Solvent Isotope Partitioning: A New Kinetic Tool for the Determination of Desorption Rates of Reactant Water from Enzyme–Substrate Complexes in Proteases†

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ABSTRACT: The rates of desorption of the substrate water from the binary enzyme–H_2O and ternary enzyme–H_2O–(peptide)substrate complexes for the two hydrolases, porcine pepsin and thermolysin, have been investigated using a novel technique, solvent isotope partitioning. The experimental design of this method was based on the protocol of Rose et al. [Rose, I. A., O'Connell, E. L., Litwin, S., & BarTana, J. (1974) *J. Biol. Chem. 249,* 5163–5168] wherein the binary enzyme–$H_2{}^{18}O$ complex established in the "pulse" solution was diluted into a "chase" solution containing variable concentrations of peptide substrates in a large pool of $H_2{}^{16}O$. The extent of trapping of $H_2{}^{18}O$ within the respective E–$H_2{}^{18}O$ and E–$H_2{}^{18}O$–(peptide)substrate complexes was determined from mass spectrometric analysis of the hydrolytic products. Our data have shown that the substrate water molecule of pepsin is not exclusively retained in the catalytic cycle and it desorbs from the apo- and substrate-bound complexes at rates that are at least 10 and 4 times faster, respectively, than that of product formation. Similarly, the low trapping of $H_2{}^{18}O$ in the carboxylic product of the thermolysin reaction is a consequence of the ready desorption of $H_2{}^{18}O$ from the ternary E–$H_2{}^{18}O$–(peptide)substrate complex and the binary E–$H_2{}^{18}O$ complex. We attribute these results to the loss of the reactant water molecule due to desolvation of the enzyme's active site upon substrate binding.

The hydrolases are unique among bireactant enzymes in that one of their substrates, H_2O, is also the reaction solvent. Accordingly, because the role of H_2O in the kinetic mechanisms of these enzymes is refractory to direct investigation, the hydrolases are treated as unireactant enzymes. Although detailed structural information of the reactant water molecules within the active sites of numerous hydrolases is available from X-ray crystallography, in particular the proteases, the rates of binding and desorption of water from the binary enzyme–H_2O and ternary enzyme–H_2O–substrate complexes remain uncharacterized for these enzymes.

Recent crystallographic data on porcine pepsin, the prototypical aspartic protease, demonstrates that, in the apo form of the enzyme, a solvent molecule, probably water, is positioned between, and perhaps hydrogen-bonds to, the catalytic aspartyl residues (Sielecki et al., 1990; Cooper et al., 1990; Abad-Zapatero et al., 1990). Water molecules also occupy the substrate binding cleft but are less well resolved than this "active-site" water molecule, which is conserved in all known structures of apo forms of the aspartic proteases. For bacterial thermolysin, a well-studied metalloprotease, the substrate water molecule is apparently coordinated to zinc, which activates it as the nucleophile for attack on the scissile carbonyl group of the peptide substrate, with simultaneous coordination of the carbonyl oxygen to the metal (Hangauer et al., 1984). One other internal solvent molecule has been implicated in the slow-binding behavior of certain transition-state inhibitors to thermolysin, particularly, the α-substituted phosphonic acids (Bartlett & Marlowe, 1987; Holden et al., 1987).

One would expect that the binding of an extended peptide substrate in the active site cleft of either pepsin or thermolysin would result in desolvation of the active site to establish hydrogen bonding of amide groups of the substrate to the amide backbone of the enzyme. What is unclear is the kinetic fate of the apparent substrate water molecule, putatively tightly bound via hydrogen bonding to the aspartyl residues in pepsin or by coordination to zinc ion in the case of thermolysin, upon binding of a substrate or inhibitor. From crystal structures of enzyme–inhibitor complexes of pepsin, the positioning of the substrate water molecule in the apoenzyme is conserved by the oxygen ligands of bound inhibitors. From this, it has been proposed that such inhibitors of this protease are merely bisubstrate analogs (Rich, 1985). Implicit in such a proposal is that the substrate water seen in the apoenzyme is retained throughout catalysis.

In this report we have examined the abilities of both porcine pepsin and thermolysin to retain their respective "structural" reactant water molecules during a catalytic cycle by modification of the isotope partitioning method of Rose et al. (1974), an extremely useful kinetic tool for measuring the desorption rates of substrates from their respective binary and ternary enzyme transitory complexes. Rose's method requires the ability to trap a radioactive, enzyme-bound substrate with variable concentrations of the second substrate upon dilution into a large pool of the unlabeled first substrate and the second substrate. Isotope partitioning can be modified for use with hydrolases by substituting $H_2{}^{18}O$ for the radioactive substrate and by diluting the binary enzyme–$H_2{}^{18}O$ complex into a solution containing variable substrate concentrations in $H_2{}^{16}O$, thereby constituting isotope partitioning of the solvent. The amount of enzyme-bound $H_2{}^{18}O$ trapped as the enzyme–$H_2{}^{18}O$ and enzyme–$H_2{}^{18}O$–substrate complexes can then be evaluated from mass spectrometric analysis of the extent of incorporation

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of ^{18}O into the hydrolase product at variable concentrations of the substrate.

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals. Crystalline porcine pepsin and thermolysin were purchased from Calbiochem and used without further purification. The protein concentration of each enzyme in solution was calculated from its UV absorption spectrum: $\epsilon^{278nm} = 50\,900\ M^{-1}\ cm^{-1}$ for pepsin (Medzihradszky et al., 1970) and $\epsilon^{280nm} = 61\,069\ M^{-1}\ cm^{-1}$ for thermolysin (Bartlett et al., 1987). The concentration of enzyme active sites was determined by active-site titration with a tight-binding inhibitor (pepstatin or phosphoramidon) using the protocol of Cha (1975). $H_2{}^{18}O$ (95% atom ^{18}O) was obtained from Aldrich Chemicals. The peptides Phe-Gly-His-(*p*-nitro)Phe-Phe-Ala-Phe-OMe (FGHF(NO_2)★FAF-OMe),[1] Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 (AcRASQNY★PVV-NH_2), *N*-[3-(2-furylacryloyl)]-Gly-Leu-NH_2 (FAG★LA), furylacryloyl-Gly-OH (FA-Gly-OH), phosphoramidon, and pepstatin were obtained in greater than 95% purity from Bachem Bioscience, Inc. Ac-Arg-Ala-Ser-Gln-Asn-Tyr-OH (AcRASQNY) was prepared by solid-phase synthesis as described (Hyland et al., 1991). The products FGHF(NO_2) and FAF-OMe were prepared by pepsin-catalyzed peptidolysis and purified by reversed-phase HPLC as described below, and their structures were confirmed by FAB-MS. All other chemicals were of the highest purity available.

Enzyme Assays. (*A*) *Pepsin.* The peptidolytic activity of pepsin with the heptapeptide substrate FGHF(NO_2)FAF-OMe was determined by using the spectrophotometric method of Medzihradszky et al. (1970). Initial velocities were measured at 25 °C by monitoring the increase in absorbance at 310 nm using a Hewlett-Packard 8450A UV–vis diode-array spectrophotometer equipped with a water bath which circulated through water-jacketted cuvettes. Each 1-mL assay mixture contained 40 mM ammonium formate, pH 3.0 or 4.0, various levels of substrate, and 10–30 nM enzyme.

The activity of pepsin with the oligopeptide substrate AcRASQNYPVV-NH_2 was measured by using an HPLC-based assay. Porcine pepsin cleaves this peptide at the tyrosyl–prolyl bond as does HIV-1 protease (Hyland et al., 1991). Reaction mixtures (50 μL) containing 40 mM ammonium formate, pH 3.0 or 4.0, and substrate (0.5–6.0 mM) were maintained at 25 °C in a water-filled temperature-regulated heating block prior to addition of enzyme (10–30 μM). The reactions were quenched at 10 min by the addition of 50 μL of 300 μM pepstatin. The product AcRASQNY was then separated and quantitated by RP-HPLC as described for HIV-1 protease by Hyland et al. (1991). A Hewlett-Packard 1090 high-performance liquid chromatograph equipped with a ternary solvent delivery system, an autosampler, a diode-array spectrophotometric detector, and a digital integrator was utilized for all analyses. The fraction of reaction was calculated as the ratio of the product formed/(product formed + substrate remaining) obtained from the digital integration of the substrate and product peaks detected at 220 nm: AcRASQNYPVV-NH_2 (10 min); AcRASQNY (5.5 min).

[1] Abbreviations: AcRASQNY★PVV-NH_2, *N*-acetyl-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 where the star (★) indicates the site of cleavage; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; ES-MS, electrospray mass spectrometry; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; FAB-MS, fast-atom bombardment mass spectrometry; FAG★LA, *N*-[3-(2-furylacryloyl)]Gly-Leu-NH_2; HPLC, high-performance liquid chromatography; HIV, human immunodeficiency virus; FGHF(NO_2)★FAF-OMe, Phe-Gly-His-(*p*-nitro)Phe-Phe-Ala-Phe-OMe; TFA, trifluoroacetic acid.

Aqueous solutions of each oligopeptide substrate or product were freshly prepared for each experiment. The concentrations of the peptide solutions were determined from their UV absorption spectra using the following extinction coefficients: $\epsilon^{278nm} = 9600\ M^{-1}\ cm^{-1}$ for FGHF(NO_2)FAF-OMe (Medzihradszky et al., 1970), $\epsilon^{278nm} = 9475\ M^{-1}\ cm^{-1}$ for FGHF(NO_2), and $\epsilon^{275nm} = 1420\ M^{-1}\ cm^{-1}$ for AcRASQNY and AcRASQNYPVV-NH_2 (Hyland et al., 1991).

(*B*) *Thermolysin.* Stock solutions of thermolysin were prepared in a buffer system composed of 50 mM Tris-HCl, pH 7.0, 10 mM $CaCl_2$, and 2.5 M NH_4Cl. All steady-state assays were performed at 25 °C by following the decrease in absorbance at 345 nm (Feder & Schuck, 1970) in a 1-mL reaction mixture containing 50 mM Tris-HCl, pH 7.0, 10 mM $CaCl_2$, 2.5 M NH_4Cl, 10% DMF, various concentrations of FAGLA, and 50–100 nM enzyme.

Solvent Isotope Partitioning Experiments. The experimental design of the solvent isotope partitioning studies was based on the protocol of Rose et al. (1974) in which the "pulse" solution (EA*), composed of enzyme dissolved in $H_2{}^{18}O$, was rapidly mixed and diluted into a "chase" solution containing variable concentrations of the "trapping substrate", a peptide (B), dissolved in a much larger volume of $H_2{}^{16}O$ (A). The partitioning of $H_2{}^{18}O$ from the EA* and EA*B complexes into product could then be assessed by analysis of the fraction of ^{18}O in the carboxylic peptidolysis product (P*) under respective conditions of very dilute or saturating levels of B. The observation of solvent isotope partitioning in the first enzyme turnover is ensured by (a) dilution of E–$H_2{}^{18}O$ into a large excess (40-fold) of $H_2{}^{16}O$ and by (b) correction of the results by a control sample at each concentration of B in which $H_2{}^{18}O$ had been previously diluted into $H_2{}^{16}O$-containing chase solution to the same extent as in the pulse–chase procedure.

Solvent isotope partitioning studies on pepsin were done using two peptide substrates of which the k_{cat}/K_m values differ by 100-fold. For the superior pepsin substrate FGHF(NO_2)-FAF-OMe, a rapid-mixing apparatus (UpDate Instruments, Madison, WI) equipped with a four-grid mixer and a 300-ms aging loop was used. For each sample, the pulse syringe contained a solution of pepsin (3.6 nmol) in 40 mM ammonium formate, pH 3.0 ($H_2{}^{18}O$), while the chase syringe contained variable concentrations of FGHF(NO_2)FAF-OMe (0.4–3.0 mM) in 40 mM ammonium formate, pH 3.0 ($H_2{}^{16}O$). The enzyme solution was spiked with [^{14}C]glycine ethyl ester (1.5–2.0 mCi/mL) as an internal standard to determine the actual amount of enzyme and $H_2{}^{18}O$ that participated in the reaction. In this apparatus, a single push forced the chase solution (320 μL) to rapidly mix with the pulse solution (8 μL), which then flowed through the reaction loop and out into the collection vessel containing a stirred solution of 0.1 N NH_4OH, which was sufficient to quench the reaction. Aliquots (25–50 μL) were removed from the quenched samples for scintillation counting, and the remainder was subjected to centrifugation at 17 000 rpm for 5 min to remove any precipitated enzyme. The peptides FGHF(NO_2)FAF-OMe, FGHF(NO_2), and FAF-OMe were separated by RP-HPLC (Beckman Ultrasphere ODS column; 4.5 mm × 25 cm, 5 μm). A mobile phase consisting of 5–40% CH_3CN (20 min) and 40% CH_3CN (5 min) in 0.05% TFA (gradient A) resulted in the resolution of the peptide peaks, detected at 220 nm, as follows: FGHF(NO_2) (14.7 min); FAF-OMe (19.5 min); FGHF(NO_2)FAF-OMe (23.7 min). Control samples for each substrate concentration contained an identical amount of $H_2{}^{18}O$ in the substrate ("chase") solution instead of in the enzyme ("pulse") solution. The separated fractions containing

FGHF(NO_2) were subsequently lyophilized prior to analysis by FAB-MS. The amount of product formed per sample (nanomoles of product) was determined from a standard curve which correlates the integrated chromatographic peak area to the nmol of FGHF(NO_2) formed. The standard curve was prepared from solutions containing stoichiometric amounts of FGHF(NO_2) and FAF-OMe obtained from a complete enzymatic digestion of numerous known concentrations of FGHF(NO_2)FAF-OMe. The enzymatic digests were then subjected to the quenching conditions as described above, and the separated aqueous layers were analyzed by RP-HPLC. In all cases, the total peptidolysis of peptide substrate was indicated by the absence of the substrate peak in the HPLC analysis.

The fraction of the carboxylic product which contained one atom of ^{18}O (nanomoles of [^{18}O]product) was calculated as the multiplicative product of the nanomoles of product formed and the fraction ([$M^{18}O$]/([$M^{18}O$] + [$M^{16}O$]) as determined from mass spectrometry of the isolated carboxylic product. The effective fraction of $H_2{}^{18}O$ in the pulse solution was determined from the fraction of ^{18}O found in the carboxylic product that resulted from complete peptidolysis of substrate in a 1:1 (by volume) mixture of pulse and chase solutions. The total amount of the enzyme–$H_2{}^{18}O$ complex that participates in the reaction (nanomoles of E–$H_2{}^{18}O$) was determined from nanomoles of E–$H_2{}^{18}O$ = (nanomoles of enzyme in the pulse solution) × (fraction of total [^{14}C]Gly-OEt delivered to pulse–chase mixture) × (effective fraction of $H_2{}^{18}O$ in pulse solution). E–$H_2{}^{18}O$ varied from 0.3–0.6 of the amount of enzyme in the original pulse solution, depending on the mixing method. Values of nanomoles of [^{18}O]product and nanomoles of E–$H_2{}^{18}O$ were determined independently for pulse–chase samples and for control samples in which $H_2{}^{18}O$ had already been diluted to the same extent as the pulse–chase mixture, and the final ratio of nanomoles of [^{18}O]product/nanomoles of E–$H_2{}^{18}O$ was calculated as the difference of these ratios.

We also sought to ensure that the lytic water molecule in the active site of pepsin was $H_2{}^{18}O$. If the solvent molecule ($H_2{}^{16}O$) were so tightly bound by pepsin in the lyophilized sample of the enzyme that it did not readily exchange with $H_2{}^{18}O$ in the pulse solutions, then we would observe no incorporation of ^{18}O in the peptidolytic products. Therefore, we "charged" the active site of pepsin by allowing the enzyme to first undergo multiple turnovers in the $H_2{}^{18}O$-containing pulse solutions with one peptide substrate, prior to solvent isotope partitioning analysis with a second substrate. Following a single turnover, pepsin should then be "$H_2{}^{18}O$-charged" with solvent water containing the same fraction of ^{18}O as the pulse solution. Preparation of "uncharged" enzyme simply involved dissolving crystalline pepsin in $H_2{}^{18}O$-containing buffer as in the experiments described above. In our case, the "charged" pulse solution (500 μM) was prepared by preincubating pepsin with an equimolar amount of AcRASQNYPVV-NH_2 in $H_2{}^{18}O$ buffer for 30 min. Solvent isotope partitioning experiments were performed by quickly adding 8 μL of either "charged" or "uncharged" enzyme solution to a vigorously agitated 320-μL solution of FGHF(NO_2)FAF-OMe (40 μM). The reactions were quenched after 3 s by the rapid addition of 0.1 N NH_4OH. Controls and analyses were performed as described above.

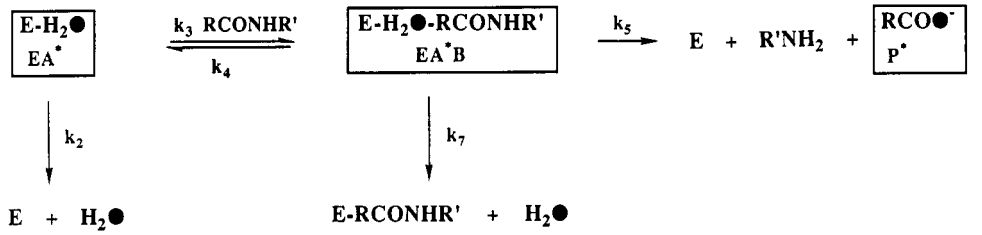
For AcRASQNYPVV-NH_2, a 0.25-mL pulse solution of 40 mM ammonium formate, pH 4.0, in $H_2{}^{18}O$ containing 72.5 nmol of pepsin was added by pipet into a rapidly stirred (by vortex mixing) 4.75-mL solution consisting of 40 mM ammonium formate, pH 4.0, and variable concentrations of AcRASQNYPVV-NH_2. The reaction was quenched at 40 s by the rapid addition of 10 mL of $CHCl_3$ and 50 μL of 62 mM pepstatin in DMSO. Control samples and analyses were done using the conditions outlined above except that the mobile phase consisted of a shallower gradient of acetonitrile in 0.05% TFA: 2% (0–7 min), 2–20% (7–15 min), 20% (15–20 min) (gradient B). The major peaks detected at 220 nm corresponded to AcRASQNY (18.5 min) and AcRASQNYPVV-NH_2 (23 min). The extent of ^{18}O incorporation into the carboxylic peptidolytic product AcRASQNY was determined by FAB-MS. Quantitation of the product formed for each sample was based on a standard curve prepared from solutions of authentic AcRASQNY.

For all ^{18}O-containing carboxylic products, exposure to acidic conditions (during chromatography and work-up) should result in little loss of ^{18}O due to acid-catalyzed exchange. Risley and Van Etten (1981) have shown that at 32 °C the rate of exchange of ^{18}O from acetic acid into solvent at a pH equivalent to our RP-HPLC conditions is low (0.0095 min^{-1}). Moreover, as shown below, control samples of ^{18}O-containing products prepared (by identical procedures) from complete peptidolysis of substrates in a 1:1 mixture of pulse and chase solutions retained ≥60% of the expected fraction of ^{18}O based on the compositions of the solutions. The inability to achieve quantitative incorporation of ^{18}O from these samples is due either to the adventitious introduction of $H_2{}^{16}O$ (via the lyophilized enzymes, for example) into the pulse solutions or to acid-catalyzed exchange, or both. Nevertheless, this result demonstrates that a large fraction of ^{18}O will remain in the peptidolysis samples.

Solvent isotope studies of thermolysin were performed using the rapid-mixing apparatus described above. The enzyme solution in $H_2{}^{18}O$ composed of 336 μM thermolysin, 50 mM Tris-HCl, pH 7.0, 10 mM $CaCl_2$, 2.5 M NH_4Cl, and [^{14}C]-glycine ethyl ester (1.5–2.0 mCi/mL) and the substrate solution in $H_2{}^{16}O$ containing 1–10 mM FAGLA, 50 mM Tris-HCl, pH 7.0, 10 mM $CaCl_2$ 2.5 M NH_4Cl, and 10% DMF were loaded into the pulse syringe and chase syringe, respectively. The reaction mixture was aged 300 ms at which point the pulse–chase solution was mixed with the quenching solution (100 mM ammonium formate, pH 5.0, 10 mM EDTA, and 3 mM phosphoramidon). Controls and analyses were done as described for pepsin. Separation of peptides was achieved by RP-HPLC using gradient A, and the peaks were detected at 300 nm: FA-Gly-OH (11.4 min); FAGLA (16 min). The fractions containing FA-Gly-OH were lyophilized prior to analysis by electrospray mass spectrometry (ES-MS). The amount of product formed for each sample was calculated from a plot of the integrated chromatographic peak area vs nmol FA-Gly-OH.

Mass Spectrometry. Fast atom bombardment (FAB) mass spectra were obtained on either (a) a VG ZAB-HF magnetic deflection mass spectrometer (accelerating voltage, 8 kV; mass range, 3000) equipped with a standard FAB ion source and a fast atom gun (Ion Tech) or (b) the first double-focusing portion (MS-1) of a VG ZAB SE-4F tandem magnetic deflection mass spectrometer (accelerating voltage, 10 kV; mass range, 12 500) equipped with a standard FAB ion source and a high voltage cesium ion gun. The Ion Tech FAB gun was operated at 8 kV, and a discharge current of 1 mA was obtained by using xenon, while the voltage cesium gun was operated at 35 kV with an emission of 2–4 μA. One to three microliters of each sample [approximately 2–5 nmol of FGHF(NO_2) or 4–16 nmol of AcRASQNY] in 10% or 20% acetonitrile containing 0.1% trifluoroacetic acid were dispersed

Scheme I



on the stainless steel target in a matrix of monothioglycerol. Data were acquired over a mass range of m/z 600–548 at 200 s/decade or m/z 810–770 at 15 s/decade with a resolution of 1500 (10% valley definition). To obtain the best signal-to-noise ratios, several sequential scans were summed in the peak profile, multichannel acquisition mode. The mass range was calibrated using the $[Cs(CsI)_n]^+$ peaks generated by FAB mass spectrometry of CsI. A VG 11-250J data system was used to acquire and process all data. Calculations were performed using peak intensity values taken from the MH^+ pseudomolecular ion species.

Electrospray (ES) mass spectra were recorded on a Sciex API-III triple-quadrupole mass spectrometer (2400 Da m/z range) fitted with a fully articulated, nebulization-assisted electrospray (Ionspray) probe and an atmospheric pressure ionization source. One microliter of a solution (approximately 2–6 nmol) of FAGLA in 20% acetonitrile containing 0.2% formic acid was introduced in the mass spectrometer by flow injection using a Rheodyne model 8125 HPLC injector with a 10-μL sample loop connected to the Ionspray probe tip by flexible fused silica tubing (100-μm i.d.). The carrier solvent (20% acetonitrile containing 0.2% formic acid) was delivered using a Harvard Apparatus infusion pump at a flow rate of either 3 or 6 μL/min. The mass spectrometer was scanned repetitively over the range m/z 184.5–204.5 with a mass step of 0.1 Da and 30-ms dwell/step (scan duration = 6 s). A mixture of polypropylene glycols 425, 1000, and 2000 (3.3×10^{-5}, 1×10^{-4}, and 2×10^{-4} M, respectively, in a 1:1 mixture of 2 mM ammonium acetate/methanol) was used to calibrate and to adjust the resolution to unity over the appropriate mass range. Data were acquired and processed with Sciex MacSpec data system. Calculations were performed using peak intensity values taken from the MH^+ pseudomolecular ion species (m/z 196) obtained from the sum of several sequential scans.

Data Analysis. Steady-state kinetic data were analyzed by using the FORTRAN programs of Cleland (1979) which weight the data by assuming equal variance for the velocities of the fitted parameter. Steady-state initial velocity data were fitted to

$$V = \frac{VB}{K_B + B} \tag{1}$$

where v is the experimentally determined initial velocity, V is the maximum velocity, B is the variable peptide substrate concentration, and K_B is the Michaelis constant. Experimental data from isotope partitioning studies were fitted to eq 2, a

$$\frac{P^*}{EA^*} = \frac{(P^*_{max}/EA^*)B}{K'_B + B} \tag{2}$$

modified version of eq 1 (Rose, 1980; Cleland, 1975). Equation 2 was derived from Scheme I in which (P^*/EA^*) is the experimentally determined fraction of $[EA^*]_o$ trapped into product, K'_B refers to the concentration of B that causes half-maximal trapping of EA*, and (P^*_{max}/EA^*) is the value of P^*/EA^* at infinite B concentration.

RESULTS AND DISCUSSION

Steady-State Kinetic Parameters. The steady-state kinetic parameters obtained for the two pepsin substrates at pH 3 (pH 4), 25 °C, by fitting to eq 1 were $V/KE_t = 100 \pm 6$ (772 ± 63) $mM^{-1}\ s^{-1}$, $V/E_t = 13.9 \pm 0.7$ (29 ± 1) s^{-1}, and $K = 0.14 \pm 0.01$ (0.037 ± 0.004) mM for FGHF(NO_2)FAF-OMe; and for AcRASQNYPVV-NH_2, $V/KE_t = 0.0056 \pm 0.0002$ (0.0092 ± 0.0004) $mM^{-1}\ s^{-1}$, $V/E_t = 0.0080 \pm 0.0002$ (0.0125 ± 0.0003) s^{-1}, and $K = 1.41 \pm 0.06$ (1.36 ± 0.08) mM. Accordingly, quenching of the solvent isotope partitioning reactions of FGHF(NO_2)FAF-OMe (pH 3) and AcRASQNYPVV-NH_2 (pH 4) at 300 ms and 40 s, respectively, constituted stopping these reactions at a maximum of 6 and 0.72 half-lives, respectively. The peptidolytic activity of thermolysin with FAGLA was examined at pH 7 in 10% DMF and resulted in $K = 3.2 \pm 0.6$ mM, $V/E_t = 17 \pm 2\ s^{-1}$, and $V/KE_t = 5.4 \pm 0.3\ mM^{-1}\ s^{-1}$. The solution concentrations of enzymatic active sites were quantified by titration of the pepsin and thermolysin activities using their respective tight-binding inhibitors, pepstatin and phosphoramidon.

Theory of Solvent Isotope Partitioning. The consequences of solvent isotope partitioning as depicted in Scheme I are derived from the general form of isotope partitioning as defined by Rose (1980). The fate of the enzyme–$H_2{}^{18}O$ complex in the pulse solution (EA*) upon dilution into an excess of $H_2{}^{16}O$ containing variable concentrations of the peptide substrate (B) depends on the relative rates of desorption of $H_2{}^{18}O$ from the binary EA* and ternary EA*B (EBA*) complexes, k_2 and k_7, respectively. For a compulsory ordered mechanism, k_7 is extremely small relative to k_{cat}, and when B is extrapolated to an infinite concentration, all of the EA* complex should be trapped as product, i.e., P^*_{max}/EA^* is unity. Using variable concentrations of the trapping substrate (B) and analysis of the trapped product in a double-reciprocal fashion permits independent assessment of desorption of $H_2{}^{18}O$ from the EA* and EA*B (EBA*) complexes by respective analyses of the slope and intercept effects. Combination of the expressions for isotope trapping studies and steady-state kinetics allows an evaluation of the respective ratios of k_2 and k_7 to k_{cat} (Rose, 1980; Cleland, 1975):

$$\frac{K'_B[EA^*]}{K_B[P^*_{max}]} \geq \frac{k_2}{k_{cat}} \geq \frac{K'_B}{K_B} \tag{3}$$

$$\frac{EA^*}{P^*_{max}} - 1 = \frac{k_7}{k_{cat}} \tag{4}$$

Again, when $H_2{}^{18}O$ is completely trapped within the ternary complex (that is, it is very "sticky"), $k_7 \ll k_{cat}$, P^*_{max} equals EA*, and the relation $[(K'_B/K_B)(k_{cat}) = k_2]$ holds.

Table I: $H_2{}^{18}O$ Isotope Partitioning Experiments on Porcine Pepsin and Thermolysin[a]

substrate (mM)	[M ^{18}O]/([M ^{18}O] + [M ^{16}O]) expt[f]	calcd[g]	no. of turnovers[b]	pulse–chase sample/control sample: nmol of E–$H_2{}^{18}O$[c]	nmol of product[d]	nmol of [^{18}O]product[e]	nmol of [^{18}O]product/nmol of E–$H_2{}^{18}O$	
(1) Substrate = FGHF(NO_2)FAF-OMe; E = Pepsin, 3.6 nmol; Reaction Time = 300 ms								
0.4	0.008	0.21	3	1.6/1.7	8.4/6.4	0.24/0.19	0.15/0.11	0.04[h]
0.6	0.007	0.11	6	0.95/1.5	10.2/11.2	0.3/0.39	0.32/0.26	0.06
1.0	0.009	0.11	6	1.3/1.9	13.8/13.9	0.40/0.41	0.30/0.21	0.09
2.0	0.011	0.13	5	1.9/1.9	16.4/16.4	0.70/0.51	0.38/0.27	0.11
3.0	0.014	0.054	15	1.2/1.8	29.3/24.3	0.68/0.76	0.56/0.42	0.14
(2) Substrate = FGHF(NO_2)FAF-OMe; E = "$H_2{}^{18}O$-Charged" Pepsin, 4.0 nmol; Reaction Time = 3 s								
0.04	0.001	0.31	2	2.4	9.7	0.048		0.02
(3) Substrate = FGHF(NO_2)FAF-OMe; E = "Uncharged" Pepsin, 4.0 nmol; Reaction Time = 3 s								
0.04	0.001	0.31	2	2.4	9.5	0.047		0.02
(4) Substrate = AcRASQNYPVV-NH_2; E = Pepsin, 72.5 nmol; Reaction Time = 40 s								
0.7	0	0.60	0.5	43.5	39.2	0.0		0.0
1.6	0	0.60	1	43.5	63.8	0.0		0.0
3.2	0	0.60	1	43.5	71.2	0.0		0.0
4.8	0	0.60	1	43.5	81.8	0.0		0.0
(5) Substrate = FAGLA; E = Thermolysin, 2.7 nmol; Reaction Time = 0.3 s								
1.0	0.001	0.10	7	1.1	12.0	0.013		0.012
1.7	0.004	0.074	10	0.96	16.0	0.064		0.067
10.0	0.006	0.057	14	1.0	23.0	0.13		0.13

[a] Experimental conditions given under Experimental Procedures. For experiment 1, data for both the pulse–chase samples and control samples are displayed for each measured parameter; for experiments 2–5, parameters for control samples are not shown, and pulse–chase samples have been corrected for controls. [b] Determined from nmol of product/nmol of enzyme added from pulse solution. [c] E–$H_2{}^{18}O$ = (nmol of enzyme in the pulse solution) × (fraction of enzyme delivered in the pulse–chase mixture) × (effective fraction of $H_2{}^{18}O$ in the pulse solution). The fraction of enzyme in the pulse–chase mixture varies between 40% and 80% of that in the pulse solution for experiments 1 and 5 and is 100% for that of experiments 2–4. [d] Determined from HPLC analysis of product in pulse–chase samples. [e] nmol of [^{18}O]product = nmol of product × [M ^{18}O]/([M ^{18}O + [M ^{16}O]); values of nmol of [^{18}O]product are uncorrected for control in experiment 1 but as shown are corrected in experiments 2–5. [f] Experimental values have been corrected for control samples for each substrate concentration. [g] Calculated from: $\{[H_2{}^{18}O]/([H_2{}^{18}O] + [H_2{}^{16}O])_{pulse} + n([H_2{}^{18}O]/([H_2{}^{18}O] + [H_2{}^{16}O]))_{pulse-chase}\}/(n + 1)$ in which $n + 1$ is the total numbers of turnovers; values of $[H_2{}^{18}O]/([H_2{}^{18}O] + [H_2{}^{16}O])_{pulse}$ and $[H_2{}^{18}O]/([H_2{}^{18}O] + [H_2{}^{16}O])_{pulse-chase}$ are typically 0.6 and 0.015, respectively. [h] Final value of nmol of [^{18}O]product/nmol of E–$H_2{}^{18}O$ is the difference of this value for the pulse–chase and control samples.

Solvent Isotope Partitioning on Pepsin. A binary complex of pepsin and $H_2{}^{18}O$ was diluted 40-fold into a solution of $H_2{}^{16}O$ containing variable levels of FGHF(NO_2)FAF-OMe, and the reactions were terminated after 300 ms. In a separate series of isotope trapping experiments, the E–$H_2{}^{18}O$ solution was diluted by rapid mixing into 19 volumes of peptide substrate solution (0.7–5.0 mM AcRASQNYPVV-NH_2), and the reaction was quenched after 40 s. Shown in Table I are the results of these solvent isotope partitioning experiments on pepsin–$H_2{}^{18}O$ and variable concentrations of the two oligopeptide substrates. The concentration of E–$H_2{}^{18}O$ in each reaction was determined from the fraction of ^{18}O found in the carboxylic product resulting from a 1:1 reaction mixture of pulse and chase solutions (which gives the actual fraction of $H_2{}^{18}O$ in the pulse solution) and from the [^{14}C]glycine-OEt present, which gives the actual molar amount of enzyme and $H_2{}^{18}O$ conveyed from the pulse solution into the reaction. The total nanomoles of the carboxylic products, FGHF(NO_2) and AcRASQNY, were determined in the quenched pulse–chase samples and control samples from standard curves of these peptides as described. The extent of ^{18}O incorporation into FGHF(NO_2) from 2 mM FGHF(NO_2)FAF-OMe as determined by FAB-MS is shown in Figure 1. The "pulse–chase" sample gave an apparent fraction of ([M^{18}O]/{[M^{16}O] + [M^{18}O]}) which exceeded that of the "control" sample by 0.01, indicating trapping of 1% of FGHF(NO_2)FAF-OMe as [^{18}O]FGHF(NO_2). The total number of turnovers for this sample (five) indicated that 80% of the product FGHF(NO_2) was formed at a $H_2{}^{18}O$-isotopic fraction identical to that of the control sample, such that a maximum of 20% of the $H_2{}^{18}O$ would be incorporated into the product if all of the $H_2{}^{18}O$ bound to enzyme were trapped in product. That only 1% of

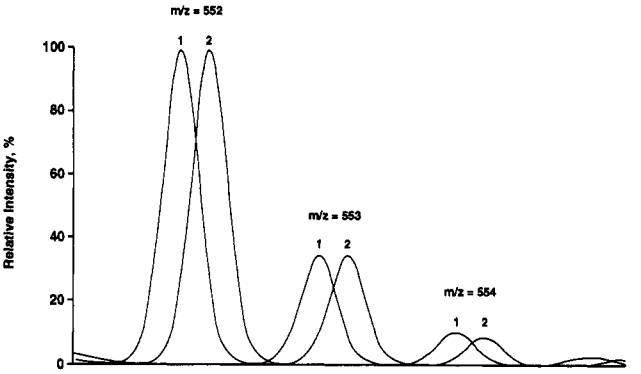


FIGURE 1: Fast atom bombardment (FAB) mass spectra of FGHF(NO_2). The relative intensities of the MH^+ pseudomolecular ion species are shown (m/z = 552 and 554 for the ^{16}O- and ^{18}O-containing peptide products, respectively). (1) Reaction sample: the pulse solution contained 3.6 nmol of pepsin in $H_2{}^{18}O$-enriched buffer, while the chase solution consisted of 2 mM FGHF(NO_2)FAF-OMe in $H_2{}^{16}O$ buffer. (2) Control sample: same as no. 1 except that the enzyme solution (pulse) was prepared in $H_2{}^{16}O$ buffer and the chase solution contained an equivalent amount of $H_2{}^{18}O$ as that found in the pulse of the reaction sample.

the $H_2{}^{18}O$ was found in the product indicates that $H_2{}^{18}O$ is not highly retained by the enzyme–substrate complexes during the reaction. As shown in Table I, the difference in the experimental fractional incorporation of ^{18}O into product vs the calculated amount based on 100% trapping further demonstrates that significant desorption of $H_2{}^{18}O$ occurs during enzymatic turnover.

The fraction of the E–$H_2{}^{18}O$ trapped into the product, [^{18}O]FGHF(NO_2)/E–$H_2{}^{18}O$, vs the corresponding concentration of the oligopeptide substrate FGHF(NO_2)FAF-OMe

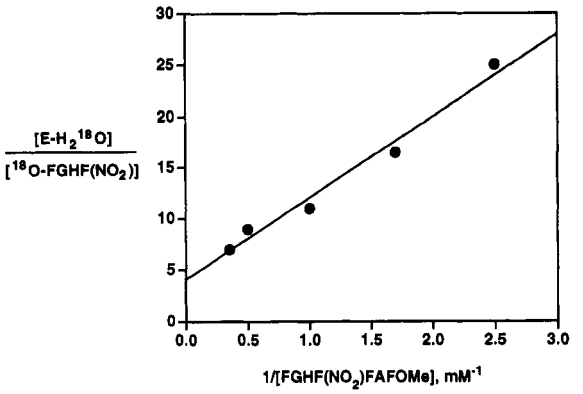


FIGURE 2: Solvent isotope partitioning for porcine pepsin as measured by the incorporation of ^{18}O from $H_2{}^{18}O$ into [^{18}O]FGHF(NO_2). The line drawn through the experimental data points is derived from a fit to eq 2. The fraction of E–$H_2{}^{18}O$ trapped into product, [^{18}O]FGHF(NO_2)/E-$H_2{}^{18}O$, falls between 0.04 and 0.14 as the concentration of the oligopeptide substrate is varied between 0.4 and 3 mM. Additional details are given in the text.

was plotted on a double-reciprocal plot as shown in Figure 2. When the data were fitted to eq 2, the ratio E–$H_2{}^{18}O$/[^{18}O]FGHF(NO_2) at infinite FGHF(NO_2)FAF-OMe equals 5.0 ± 0.5. Thus, only 20% of E–$H_2{}^{18}O$ was trapped as [^{18}O]FGHF(NO_2) upon extrapolation to infinite substrate concentration. The half-maximal trapping concentration, K_B', equals 1.4 ± 0.3 mM. Evaluation of our isotope trapping data with pepsin–$H_2{}^{18}O$ as the pulse and FGHF(NO_2)FAF-OMe in $H_2{}^{16}O$ as the chase using eqs 2–4 and the steady-state parameters K_B = 0.14 mM and k_{cat} = 13.9 s^{-1} resulted in 695 $s^{-1} \geq k_2 \geq$ 139 s^{-1} and k_7 = 56 s^{-1}. The substrate water molecule is not sticky in either the binary pepsin–$H_2{}^{18}O$ or the ternary pepsin-$H_2{}^{18}O$–FGHF(NO_2)FAF-OMe complex. It desorbs from the ternary and binary complexes at rates which are, respectively, at least 4 and 10 times faster than k_{cat}. These results suggest that the kinetic mechanism of porcine pepsin is random sequential for the substrates H_2O and FGHF(NO_2)FAF-OMe. However, an alternate explanation is that the kinetic mechanism is compulsory ordered with peptide substrate binding before the reactive water molecule (EB + A in Scheme I), but this seems unlikely since the enzyme is immersed in the second substrate to bind. The rather low amount of ^{18}O incorporated into the product is also a consequence of the multiple enzymatic turnovers during the course of the isotope partitioning reaction (Table I). A more intense ^{18}O signal in the product, as expected for a single-turnover event is, therefore, diluted by the subsequent formation of product, during multiple turnovers, in a milieu in which the fraction of $H_2{}^{18}O$ has been diluted 40-fold. However, the inclusion of control reactions in the analyses eradicates any discrepancy introduced by several catalytic cycles, and as shown in Table I, measurable levels of [^{18}O]product were present after substraction of control samples.

In the case where AcRASQNYPVV-NH_2 was used as the variable substrate, no trapping of E–$H_2{}^{18}O$ into AcRASQNY was detected at any concentration of substrate, even at greater than its steady-state value of K_m. The use of this poor substrate to greatly reduce the number of turnovers in the pepsin-catalyzed peptidolysis produced no observable ^{18}O signal in the product at all the concentrations of the variable substrate examined. Therefore, the desorption of H_2O from both the E–H_2O and E–H_2O–substrate complexes occurred at an exceedingly higher rate than the k_{cat} for this oligopeptide substrate such that the first enzymatic turnover in the partitioning was actually indistinguishable from reaction conditions after isotopic dilution. Therefore, the choice of the "trapping" substrate (B) is critical to the observation of $H_2{}^{18}O$ incorporation. The concentration of B needed to trap half of EA* is estimated to be less than $2 \times K_{dis(EA)} \times 10^8$ M^{-1} $s^{-1}/(V_{max}/K_B)$, where $K_{dis} = k_{off}/k_{on}$ and k_{on} is $\sim 10^8$ M^{-1} s^{-1} when the binding of A to E is diffusion-limited (Rose, 1980). If A desorbs rapidly from EA, then we would expect a poor "trapping" substrate, that is, one in which V/K_B is much lower that the diffusion-controlled maximum value, to have an impractically large K_B' value. This is certainly true for AcRASQNYPVV-NH_2 with pepsin ($V/K_B = 10^1$ M^{-1} s^{-1}) wherein $H_2{}^{18}O$ is not trapped because it "leaks out" of the active site much faster than catalytic turnover. On the other hand, the use of a very good "trapping" substrate ($V/K_B > 10^5$ M^{-1} s^{-1}) is accompanied by the diminution of ^{18}O signal due to multiple turnovers subsequent to isotopic dilution after the initial turnover. This drawback can be minimized by the use of rapid-quench procedures.

Recent crystallographic data on porcine pepsin have identified a "structurally ordered" water molecule within hydrogen-bonding distance of both the catalytic aspartyl residues (Sielecki et al., 1990; Cooper et al., 1990; Abad-Zapatero et al., 1990). This water molecule is highly resolved and is conserved in the structures of all aspartic proteases solved to date. If this solvent molecule were very tightly bound between the two aspartyl residues, this would pose a concern regarding the ability of this active-site H_2O to undergo facile exchange with the bulk solvent, in which case a competent E–$H_2{}^{18}O$ pulse solution would not have been established in our isotope partitioning studies. Accordingly, a "$H_2{}^{18}O$-charged" enzyme solution was prepared by prereacting pepsin with AcRASQNYPVV-NH_2 in $H_2{}^{18}O$ buffer prior to its use as the pulse solution in isotope trapping experiments with FGHF(NO_2)FAF-OMe. During several catalytic cycles with the first peptide substrate, a tightly bound and unexchangeable $H_2{}^{16}O$ molecule would be replaced by solvent $H_2{}^{18}O$. If the substrate water molecule is indeed unexchangeable with bulk solvent such that a catalytic cycle is required for substitution of $H_2{}^{18}O$ for $H_2{}^{16}O$ in the active site, the subsequent solvent isotope partitioning of the "$H_2{}^{18}O$-charged" pepsin with the second peptide substrate will result in 100% trapping of ^{18}O in the peptidolytic product and a corresponding enhancement in the [$M^{18}O$] signal of the mass spectrum. However, no enhancement in the amount of [^{18}O]product was observed by using "charged" enzyme instead of "uncharged" enzyme in the isotope trapping experiments (Table I). It becomes apparent then from these results and all other partitioning data that the lytic water molecule, observed to be bound at the active site of pepsin, is not exclusively retained in the catalytic cycle and readily desorbs from the apo and substrate-bound complexes.

Solvent Isotope Partitioning on Thermolysin. In the proposed mechanism of action of thermolysin (based largely on crystallographic characterization), binding of the peptide substrate triggers the displacement of the zinc-coordinated water toward a catalytic glutamyl residue to form a penta-coordinate complex (Hangauer et al., 1984; Holden et al., 1987). This positions and activates the lytic water to attack the scissile carbonyl carbon of the substrate to form a tetrahedral intermediate. We have endeavored to examine the stickiness of this active-site water molecule in the competent enzyme–substrate complexes by solvent isotope partitioning. The approach paralleled the one used for pepsin in which a binary complex of thermolysin and $H_2{}^{18}O$ was diluted 40-fold

into a solution of $H_2{}^{16}O$ containing variable concentrations of FAGLA (1–10 mM). The reactions were terminated at 300 ms, which constituted a maximum quenching time of seven half-lives. A high ionic strength was employed in the buffer system to enhance the stability and solubility of thermolysin (Bartlett & Marlowe, 1987).

The observed fractions of ^{18}O-labeled product in the solvent isotope partitioning studies on thermolysin were found to be as much as 100-fold lower than the values expected for 100% trapping of E–$H_2{}^{18}O$ (Table I). Fitting of these data in double-reciprocal fashion did not provide an accurate determination of the isotope partitioning parameters, K'_B and P^*_{max}/EA^*. However, it is evident from the low amount of E–$H_2{}^{18}O$ trapped as FA-Gly-^{18}OH from high and low concentrations of FAGLA that the lytic water molecule is not sticky in either the binary thermolysin–$H_2{}^{18}O$ or the ternary thermolysin–$H_2{}^{18}O$–FAGLA complex.

X-ray crystallographic analysis in concert with kinetic analysis has implicated the involvement of active-site water molecules in the inhibitory behavior of certain transition-state analogs of thermolysin (Holden et al., 1987; Bartlett & Marlowe, 1987). The establishment of stable enzyme–inhibitor complexes is hitherto attributed to the displacement of water molecule(s), thereby facilitating main-chain hydrogen-bonding interactions between the inhibitor and the enzyme. It is likely that the productive binding of peptide substrate to thermolysin similarly involves displacement of solvent molecules (Kester & Matthews, 1977; Hangauer et al., 1984). The most conspicuous "ordered" solvent molecule in thermolysin is a zinc-coordinated water which is most likely to be the substrate molecule. The metal-bound solvent molecule in this metalloprotease is analogous to the "ordered" solvent molecule found proximal to the catalytic aspartyl residues of the aspartic proteases, which is also thought to be the reactant water molecule.

Our results have indicated that the putative reactant water molecules seen in the structures of both porcine pepsin and thermolysin are not exclusively retained in the catalytic cycle and readily desorb from the apo and/or the substrate-bound complexes. We attribute the rapid desorption of the reactant water molecules of these proteases to the desolvation of the active sites which accompanies the binding of the peptide substrates. In support of this, Sali et al. (1989) have demonstrated that the binding of an hexapeptide analog inhibitor to endothiapepsin results in the displacement of 23 solvent molecules, including the putative reactant water. However, seven other water molecules which are more deeply buried or extensively hydrogen-bonded are apparently retained in the enzyme–inhibitor complex in positions which are approximately equivalent to those in the uncomplexed endothiapepsin structure. Our present findings suggest that in these proteases the substrate solvent molecules, while highly ordered in the crystal structures, are subject to facile displacement and may bind to the enzymes with affinities not appreciably greater than those of other solvent molecules.

A reviewer has suggested an interesting alternate explanation for our results: failure to trap $H_2{}^{18}O$ from E–$H_2{}^{18}O$ as peptidolytic product is due to the fact that the true substrate of these proteases is a hydrated peptide, such that binding of the peptide includes binding of the coreactant water molecule. None of our present data address this possibility, but it may be possible to interrogate this proposal by reconfiguring the solvent isotope partitioning method such that the pulse solution contains peptide and $H_2{}^{18}O$ and is "chased" with enzyme in $H_2{}^{16}O$. If applied to other classes of hydrolases, such as phosphatases or the serine proteases, the present procedure of solvent isotope partitioning could prove to be a useful and general method for determining the kinetic order and "stickiness" of the water substrate in these enzymes.

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