms.

Enzymatic Reactions. The limited solubility of *p*-NPA in purely aqueous solutions necessitated the use of 20% (v/v) methanol in the reaction mixture. Solutions of similar (or even higher) organic content were used in previous studies on the chymotrypsin-catalyzed conversion of *p*-NPA.[37,38] The activity of chymotrypsin does not seem to be affected by the presence of organic cosolvents at these concentrations.[39] Solutions containing 40% methanol and 1−10 mM *p*-NPA were brought to pH 8.1 using ammonium hydroxide. These solutions were mixed in a 1:1 ratio with 32 μM chymotrypsin in water for a final pH of 7.8, which corresponds to the pH optimum of the enzyme. A makeup solvent consisting of 5 mM HCl was found to produce the best signal-to-noise ratio for these *p*-NPA studies. Experiments on bradykinin were carried out in an analogous manner, but in purely aqueous solution, and by using 20% (v/v) acetic acid in water as makeup solvent. Control experiments showed the pH of the solutions to be stable for at least 5 s after mixing. Substrate concentrations given below represent the values in the reaction mixture, i.e., after the first mixing step. Burst phase kinetics observed by stopped-

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flow UV−visible spectroscopy for *p*-NPA hydrolysis showed that the chymotrypsin used had an active enzyme content of 80 wt %. This factor was taken into account for calculations involving enzyme concentrations. All experiments were carried out at room temperature (22 ± 1 °C). pH measurements were performed with an AB15 pH meter (Fisher Scientific). Reported pH values are "as read"; i.e., no corrections were made for solutions that contained methanol.

RESULTS AND DISCUSSION

Forms of Chymotrypsin in Commercially Supplied Samples. α-Chymotrypsin consists of three polypeptide chains that are covalently linked by disulfide bridges. The enzyme is generated from chymotrypsinogen, which is an inactive precursor with a continuous polypeptide backbone.[1,2,40] The formation of α-chymotrypsin from chymotrypsinogen can proceed by at least two mechanisms and involves multiple intermediate species.[41] In one of these scenarios, chymotrypsinogen is first converted to an active enzyme through cleavage of the Arg_{15}−Ile_{16} bond. Subsequent hydrolysis of the Ile_{13}−Ser_{14} bond releases a dipeptide. The resulting enzyme is known as *δ*-chymotrypsin. The α form of the enzyme (MW 25 234) is formed through two additional hydrolysis steps that release a second dipeptide consisting of Thr_{147} and Asn_{148}. Incomplete removal of the second dipeptide, by hydrolysis of just one of the peptide bonds, yields a form that can be referred to as *δ′* (MW 25 449).[41,42]

The deconvoluted ESI mass spectra of the chymotrypsin samples used for this work show two major components (Figure 1A). The observed peaks at 25 234 ± 1 and 25 450 ± 1 Da correspond to the masses of α- and *δ′*-chymotrypsin, respectively. The intensity ratios of the two species were found to be highly variable in the different lots used for the current study (compare, e.g., Figures 1A and 4). These observations are in line with previous work by Ashton et al.[43]

Hydrolysis Kinetics of *p*-NPA. The chymotrypsin-catalyzed hydrolysis of *p*-NPA generates *p*-NP and acetate. In the framework of Scheme 1, *p*-NP corresponds to P_1 and acetate corresponds to P_2.[1−3,33,39] *p*-NPA was chosen as substrate for these studies, because the released *p*-NP is an intense yellow, thus providing a convenient way to compare the ESI-MS-based kinetic experiments with the results of optical control experiments.[36,38] ESI mass spectra were generated at various times after mixing the enzyme solution with *p*-NPA. Figure 1 shows deconvoluted mass distributions, obtained at a *p*-NPA concentration of 2 mM, for three different reaction times. The two major peaks observed at $t \approx 30$ ms (Figure 1A) are assigned to the α and *δ′* forms of chymotrypsin. For a reaction time of 700 ms (Figure 1B), both forms of the protein show pronounced satellite peaks that correspond to a mass increase of 43 Da. At $t = 3$ s, these satellite peaks have become the dominant features in the mass distribution (Figure 1C). The observed mass increase of 43 Da is attributed to the acetylation of Ser_{195} in the active site of the enzymes. The three spectra depicted in Figure 1, therefore, represent the pre-steady-state accumulation of the EP_2 complex in Scheme 1. The fact that both forms of the protein undergo acetylation confirms that both of them are catalytically active, as previously observed by Ashton et al.[43] Figure 2 shows pre-steady-state intensity−time profiles of the unmodified and the acetylated forms of α-chymotrypsin. As predicted by eq 5, the acetylation rate depends on the substrate concentration. Consequently, the measured kinetics are markedly slower at 1 mM *p*-NPA (Figure 2A) than at 5 mM *p*-NPA (Figure 2B). Very similar kinetics were observed for *δ′*-chymotrypsin (data not shown).

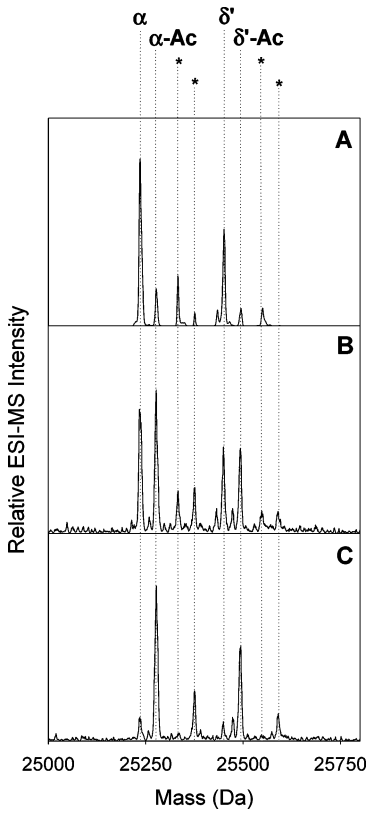

Figure 1. Deconvoluted ESI mass distributions recorded during the pre-steady-state of *p*-NPA hydrolysis by chymotrypsin. Peaks labeled with α and *δ′* correspond to α- and *δ′*-chymotrypsin, respectively. α-Ac and *δ′*-Ac refer to acetylated forms of the two enzyme species, corresponding to the covalent EP_2 complex in Scheme 1. Spectra were recorded at reaction times of 30 ms (A), 700 ms (B), and 3 s (C). The *p*-NPA concentration was 2 mM. All four forms of the protein (α, α-Ac, *δ′*, *δ′*-Ac) show minor satellite peaks corresponding to masses that are 98 ± 2 Da higher than those of the corresponding proteins (labeled with ∗). For α- and *δ′*-chymotrypsin, these peaks are also observed in the absence of substrate (data not shown). We tentatively assign these satellite peaks to adducts of the protein with an unidentified low molecular weight contaminant. Adduct formation is a common occurrence in ESI-MS.[24,52] As expected,[53] the magnitude of the satellite peaks depends on the declustering voltage in the ion sampling interface of the mass spectrometer (data not shown).

Exponential fits to the measured intensity−time profile provide the parameter k_{obs} (see eqs 3 and 4). Plots of these k_{obs} values as a function of *p*-NPA concentration are depicted in Figure 3 for both forms of the enzyme. The values measured for *δ′*-chymotrypsin are slightly higher than those for α-chymotrypsin. However, the differences are small, and the error bars overlap for most data points. These observations are consistent with previous studies on chymotrypsin that suggest that the various forms

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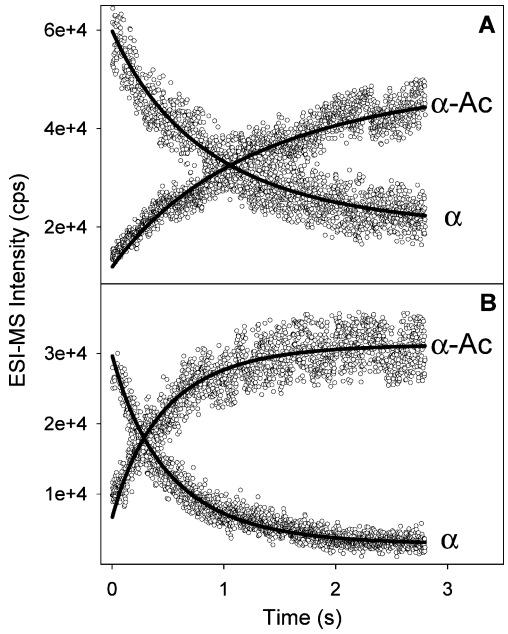

Figure 2. Pre-steady-state hydrolysis of *p*-NPA by chymotrypsin monitored by ESI-MS in kinetic mode. The two panels depict the depletion of unmodified α-chymotrypsin (α) and the formation of the acetylated α-chymotrypsin (α-Ac) at *p*-NPA concentrations of 1 (A) and 5 mM (B). The data were obtained by monitoring the 12^+ charge state of free and acetylated enzyme at *m*/*z* 2103 and 2107, respectively. Solid lines are fits to the experimental data based on eqs 3 and 4.

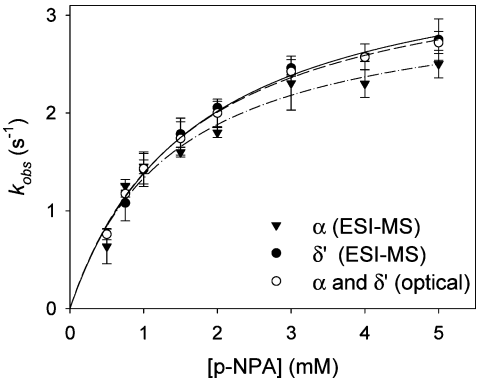

Figure 3. Measured k_{obs} values for *p*-NPA hydrolysis by chymotrypsin as a function of substrate concentration. Solid triangles represent ESI-MS measurements for α-chymotrypsin, and solid circles depict the corresponding data for *δ′*-chymotrypsin. Each point represents the average of four fits (two intensity−time profiles for the formation of the acetylated enzymes, and two traces for the depletion on the nonacetylated forms). Open circles depict k_{obs} values determined by optical stopped-flow spectroscopy in triplicate measurements. Error bars indicate standard deviations. Fits to these k_{obs} values based on eq 5 are given as solid line for the *δ*-chymotrypsin ESI-MS kinetics, as dashed line for the optical data, and as dash−dotted line for the α-chymotrypsin ESI-MS kinetics.

generated during processing of the enzyme have very similar structures and reaction kinetics.[1,40,44]

Fits to the measured k_{obs} data based on eq 5 yield k_2 values of 3.2 ± 0.3 and 3.7 ± 0.3 s^{-1} for α- and *δ′*-chymotrypsin, respectively. The corresponding dissociation constants K_d are 1.4 ± 0.2 and 1.7 ± 0.2 mM. Unfortunately, the value of k_3 is too small for an accurate determination by this method. This is entirely consistent with the accepted mechanism of *p*-NPA hydrolysis by chymotrypsin, according to which k_3 corresponds to the rate-determining step in Scheme 1. Previous work has shown k_3 to be orders of magnitude smaller than k_2.[1−3] This difference in rate constants is responsible for the fact that the EP_2 complex accumulates during *p*-NPA hydrolysis, which is a prerequisite for meaningful pre-steady-state measurements. For this scenario ($k_3 \ll k_2$), the rate constant k_3 can be approximated by k_{cat}. Based on optical steady-state measurements, we found $k_3 \approx k_{cat}$ to be 0.034 ± 0.003 s^{-1} (data not shown).

Measurements of k_{obs} as a function of substrate concentration were also carried out by stopped-flow spectroscopy, using the release of the yellow *p*-NP moiety as optical probe. In contrast to the ESI-MS experiments, these optical studies cannot discern the two forms of the enzyme, and therefore, the measured data represent a weighted average of the substrate conversion caused by α- and *δ′*-chymotrypsin. The analysis of the optical kinetics was carried out based on eq 2 (data not shown), and the results obtained are included in Figure 3, yielding k_2 and K_d values of 3.6 ± 0.2 s^{-1} and 1.6 ± 0.1 mM, respectively. These results are in good agreement with those reported above, thus confirming the reliability of our ESI-MS-based method as a tool for monitoring the kinetics of enzymatic reactions.

The k_2 values obtained in the different experiments described here are close to the corresponding rate constant of 3 s^{-1} that was previously reported by Gutfreund and Sturtevant.[37] Also, our estimate of k_3 is in line with their reported value of 0.03 s^{-1}. However, the K_d measurements in that work resulted in a value of 7 mM, which is substantially higher than the results obtained here. This discrepancy is not entirely unexpected, however, considering the much higher salt concentrations used by those authors, together with the known dependence of K_d on ionic strength.[39]

In summary, the pre-steady-state data on the hydrolysis of *p*-NPA clearly establish the viability of our ESI-MS-based method for mechanistic and kinetic studies on enzymatic processes. In the described experiments, the use of a chromophoric substrate allowed the independent confirmation of the measured kinetics by optical stopped-flow spectroscopy. We will now examine the conversion of a nonchromophoric compound, bradykinin, that cannot be followed by standard optical methods.

Hydrolysis Kinetics of Bradykinin. Bradykinin is a peptide consisting of nine amino acids (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, MW 1060). Based on the known preference of chymotrypsin to induce hydrolysis on the C-terminal side of phenylalanine,[33] both Phe_5−Ser_6 and Phe_8−Arg_9 represent potential cleavage sites. Preliminary studies showed the second of these possibilities to be preferred by a ratio of at least 100:1 (data not shown). Thus, P_1 in Scheme 1 corresponds to Arg_9, whereas P_2 is represented by the remainder of the peptide, i.e., Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe (MW 904).

Figure 4 shows the deconvoluted ESI mass distribution of chymotrypsin, 0.2 s after mixing with 2 mM bradykinin. The spectrum shows peaks corresponding to α- and *δ′*-chymotrypsin, the latter being the dominant species in the enzyme lot that was used for these bradykinin experiments. In contrast to the kinetic measurements performed on *p*-NPA, neither the α nor the *δ′* form

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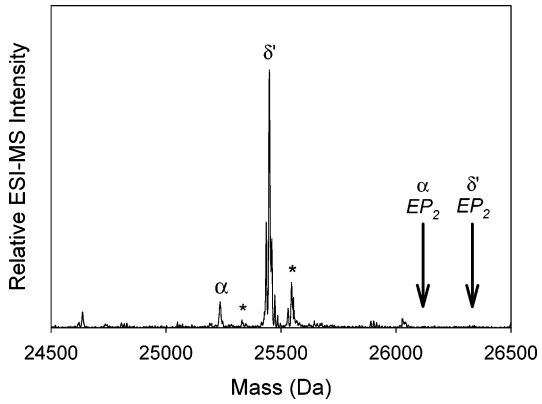

Figure 4. Deconvoluted ESI mass distribution obtained 0.2 s after mixing chymotrypsin with 2 mM bradykinin. α and δ' represent the two forms of the enzyme, and ∗ indicates adduct peaks, as in Figure 1. Arrows indicate the masses were the EP_2 complexes (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-[Ser_{195}-enzyme]) of α- (26 120 Da) and δ'-chymotrypsin (26 335 Da) would be expected.

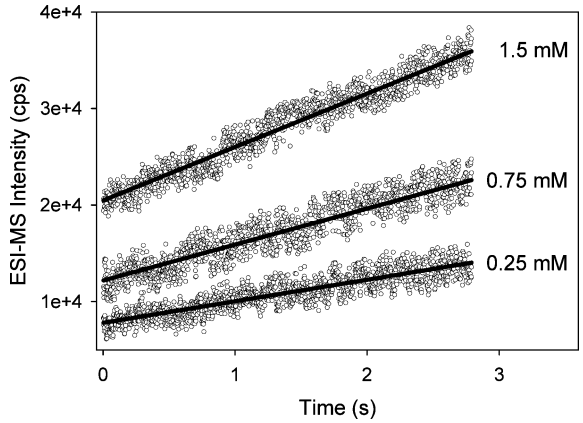

Figure 5. Chymotrypsin-catalyzed hydrolysis kinetics of bradykinin at three substrate concentrations. The signal intensity of the $[M + H]^+$ ion, corresponding to the hydrolysis product P_2 (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe, *m*/*z* 905), was monitored as a function of time. Solid lines are fits to the experimental data.

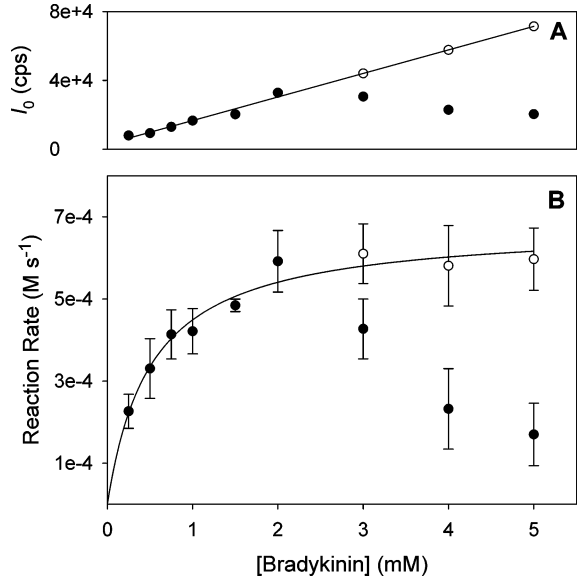

Figure 6. Data obtained for the hydrolysis of bradykinin by chymotrypsin, determined from kinetic profiles similar to those depicted in Figure 5. (A) Dependence of the initial product intensity, I_0, on the bradykinin concentration. Open symbols for concentrations of 3, 4, and 5 mM correspond to values corrected for signal suppression effects. These corrected data points are based on a linear extrapolation of the I_0 values measured at bradykinin concentrations of up to 2 mM (solid line). (B) Michealis−Menten plot of the reaction rate vs substrate concentration. Closed symbols represent measured values. Open symbols correspond to reaction rates adjusted for signal suppression; they were obtained by multiplication of the measured values with correction factors obtained from panel A. Slopes of the measured ESI-MS intensity profiles (in units of counts/s) were converted to reaction rates (in units of M s^{-1}) by using a conversion factor of 84 counts/s μM^{-1}. This factor was determined in separate experiments, using standardized solutions of purified P_2 ($\epsilon_{258} = 390$ M^{-1} cm^{-1}). The solid line in (B) is a fit to the corrected data set based on eq 6.

shows any accumulation of an EP_2 complex. The same observation was made in experiments that used different reaction times, different substrate concentrations, and samples that had a different ratio of α- to δ'-chymotrypsin. The absence of an observable EP_2 complex in this case is *not* due to a lack of enzyme activity; on the contrary, it will be seen that the enzyme undergoes rapid turnover under the conditions of Figure 4 (see below). It has previously been established that in the case of peptide bond hydrolysis by chymotrypsin, k_3 is much larger than k_2. In other words, acylation of the enzyme is the rate-determining step in Scheme 1 under these conditions.[1−3,45,46] EP_2 is being formed slowly and hydrolyzed quickly, and therefore, it does not become significantly populated at any point in the reaction. A pre-steady-state analysis, based on the concepts used above, is not possible under these conditions. Instead, it will be demonstrated how the ESI-MS-coupled capillary mixing setup can be applied to study the reaction kinetics under *steady-state* conditions.

The formation of P_2 was monitored at different bradykinin concentrations. Typical intensity−time profiles are depicted in Figure 5, together with the corresponding linear fits. As predicted by eq 6, the reaction rate increases with increasing substrate concentration. An unexpected feature of Figure 5 is the observation of a concomitant increase of the initial signal intensities I_0. This effect is caused by the presence of a small amount of P_2 as an impurity in the commercially supplied bradykinin substrate. A plot of I_0 for different bradykinin concentrations is depicted in Figure 6A. This figure shows a linear increase of I_0 up to substrate concentrations of ∼2 mM. Surprisingly, this is followed by a range where I_0 *decreases* with increasing bradykinin concentration. This observation is attributed to a suppression of P_2 ions, caused by the very high concentration of bradykinin in the solution. Effects of this kind are a well-known occurrence in ESI-MS.[47,48]

The dependence of the reaction rate on the bradykinin concentration was determined from the measured ESI-MS kinetic profiles, resulting in the data depicted in Figure 6B. The measured rates increase up to a substrate concentration of 2 mM, followed by a decrease. This decrease is ascribed to the same signal suppression effect discussed for I_0. Using the P_2 impurity in the bradykinin solution as an internal calibrant, the measured reaction rates were corrected for this effect, employing the procedure

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outlined in the caption of Figure 6. Thus, a Michaelis−Menten plot was produced (Figure 6B), from which the steady-state parameters $K_M = 0.51 \pm 0.08$ mM and $k_{cat} = 43 \pm 2$ s^{-1} were determined, resulting in a specificity constant of $k_{cat}/K_M = 8.4 \times 10^4$ s^{-1} M^{-1}.

Given the fact that bradykinin is a nonchromophoric substrate, it is not surprising that there seems to be a lack of literature data for direct comparison with the steady-state kinetics reported here. DelMar et al.[49] compiled parameters for a number of chromophoric oligopeptide substrate analogues of chymotrypsin. Many of these compounds show K_M values in the range around 0.5 mM, which is consistent with our results. The k_{cat} values of those substrate analogues show a large spread, from 0.01 s^{-1} up to more than 100 s^{-1} and specificity constants between 10 and 10^7 s^{-1} M^{-1}. The corresponding results obtained in the current study for a "natural" chymotrypsin substrate, therefore, are located in the midrange of the parameters determined for those chromophoric compounds.

CONCLUSIONS

This work employs a newly developed rapid mixing apparatus for time-resolved studies by ESI-MS. This methodology opens a wide area for kinetic studies on enzyme reactions, without requiring chromophoric or radioactively labeled substrates. It is anticipated that future uses of this approach will be particularly powerful for pre-steady-state kinetic measurements, because these experiments provide direct information on the reaction mechanisms of enzyme-catalyzed reactions. In cases where pre-steady-state measurements are not feasible, as for bradykinin, ESI-MS-based studies can still provide valuable kinetic information based on steady-state measurements.

Signal suppression artifacts represent a potential problem in assays that monitor the release of a reaction product at different substrate concentrations. The present work illustrates a simple method to correct for this effect. In the case of bradykinin, it was fortuitous that the substrate contained a trace amount of reaction product that could be used as an internal calibrant. In other cases, it may be necessary to add such a calibrant to the reaction mixture. It is noted that potential suppression effects are of no concern for the pre-steady-state experiments on *p*-NPA, because the measured k_{obs} values do not depend on absolute signal intensities.

An interesting feature of the ESI-MS-based method used in this study is the potential to measure the kinetics for individual components of an enzyme mixture. This was demonstrated in the *p*-NPA experiments, where the accumulation of two different reaction intermediates, corresponding to the α and δ' forms of chymotrypsin, could be monitored. The capability to probe the activity of coexisting enzyme species is reminiscent of recently developed approaches for monitoring the thermodynamic stabilities of unpurified proteins in mixtures by hydrogen/deuterium exchange methods and MS.[50] In both cases, advantage is taken of the inherent selectivity of MS-based techniques.

Both "spectral" and "kinetic" MS data are required for the characterization of enzymatic reaction mechanisms. Measurements in spectral mode allow an analysis of the components of the reaction mixture, in particular the identification of mechanistically important intermediates. Data recorded in kinetic mode provide the rate constant(s) of the reaction. Previous MS-based techniques were capable of providing data in either kinetic[20,21] or spectral mode,[12,23,27] but not both. In principle, it is possible to obtain intensity−time profiles for the determination of rate constants by "piecing together" spectral mode data.[24] However, that approach is very tedious, and the resulting kinetic traces consist of only relatively few data points. It is an inherent advantage of the ESI-MS-coupled capillary mixing setup used here that it is capable of providing mass spectra recorded at selected reaction times, as well as detailed intensity−time profiles with an extremely high density of data points.

For this study, a key modification was introduced to our previously described instrumental setup.[29] By using a second mixing step at the outlet of the reaction capillary it is possible to add a makeup solvent to improve the signal intensity and stability in ESI-MS. In the present case, meaningful kinetic experiments without this modification would not have been possible. The addition of acid in the current experiments leads to quenching of the reaction, ~30 ms before ionization occurs. Unfortunately, the use of these non-native solution conditions at the ESI source likely precludes the observation of noncovalently bound intermediates, such as the ES complex in Scheme 1. One limitation of the current on-line ESI-MS method is its incompatibility with high concentrations of salts, buffers, and other solvent additives that have detrimental effects on the ionization process.[51] We are confident that it will be possible to overcome this problem in future studies, by including a rapid on-line desalting device between the outlet of the mixing capillary and the inlet of the ESI source. It is hoped that modifications of this kind will further broaden the applicability of this method to study the mechanisms of various (bio)chemical processes.

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