Published in final edited form as:

Anal Biochem. 2017 April 01; 522: 30–36. doi:10.1016/j.ab.2017.01.020.

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.

Keywords

Fluorescence quenching; enzyme kinetics; proteases; enzyme assays; membrane proteins; Ste24p/ZMPSTE24

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

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Contributions

M.C.W. conceived the study. B.T.A. performed the experiments. B.T.A. and M.C.W. analyzed the data. B.T.A. and M.C.W. made the figures and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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 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 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 $\sim 14,000 \text{ \AA}^3$ “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-*zmpste24* cys. Mutagenesis was carried out using 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 ZMPSTE24 cys expression

Plasmid pSPG47-*zmpste24* cys was transformed into *S. cerevisiae* strain BJ5460 [25] (ATCC 208285) (*MATa ura3-52 trp1 lys2-801 leu2- 1 his3- 200 pep4::HIS3 prb1- 1.6R 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 hours 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 1L of sterile CSM-ura medium, and the yeast cultures were allowed to grow at 30 °C, 220 rpm shaking for ~ 24 hours, corresponding to a cell density absorbance $A_{600\text{ nm}}$ value of 1.4. Cells were pelleted ($3,500 \times g$) for 15 minutes 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 YPD. Cultures in YPD medium were grown at 26 °C (220 rpm shaking) for an additional 24 hours, after which cells were harvested by centrifugation ($3500 \times g$), flash frozen in liquid nitrogen and stored at -80 °C until use. The control strain, plasmid-free BJ5460, was grown for 48 hours in YPD medium at 26 °C in a fermenter (12L) with 400 rpm agitation. BJ5460 cells were harvested by centrifugation ($3,500 \times g$), flash frozen in liquid nitrogen as small individual “beads,” and stored at -80 °C.

2.4 Membrane preparation

Thirty grams of BJ5460 cells, with or without overexpressed ZMPSTE24 cys, were thawed on ice and resuspended in Membrane Buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 15% w/v glycerol, 100 μ M PMSF, 2 mM β -mercaptoethanol, 2.5 μ g/mL leupeptin), using a hand-held glass homogenizer. Cells were then lysed by three passes at 26,000 psi through a microfluidizer (MP-110 Microfluidics, Westwood, MA), with the outlet coil immersed in an ice bath. Lysate was centrifuged at $3,500 \times g$ for 30 minutes at 4 °C. The resulting supernatant from this step was spun at $205,000 \times g$ for 90 minutes at 4 °C to isolate membranes. The pelleted membranes from this high speed spin was resuspended in Membrane Buffer in a mass to volume ratio of 1 gram to 6 mL, using a hand held glass homogenizer. Resuspended membranes were divided into 1 mL aliquots, flash frozen in liquid nitrogen and stored at -80 °C until use for assays.

2.5 Preparation of peptide stocks

Stock solutions of the peptide substrate and products were prepared by adaptations of published methods [23, 27]. For the substrate Abz-KSKTKC(farnesyl)VIK-Dnp, 26 μ L of 100% DMSO was added to a vial containing 1 mg peptide, followed by gradual addition of 974 μ L sterile 0.2 μ M filtered deionized water in 50 μ L increments to yield 1 mL of 1 mg/mL peptide stock in 2.6% v/v DMSO. For the product Abz-KSKTKC(farnesyl), 13 μ L of 100% DMSO was added to 0.5 mg of peptide, followed by gradual addition of 487 μ L of sterile deionized water to yield 0.5 mL of 1 mg/mL peptide stock in 2.6% v/v DMSO. For the product VIK-Dnp, which is more hydrophobic than Abz-KSKTKC(farnesyl)VIK-Dnp and Abz-KSKTKC(farnesyl), 25 μ L of 100% DMSO was added to 0.25 mg of peptide, followed by gradual addition of 475 μ L of sterile deionized water to yield 0.5 mL of 0.5 mg/mL peptide stock in 5.0% v/v DMSO. Peptides were sonicated for 3 minutes in a water bath (~ 25 °C, Solid State Ultrasonic FS-9, Fisher Scientific, Birmingham, AL), maintained on ice and used in assays within two hours of preparation. The molar concentrations of Abz-KSKTKC(farnesyl)VIK-Dnp and VIK-Dnp were determined by measurement of $A_{360\text{nm}}$ and use of a molar extinction coefficient value of $\epsilon = 17,530 \text{ M}^{-1}\text{cm}^{-1}$ for ϵ -DNP-lysine [28]. The molar concentration of Abz-KSKTKC(farnesyl) was determined by measurement of $A_{320\text{nm}}$ and use of a molar extinction coefficient value of $\epsilon = 21,870 \text{ M}^{-1}\text{cm}^{-1}$ for Abz [29].

2.6 Fluorescence measurements

All data were collected at 25 °C. Peptide solutions and membrane suspensions were maintained at 4 °C, prior to addition to Assay Buffer (100 mM HEPES pH 7.5, 5 mM MgCl_2), which had been pre-warmed to 25 °C. Samples used for fluorescence measurement consisted of 0.65 mg/mL membranes in Assay Buffer, with variable peptide concentrations. Total sample volume was 200 μ L. Measurements were made in triplicate from 96-well flat bottom fluorescence microplates (Cat. #7605, Thermo Scientific, Rochester, NY), using a plate-reader (SpectraMaxM5, Molecular Devices, Sunnyvale, CA). Excitation and emission wavelengths were 320 nm and 420 nm, respectively.

2.7 Determination of $\alpha([S], C_o)$

Samples were prepared by combination of substrate and product peptides with BJ5460 membranes lacking over-expressed ZMPSTE24 cys. After one minute equilibration in the dark, fluorescence was measured at $t = 180$ s. The correction factor $\alpha([S], C_o)$ is the ratio of fluorescence of samples identical in composition except for the presence or absence of substrate. Error-weighted linear fits to $\alpha([S], C_o)$ vs. $[P]$ and $\alpha([S], C_o)$ vs $[S]$ were performed with Prism (GraphPad Software, Inc., La Jolla, CA).

2.8 IQF enzyme kinetics assay of ZMPSTE24 cys

Samples were prepared by combination of substrate peptide with BJ5460 membranes containing over-expressed ZMPSTE24 cys. After one minute equilibration in the dark, fluorescence was measured at ten second intervals for a duration of $t = 180$ s. Following application of the $\alpha([S], C_o)$ correction, initial velocities V_i were determined by linear fits to the fluorescence vs. time data. We observed that control BJ5460 membranes exhibit low-level residual activity with the Abz-KSKTKC(farnesyl)VIK-Dnp substrate peptide, likely due to the presence of chromosomally encoded *S. cerevisiae* Ste24p and Rce1p. Therefore, initial velocities V_i were determined for these membranes at the substrate concentrations utilized for the assay, and subtracted from ZMPSTE24 cys V_i values prior to analysis of kinetics. Error-weighted fits of Michaelis-Menten kinetic models to the V_i vs. $[S]$ data were performed with Prism (GraphPad Software, Inc., La Jolla, CA). A product calibration curve, enabling placement of V_i upon an absolute scale, was generated by fluorescence measurement of equimolar concentrations (0.00–5.00 μ M) of the product peptides in Assay Buffer containing BJ5460 membranes at the same concentration as that used in the IQF assay.

3. Results and Discussions

3.1 Selection of protease assay conditions

Proteolytic activity of yeast membranes containing overexpressed ZMPSTE24 cys (cysteine-free human Ste24p, with the five nonconserved cysteines mutated to serine) was characterized with a modified version of existing IQF assays previously utilized for several yeast orthologs of Ste24p [23, 30]. The peptide substrate utilized, Abz-KSKTKC(farnesyl)VIK-Dnp, is cleaved by ZMPSTE24 to yield Abz-KSKTKC(farnesyl) and VIK-Dnp products [23]. To determine the membrane concentration to be used for the assay, a series of two-fold dilutions of ZMPSTE24 cys enriched membranes were examined in the presence of 1, 10 and 40 μ M of Abz-KSKTKC(farnesyl)VIK-Dnp. These concentrations are approximately 0.1x, 1x and 4x multiples of published K_M values for this substrate in several yeast orthologs of Ste24p: 16.4 ± 1.0 μ M (*S. cerevisiae* Ste24p in yeast membranes) [23] and 11 ± 2 μ M (purified *S. mikatae* Ste24p in detergent) [16].

Selection of an appropriate membrane concentration balances two competing factors: maximization of fluorescence signal by having more enzyme present, and minimization of sample absorbance by having less membrane present. A membrane concentration of 0.65 mg/mL (total membrane mass per volume) was determined to be a suitable concentration. Selection of an appropriate time interval t for measurement of initial velocities V_i also

balances two competing factors: maximization of t to increase fluorescence signal, and minimization of t to accurately measure initial velocity. Examination of straight-line fits over a range of time intervals was used to determine t for the assay (Fig. 1a). Longer t values yield higher amounts of product, with higher fluorescence signal and improved signal to noise (S/N). However, if t is longer than the “initial velocity regime,” the slope of the fluorescence vs. time curve (i.e., V_i) will be systematically lower than the slope at shorter t . A time interval of $t = 180$ seconds was determined to be a suitable value (Fig. 1b). The fluorescence of equimolar concentrations of the cleavage products, Abz-KSKTKC(farnesyl) and VIK-Dnp, was utilized to generate an absolute scale for the assay (Fig. 2). During measurement of V_i , ~ 0.2 to 4.2% product is generated.

3.2 Determination of $\alpha([S], C_o)$

Conceptually, an IQF sample contains components with time-dependent concentrations (substrate $[S(t)]$, product $[P(t)]$) and other components, such as buffer, with time-independent concentration C_o . The initial substrate and product concentrations, at $t = 0$, are $[S(t=0)] = [S]_i$ and $[P(t=0)] = [P]_i = 0$, respectively. The final substrate and product concentrations, at $t = t$, are $[S(t=t)] = [S]_f = [S]_i - [P]_f$ and $[P(t=t)] = [P]_f$, respectively. If $[S]_i \gg [P]_f$, then $[S]_f \sim [S]_i = [S]$. If measurement of initial velocity V_i can be performed under the above conditions and, critically, if the IF and NSQ effects do not vary significantly over the range of product concentration $[P(t)]$ generated during the t collection period, then the correction factor α is a simple time-independent function of only the substrate concentration $[S]$, i.e., the fundamental form of $\alpha([S(t)], [P(t)], C_o)$ is well-approximated by $\alpha([S], C_o)$. Experimentally, then, what is $\alpha([S], C_o)$? Consider a mixture comprising product at concentration $[P]$ and other components at concentration C_o , in the absence of substrate ($[S] = 0$); this mixture will emit fluorescence of intensity $F([S] = 0, C_o)$. The addition of substrate at a concentration $[S]$ to this mixture will reduce the observed fluorescence, $F([S] > 0, C_o)$, due to IF and NSQ arising from the presence of substrate. Therefore, at a given substrate concentration $[S]$, the fluorescence correction factor $\alpha([S], C_o)$ is simply the ratio of $F([S] = 0, C_o)/F([S] > 0, C_o)$. To confirm this theoretical treatment, $\alpha([S], C_o)$ vs. $[P]$, in the absence of ZMPSTE24 cys, was determined for individual substrate $[S]$ concentrations ranging from 0.625 to 80.0 μM , with product $[P]$ concentrations varying from 0.250 to 5.00 μM for each invariant substrate concentration (Fig. 3a–h). For seven of the eight substrate concentrations (all but $[S] = 40.0 \mu\text{M}$), the correction factor shows no statistically-significant dependence upon product concentration $[P]$; thus, under the conditions of the assay, there is no product dependence of the correction factor, i.e., $\alpha([S(t)], [P(t)], C_o) = \alpha([S], C_o)$. Below 15.0 μM substrate concentration, $\alpha([S], C_o)$ is approximately constant (1.118 ± 0.011) and then increases linearly to a maximum value of 1.547 ± 0.019 at $[S] = 80.0 \mu\text{M}$ (Fig. 3i). Thus, under the conditions of these experiments, the systematic reduction of observed fluorescence due to inner filter effect (IF) and non-specific intermolecular quenching (NSQ) ranges from $\sim 12\%$ to 55%.

3.3 IQF enzyme kinetics of ZMPSTE24 cys

ZMPSTE24 cys was expressed in yeast (*S. cerevisiae*), and isolated yeast membranes enriched in ZMPSTE24 cys were subjected to IQF assays. The uncorrected and corrected fluorescence vs. time data for substrate concentrations of 0.00 to 80.0 μM , from which initial

velocities V_i are obtained, illustrate the effect of $\alpha([S], C_o)$ upon individual data points in the time series (Fig. 4a,b). The uncorrected and corrected initial velocity V_i vs. substrate concentration $[S]$ data indicate the systematic error in measured initial velocity V_i , which increases linearly with substrate concentration $[S]$ (Fig. 4c, Table 1). Fits to Michaelis-Menten kinetics, $V_i([S]) = V_{\max} * ([S]/(K_M + [S]))$, yield K_M values of $2.25 \pm 0.46 \mu\text{M}$ (uncorrected) and $3.46 \pm 0.22 \mu\text{M}$ (corrected), a difference of 35%, and $V_{\max}$ values of $0.177 \pm 0.008 \text{ nM/s}$ (uncorrected) and $0.238 \pm 0.004 \text{ nM/s}$ (corrected), a difference of 26%. The improved quality of the fit, and concomitantly increased confidence in the accuracy of the specific kinetic model, is also seen in the smaller uncertainties of K_M and $V_{\max}$, as well as in the improved R^2 (0.9612 uncorrected vs. 0.9970 corrected).

We have developed a straightforward approach for obtaining accurate Intramolecular Quenched Fluorescence (IQF) data that does not require iterative solution of kinetic rate equations for proper inner-filter absorbance (IF) and non-specific intermolecular quenching (NSQ) corrections. Rather, early-stage assay design and characterization is utilized to determine the time interval Δt for accurate initial velocity V_i measurement, followed by determination of the range of product concentration $[P]$ in which the IF and NSQ corrections are invariant with respect to substrate concentration $[S]$. If good signal-to-noise (S/N) data can be obtained, during a suitable time interval Δt in which an appropriate product concentration $[P] \ll [S]$ is generated, then a correction factor $\alpha([S], C_o)$ can be readily determined. For assays in which these conditions are not met, use of an alternative instrument more sensitive to low product concentration may be the remedy. Alternatively, redesign of the IQF substrate to reduce IF and/or NSQ may be warranted.

Acknowledgments

We thank Walter K. Schmidt (University of Georgia) for guidance on technical aspects of this study and comments on the manuscript, Brandon Goblirsch (University of Virginia) for critical discussion and reading of the manuscript prior to submission, Daniel Councill (University of Virginia) for preparation of the ZMPSTE24 cys expression construct, and Christopher Stroupe (University of Virginia) for use of his plate reader.

Funding

This work was supported by the National Institutes of Health [R01GM108612].

Abbreviations

IQF	intramolecular quenched fluorescence
IF	inner filter absorbance
NSQ	non-specific intermolecular quenching
FRET	fluorescence resonant energy transfer

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Figure 1.

Determination of optimal time interval Δt for determination of initial velocity V_i . (a) Fluorescence (RFU, Relative Fluorescence Units) vs. time for $[S] = 0.00$ to $80.0 \mu\text{M}$ (as indicated by symbols). (b) Initial velocity V_i vs. time interval Δt for $[S] = 0.00$ to $80.0 \mu\text{M}$ (as indicated by symbols), indicating $\Delta t = 180$ s as an appropriate time interval for these particular IQF experiments.

Figure 2. Product calibration curve. Equimolar concentrations of the reaction products Abz-KSKTKC and VIK-Dnp were mixed with 0.65 mg/mL membranes from the yeast expression strain BJ5460 in standard assay conditions. Fluorescence intensity was measured using a SpectraMax M5 microplate reader at excitation and emission wavelengths of 320 nm and 420 nm, respectively. Each data point is in triplicate. $RFU = (1459 \pm 11 \text{ RFU}/\mu\text{M}) * [\text{Product}]$.

Figure 3.

Determination of intramolecular quenched fluorescence (IQF) correction factor $\alpha([S], C_0)$.

(a–h) $\alpha([S], C_0)$ vs. product concentration $[P]$ for indicated substrate concentrations $[S]$.

Each point is an average of three determinations of $\alpha([S], C_0)$ for the indicated value of $[P]$, with estimated errors propagated from experimental uncertainties. Solid lines indicate error-weighted linear fits. Dashed lines represent the slope of each line $\pm$ twice its error, depicting, effectively, the range of slopes consistent with the data. (i) Correction factor $\alpha([S], C_0)$ vs. $[P]$. $\alpha([S], C_0) = 1.118 \pm 0.011$ for $[S] \leq 15.0$ μM; $\alpha([S], C_0) = (1.118 \pm 0.011) + ([S] - 15.0) \times (6.593 \pm 0.855) \times 10^{-3}$ for $[S] > 15.0$ μM.

Figure 4.

Effect of correction factor $\alpha([S], C_0)$ on IQF data from ZMPSTE24p cys with substrate Abz-KSKTKC(farnesyl)VIK-Dnp. **(a)** Uncorrected fluorescence (RFU, Relative Fluorescence Units) vs. time for [S] = 0.00 to 80.0 μ M (as indicated by symbols). **(b)** Corrected fluorescence (RFU, Relative Fluorescence Units) vs. time for [S] = 0.00 to 80.0 μ M (as indicated by symbols). **(c)** Comparison of initial velocity V_i vs. [S] for uncorrected

(closed symbols) vs. corrected (open symbols) IQF data. V_i were obtained by linear fits to the data of (a) and (b), using the time interval $\Delta t = 180$ s.

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Table 1Effect of $\alpha([S])$ IQF correction upon initial velocity V_i data of ZMPSTE24 cys

S (μ M)	Uncorrected V_i (nM/s)	Corrected V_i (nM/s)	% Reduction
0.625	0.0281 ± 0.0038	0.0314 ± 0.0042	12
1.25	0.0572 ± 0.0015	0.0640 ± 0.0016	12
2.5	0.0896 ± 0.0067	0.1001 ± 0.0075	12
5	0.1275 ± 0.0070	0.1425 ± 0.0078	12
10	0.1615 ± 0.0087	0.1805 ± 0.0097	12
20	0.1719 ± 0.0095	0.1978 ± 0.0109	15
40	0.1653 ± 0.0082	0.2116 ± 0.0105	28
80	0.1517 ± 0.0054	0.2351 ± 0.0083	55