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Acquisition of accurate data from intramolecular quenched fluorescence protease assays

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ABSTRACT

The Intramolecular Quenched Fluorescence (IQF) protease assay utilizes peptide substrates containing donor-quencher pairs that flank the scissile bond. Following protease cleavage, the dequenched donor emission of the product is subsequently measured. Inspection of the IQF literature indicates that rigorous treatment of systematic errors in observed fluorescence arising from inner-filter absorbance (IF) and non-specific intermolecular quenching (NSQ) is incompletely performed. As substrate and product concentrations vary during the time-course of enzyme activity, iterative solution of the kinetic rate equations is, generally, required to obtain the proper time-dependent correction to the initial velocity fluorescence data. Here, we demonstrate that, if the IQF assay is performed under conditions where IF and NSQ are approximately constant during the measurement of initial velocity for a given initial substrate concentration, then a simple correction as a function of initial substrate concentration can be derived and utilized to obtain accurate initial velocity data for analysis.

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1. Introduction

Proteases are abundant, with the mammalian degradome containing approximately 2% of genome-encoded proteins [1]. Proteases are found in virtually every cell and tissue type, and conduct a remarkably broad range of biological functions. As such, proteases are of tremendous interest as both specific targets for drug discovery [2–4] as well as objects of fundamental mechanistic interest. In addition to substrate specificity, a critical aspect of the characterization of proteases is analysis of their kinetics. The data used for this analysis, typically initial velocity V_i data at single or multiple substrate concentrations, can be used to both screen chemical libraries for inhibitory lead compounds and to fit functional kinetic models. A commonly utilized assay for proteases utilizes changes in fluorescence of *in vitro* peptide substrates [5]. Typically, the peptide contains a fluorescent moiety on one side of the scissile bond, often located at or near the peptide terminus so as to have minimal effect upon substrate recognition, binding and processing. Following excitation, emission of the fluor is quenched by a moiety located on the other side of the scissile bond. While the quenching can occur by a collisional mechanism, the vast majority of Intramolecular Quenching of Fluorescence (IQF) substrates utilizes fluorescence resonant energy transfer (FRET) to an acceptor that does not emit [6] (sometimes referred to as a "dark" quencher). Cleavage of the substrate by the protease physically separates the FRET pair, dequenches the fluorescence emission, and measurement of fluorescence emission versus time yields the initial velocity V_i at that substrate concentration. The initial velocity V_i is monitored at a single substrate concentration in the presence of a compound from a chemical library, with reduction of V_i as the readout for enzyme inhibition by a putative lead compound. Alternatively, a set of V_i values as a function of substrate concentration [S] is the primary input data for fitting kinetic models, typically yielding values of the Michaelis constant K_M, maximum velocity V_{max} and Hill coefficient n.

Classically, several sources of systematic error exist in fluorescence measurements [7]. Among them is the inner-filter effect (IF), where absorbance of the sample can attenuate the intensity of incident and/or emitted light. Also, in the idealized IQF schema, quenching of a fluor in a given peptide substrate only occurs via the quencher in that uncut peptide, i.e., only intramolecular quenching occurs. Generally, this tacit assumption of zero non-specific intermolecular quenching (NSQ) is not true. Moreover, during the time-course of determination of V_i, the concentration of substrate and

Abbreviations: IQF, intramolecular quenched fluorescence; IF, inner filter absorbance; NSQ, non-specific intermolecular quenching; FRET, fluorescence resonant energy transfer.

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products is changing, so that, in principle, both the IF and NSQ effects will manifest as time-dependent corrections. A spectacular example of the effects of inadequate treatment of IF and NSQ in IQF assays is the determination, via a non-fluorescence reversed-phase HPLC assay [8], of K_M and k_{cat} values 300-fold and 12,000-fold higher, respectively, than those observed via an IQF assay [9] for a bacterial sortase transpeptidase. The importance of correcting for IF and NSQ artifacts is generally recognized, with a 2007 paper describing five different categories of correction methods [10]. Also, this paper states, correctly, that fitting and solution of the kinetic rate equations enables one to iteratively determine the time- and concentration-dependent IF and NSQ corrections [10]. This iterative approach is the rigorous and correct approach, and a deficiency of many published correction approaches is that IF and NSQ are determined for a small set of substrate [S] and product [P] concentrations compared to those of the entire assay, with statistically-unsupportable inferences and extrapolations used for global corrections.

Conceptually, enzyme characterization is a two-step process consisting of acquisition of initial velocity V_i data, followed by theoretical analysis of this V_i data. In this work, we demonstrate that, under suitable assay conditions, the IF and NSQ corrections to IQF data are product-independent (i.e., time-independent) and can be experimentally determined and parameterized as a simple function of initial substrate concentration [S]. The approach to acquisition of accurate IQF data that we describe is based upon fundamental considerations of fluorescence and fluorescence quenching, and has nothing to do with the "downstream" theoretical enzymological analysis. We develop and present no new kinetic or other functional models for enzyme activity; rather, we develop and present a heuristic approach to obtaining the most accurate V_i data for whatever "downstream" analysis is pursued for the specific IQF application at-hand. Our approach permits IF and NSQ corrections without requirement of iterative solution of rate equations, enables a more consistent and facile measurement of accurate IQF data, and thus, yields both improved kinetic models and more robust results of compound library screening. As an example, we develop and apply these corrections to kinetic analysis of the integral membrane protein protease Ste24p.

Ste24p is a zinc metalloprotease initially discovered and characterized via yeast genetics by its role in mating and maturation of the **a**-factor mating pheromone [11,12]. Human Ste24p (ZMPSTE24) processes prelamin A, a component of the nuclear lamina; mutations in ZMPSTE24 that diminish its activity give rise to genetic diseases of accelerated aging (progerias) [13,14]. Additionally, lipodystrophy, acquired from the standard highly active antiretroviral therapy (HAART) used to treat AIDS patients, likely results from off-target interactions of HIV (aspartyl) protease inhibitor drugs with ZMPSTE24 [15]. The structure of Ste24p possesses a novel membrane protein fold, consisting of seven long kinked transmembrane helices that surround a membrane-bound ~14,000 $Å^3$ "reaction chamber" containing the zinc metalloprotease active site [16,17], and we have proposed a "processive processing" model for its function [16]. Ste24p cleaves the farnesylated "CaaX" motif (CaaX = cysteine-aliphatic-aliphatic-any residue) between the isoprenylated cysteine and the adjacent aliphatic residue [11]. Ste24p has generally been consigned to be a "CaaX protease" component of a multi-enzyme pathway mediating isoprenylation, proteolytic processing, and methylation of protein substrates for increased membrane-binding affinity [18–20]. However, a recent series of papers provides significant evidence for broader biological function. Ste24p is a key factor in several endoplasmic reticulum (ER) processes, including the unfolded protein response, a cellular stress response of the ER [21], and removal of misfolded proteins from the translocon [22]. Also, substrate prenylation has been demonstrated to be dispensable for cleavage by Ste24p [23].

2. Materials and methods

2.1. Peptides and other reagents

The peptides Abz-KSKTKC(farnesyl)VIK-Dnp, Abz-KSKTKC(farnesyl) and VIK-Dnp were purchased as lyophilized powder at 98+% purity from AnaSpec (Fremont, CA). Abz, *ortho*-aminobenzoic acid, is located at the N-terminus of Abz-KSKTKC(farnesyl)VIK-Dnp. Dnp, 2,4-dinitrophenol, located in the modified sidechain of ε-DNP-lysine at the C-terminus of Abz-KSKTKC(farnesyl)VIK-Dnp and VIK-Dnp, is a non-emitting FRET acceptor, i.e., quencher, for Abz fluorescence. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium chloride (NaCl), β-mercaptoethanol (BME), glycerol and dimethylsulfoxide (DMSO) were purchased from Fisher Scientific. Magnesium chloride and phenylmethylsulfonylfluoride (PMSF) were from Sigma. Leupeptin was purchased from Roche Diagnostics.

2.2. Subcloning and mutagenesis

The human *ste24* (*zmpste24*) gene (HsCD00075979) was obtained from the DNASU Plasmid Repository (Arizona State University, Tempe, AZ). The *zmpste24* gene was amplified by polymerase chain reaction and inserted, using the In-Fusion HD Cloning kit (Clontech Laboratories, Mountain View, CA), into the yeast (*S. cerevisiae*) expression vector pSPG47. pSPG47 utilizes an *ADH2* promoter, with protein expression induced when glucose is depleted from the medium [24]. Because future spectroscopic experiments, beyond the scope of this paper, will utilize specific labeling of inserted cysteines, the resultant plasmid pSPG47-zmpste24 was then used as template to construct a cysteine-free variant, pSPG47-zmpste24Δcys. Mutagenesis was carried out using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA), with some modification of the provided protocol by adjustment of reaction component concentrations and PCR cycling parameters. Following template DNA digestion by DpnI (Agilent Technologies, Santa Clara, CA) and heat-shock transformation into XL10 Gold UltraCompetent Cells (Agilent Technologies, Santa Clara, CA), individual colonies were isolated and sequenced (Genewiz, South Plainfield, New Jersey) for verification of mutation.

2.3. ZMPSTE24Δcys expression

Plasmid pSPG47-zmpste24Δcys was transformed into *S. cerevisiae* strain BJ5460 [25] (ATCC 208285) (*MAT***a** *ura3-52 trp1 lys2-801 leu2-Δ1 his3-Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL*+), by the lithium acetate-PEG method [26]. Fifty mL of starter yeast cultures in sterile CSM-ura medium (0.77 g/L Complete Supplement Mixture, MP Biochemicals, LLC, Solon, OH; 1.7 g/L yeast nitrogen base, Thomas Scientific, Swedesboro, NJ; 5 g/L ammonium sulfate, Fisher Scientific, Fair Lawn, NJ; 20 g/L dextrose, Fisher Chemical, Fair Lawn, NJ) were incubated for 18 h at 30 °C with 220 rpm shaking (OD ~ 1.8). Ten mL of yeast starter cultures were then transferred into a 2.8 L flask containing 1 L of sterile CSM-ura medium, and the yeast cultures were allowed to grow at 30 °C (220 rpm shaking) for ~24 h, corresponding to a cell density absorbance A_{600} nm value of 1.4. Cells were pelleted (3500 × g) for 15 min and were subsequently resuspended in 100 mL of sterile YPD medium (10 g/L yeast extract, Fisher Chemical, Fair Lawn, NJ; 20 g peptone/L Becton, Dickinson and Company, Sparks, MD; 20 g/L dextrose, Fisher Chemical, Fair Lawn, NJ). The resuspended pellet was then transferred into a sterile 2.8 L flask containing 900 mL of

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