Mass-Dependent Bond Vibrational Dynamics Influence Catalysis by HIV-1 Protease

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Materials and Methods

Materials

Isotopic compounds were purchased from Cambridge Isotope Laboratories. The fluorescent peptide (2-Aminobenzoyl-Ile-Thr-Nle-pNO_2-Phe-Gln-Arg-NH_2) was purchased from Bachem. Competent BL21(DE3) cells were purchased from Invitrogen. All other chemicals were obtained from Fisher or Sigma.

Enzyme Preparation

HIV-1 protease constructs were provided in pET11a expression vectors with the following background mutations: Q7K, L33I, L63I (for restricted autoproteolysis), and C67A and C95A (for restricted thiol oxidation). HIV-1 protease plasmids were transformed into BL21(DE3) competent cells. Natural isotope-abundant (light) enzyme was expressed in LB according to an established protocol.[1]

Heavy (^{15}N, ^{13}C, ^{2}H) HIV-1 protease was expressed in M63 media with 1.0 g/L [^{15}N, 99%] NH_4Cl, 3.0 g/L [$^{13}C_6$, 99%; 1,2,3,4,5,6,6-$^{2}H_7$, 97-98%] glucose, and [$^{2}H_2$, 99.8%] H_2O (heavy media). Growth cultures were started from glycerol stocks in 10 mL LB + 100 mg/L carbenicillin and grown at 37 °C for 5 hours. Cells from 5 mL of the starter LB culture were pelleted, the LB supernatant was discarded, and the pellet was resuspended in 1 L pre-warmed heavy media media and shaken at 200 rpm

at 37 °C for approximately 20 hours (OD reading 1.0-1.2). 2 mM IPTG was then added and the solution was shaken at 200 rpm at 37 °C for 4 hours. The enzyme was then purified from inclusion bodies according to methods previously described[1] and stored denatured in 35% acetonitrile in H_2O + 0.1 % TFA. ^{15}N HIV-1 protease was expressed and purified as described for the heavy enzyme in the M63 media with [^{15}N, 99%] NH_4Cl and natural isotope-abundance glucose and H_2O. Approximately 50 mg of enzyme was obtained per 1 L of media. Purity was confirmed by SDS-PAGE. Mass spectrometry confirmed the molecular weight for the light, ^{15}N, and heavy enzymes as 10,732 kDa, 10,865 kDa, and 11,972 kDa, respectively (Figure S1).

Prior to all enzymatic assays, light, ^{15}N, and heavy enzymes were refolded from their denatured solutions and dialyzed sequentially in 25 mM formic acid, 50 mM sodium acetate pH 5.0, followed by the appropriate reaction buffer. In order to ensure that enzymatic characteristics remained directly comparable, enzyme concentrations were kept within 0.01 mg/ml of one another as measured by a minimum of 10 absorbance readings at 280 nm using a Nanodrop spectrophotometer. If concentrations fell outside of the designated range, volumes were adjusted and the process was repeated until the concentrations were exact. After refolding, buffer exchange, and prior to every assay, the enzymes were put through the same process. When necessary, enzymes were concentrated with Centriprep centrifugal filter units (Ultracel YM-3, 3,000 MWCO).

Saturation Kinetics

Saturation curves were obtained using a Horiba FluoroMax-3 spectrofluorometer. Excitation and emission wavelengths were set to 340 nm and 420 nm, respectively, with 1 nm slit widths. Reactions were run in 50 mM MES-Tris pH 6.0, 1.25 M NaCl, and 5% DMSO at 25 °C. Peptides substrates were maintained in 100% DMSO stocks at 0.8 mM. Varying concentrations of the fluorescent peptide substrate (4.4-70 μM) were reacted with 10 nM enzyme in 600 μl reaction volumes in 0.5 cm path length quartz cuvettes. Initial rates were measured as relative fluorescence units per second (RFU s^{-1})

by taking the slope of the first 50 seconds of the reaction. Rates were converted using the response factor, which was measured to be 24,000 RFU $nmol^{-1}$. Saturation curves were obtained from a minimum of three measurements per concentration of substrate and the errors listed are standard errors of the mean. For the light, ^{15}N, and heavy enzyme, the standard error results from 17, 15, and 15 independent measurements, respectively.

Stopped-Flow Fluorescence

Pre-steady-state experiments were carried out using an Applied Photophysics SX20 stopped-flow spectrofluorometer set to measure fluorescence with a 2 mm path length. An excitation wavelength of 280 nm was applied using a Xe lamp and reactions were monitored using a 400 nm emission filter. Excitation and emission slit widths were set to 0.5 mm. Reactions were run in 50 mM MES-Tris pH 6.0, 1.25 M NaCl, and 5% DMSO. For each reaction, 60 µl of 200 µM peptide was mixed with with 60 µl of 20 µM enzyme, giving a final reaction volume of 120 µl and peptide and enzyme concentrations of 100 µM and 10 µM, respectively. The light and heavy enzyme traces each represent an average of 6 independent measurements and the reported errors represent the standard error of the mean.

Circular Dichroism (CD) Spectrometry

CD spectral analysis was performed using a Jasco J-810 spectrometer. A 0.1 cm quartz cuvette was used for each sample and measurements were made with a 50 nm/min scanning speed, 0.5 nm data pitch, 1 nm bandwidth, and four-second response time. 10 µM light and heavy enzyme was prepared as described above in a final solution of 50 mM MES-Tris pH 6.0 and 1.25 M NaCl. Three independent samples were measured for the light and heavy enzyme and each sample was scanned five times and the spectra were averaged. The data for both enzymes is presented as three independent traces of five-scan averages.

Supplementary Figures

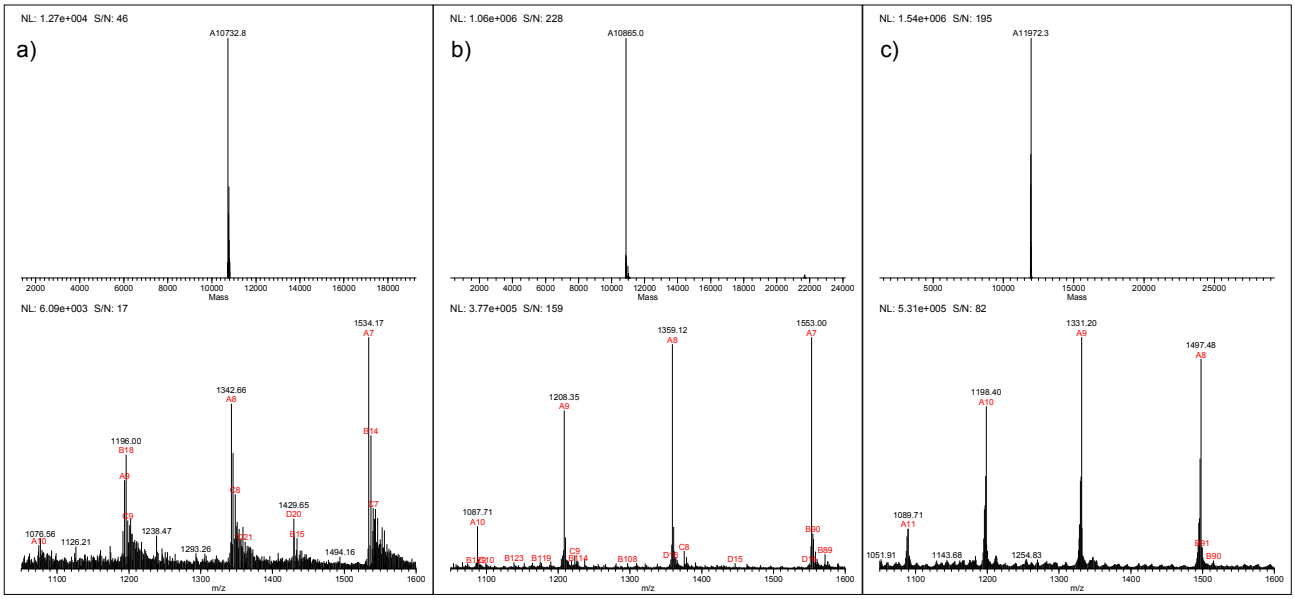

Figure S1. Mass spectra of (a) light, (b) ^{15}N, and (c) heavy HIV-1 protease.

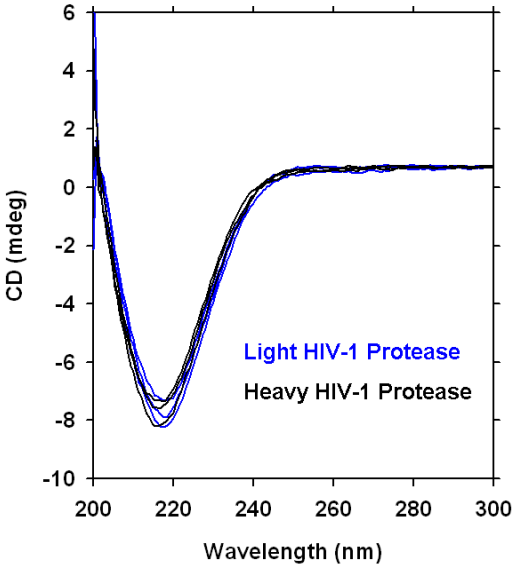

Figure S2. Heavy vs. Light CD spectra.

References

(1) Sayer, J. M.; Agniswamy, J.; Weber, I. T.; Louis, J. M. *Protein Sci* **2010**, *19*, 2055.