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Stoichiometry and Absolute Quantification of Proteins with Mass Spectrometry Using Fluorescent and Isotope-labeled Concatenated Peptide Standards*[S]

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We have explored a general approach for the determination of absolute amounts and the relative stoichiometry of proteins in a mixture using fluorescence and mass spectrometry. We engineered a gene to express green fluorescent protein (GFP) with a synthetic fusion protein (GAB-GFP) in *Escherichia coli* to function as a spectroscopic standard for the quantification of an analogous stable isotope-labeled, non-fluorescent fusion protein (GAB*) and for the quantification and stoichiometric analysis of purified transducin, a heterotrimeric G-protein complex. Both GAB-GFP and GAB* contain concatenated sequences of specific proteotypic peptides that are derived from the α, β, and γ protein subunits of transducin and that are each flanked by spacer regions that maintain the native proteolytic properties for these peptide fragments. Spectroscopic quantification of GAB-GFP provided a molar scale for mass spectrometric ratios from tryptic peptides of GAB* and defined molar responses for mass spectrometric signal intensities from a purified transducin complex. The stoichiometry of transducin subunits α, β, and γ was measured to be 1:1.1:1.15 over a 5-fold range of labeled internal standard with a relative standard deviation of 9%. Fusing a unique genetically coded spectroscopic signal element with concatenated proteotypic peptides provides a powerful method to accurately quantify and determine the relative stoichiometry of multiple proteins present in complexes or mixtures that cannot be readily assessed using classical gravimetric, enzymatic, or antibody-based technologies. ***Molecular & Cellular Proteomics 7:442–447, 2008.***

We describe a general method to determine the stoichiometry and absolute quantification of proteins, a method that substantively extends previously developed techniques. Many proteins assemble to form protein complexes to fulfill unique functional roles. Protein assemblies provide a functional diversity that can contextually change with time and space during a cellular lifecycle. Many of these complexes have been dissected and catalogued in different organisms using the combination of tandem affinity purification followed by identification of individual members by mass spectrometry (1, 2). Comprehensive identification of individual members in protein assemblies is now performed routinely using mass spectrometry (3, 4). However, the determination of stoichiometry and quantification of individual proteins in complexes or mixtures usually requires the use of analytical ultracentrifugation.

Several techniques for protein quantification using mass spectrometry depend on quantification of peptides generated during their proteolytic digestion. Chemical labeling of peptides after digestion (5) or metabolic labeling of growing cells in the presence of labeled substrate are useful for relative or comparative analyses of different sample groups (6). It has been proposed that the absolute quantification of proteins in complex protein mixtures can be accomplished by isotope dilution using synthetic labeled internal standards that are chemically identical to the proteotypic peptides generated by proteolysis of proteins of interest (7). Chemical synthesis of labeled peptides has inherent limitations that may restrict proteotypic peptide selection. Beynon *et al.* (8) circumvented these by creating a synthetic gene to code for a protein expressing a concatenated series of proteotypic peptides within a synthetic standard fusion protein. The authors quantified seven proteins in chick skeletal muscle tissue using this approach. Kito *et al.* (9) refined this approach by incorporating flanking sequences into the fusion protein to account for sequence difference effects on proteolytic efficiency and demonstrated their method by determining the stoichiometry of elongation factor eIF2B-eIF2 complex in yeast *Saccharomyces cerevisiae*.

In this work, we introduce an absolute quantification element to the peptide fusion standard enabling both absolute quantification and stoichiometric analysis of proteins. We designed a gene coding for green fluorescent protein fused to a concatenated series of proteotypic peptides. A fluorescent fusion protein embodies a unique property for its quantifica-

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tion and provides either unlabeled or labeled proteotypic peptides for the determination of the stoichiometry of individual components in multiprotein mixtures. We chose to demonstrate this method by the analysis of transducin, a heterotrimeric G-protein that is activated upon absorption of light by rhodopsin in the rod photoreceptor cell. The transducin complex is membrane-associated through fatty acyl and prenyl groups on α and γ subunits, respectively. Stringent conditions for isolation of transducin have been developed (10) that result in a purified protein complex with the stoichiometry of 1:1:1 for the α, β, and γ chains (11). Transducin provides a challenging test of a method for absolute quantification of proteins because it is a proteolytically resistant trimer. We successfully quantified a stable isotope-labeled fusion protein and purified transducin and confirmed earlier observations that α, β, and γ chains are in 1:1:1 ratio in transducin complex similar to other heterotrimeric G-protein complexes using both the external and internal stable isotope standard methods (12).

MATERIALS AND METHODS

Design, Expression, and Purification of Fusion Protein Standard—A synthetic gene encoding 67 amino acids composed of the sequence PVINIEDLTEKDKLKMELSDLERLVTPGYVPTEQDVLRSRVKTTASQDGKLIIWDSYTTNKVHAIPL was constructed by synthetic overlap PCR (12). The underlined segments represent the signature proteotypic peptides of γ, α, and β subunits of transducin (GAB)[1], respectively. The synthetic 67-mer GAB was cloned into expression vector pEXP-5CT/TOPO (Invitrogen) in-frame to C-terminal His_6 tag. For an external standard, synthetic GAB and GFP genes (GI:155662) were fused by standard overlap PCR such that GAB is fused to the N-terminal end of GFP. The GAB-GFP gene was cloned in-frame to C-terminal Strp2 tag (WSDPQFEK) in the expression vector.

Stable isotope-labeled GAB* was expressed using an *in vitro* translation kit (Invitrogen) according to the manufacturer's protocol. Unlabeled arginine and lysine in the amino acid module from the kit were replaced by L-[$^{13}C_6$,$^{15}N_2$]lysine and L-[$^{13}C_6$,$^{15}N_4$]arginine (>95% purity, Spectral Stable Isotopes). After *in vitro* translation, the product labeled fusion proteins were purified by nickel-nitrilotriacetic acid resin in batch mode (Qiagen, Valencia, CA). Finally the purified GAB* was filtered through ultracentrifugal spin filters (7500 × *g*, 30,000 molecular weight cutoff, Amicon Ultra, Millipore, Billerica, MA) to remove high molecular weight contaminants. The supernatant from ultrafiltration consisted of 99% pure GAB* fusion protein as analyzed by silver-stained SDS-PAGE gel and MALDI (supplemental Fig. 1, *A* and *B*).

The expression of unlabeled GAB-GFP fusion protein standard was under control of T7 promoter and was expressed in *Escherichia coli* Rossetta DE3 strain (Novagen, San Diego, CA). Cells expressing the GAB fusion were grown at 16 °C in Luria-Bertani medium containing ampicillin (0.1 mg/ml) to OD of 0.3 at 600 nm and induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside for 45 min. The cell pellet was resuspended in 100 mM Tris, 150 mM NaCl, 1 mM EDTA at pH 8.0 and sonicated at 4 °C. The cell extract was centrifuged at 4 °C for 20 min to remove insoluble cell debris. The clear supernatant was loaded onto a Strep2 column (IBA, Gottingen, Germany). The column was washed with 3 column volumes of the wash buffer (100 mM Tris, 300 mM NaCl, pH 8.0) followed by 2 column volumes of wash buffer with 1 M NaCl and a final wash of 2 column volumes of wash buffer. The recombinant protein was eluted with 2.5 mM desthiobiotin in wash buffer. Silver-stained SDS-PAGE of the eluent revealed two bands, GAB-GFP and BCCP_ECOLI (Swiss-Prot accession number P0ABD8, identified by LC/MS/MS, data not shown) (supplemental Fig. 1C). Western blot with anti-GFP antibody (supplemental Fig. 1*D*) revealed a single component indicating that the fluorescent protein is of suitable purity for an external standard. The aliquots of purified protein standard in elution buffer were stored at −80 °C until further use.

Quantification of Fusion Protein Standard—The external fusion protein standard GAB-GFP was quantified by measuring the intrinsic fluorescence of GFP (TD700 fluorometer, Turner Biosystems, Sunnyvale, CA). The excitation and emission wavelengths were set to 395 and 509 nm, respectively.

Proteolytic Digestion of Transducin and Fusion Protein Standards—Bovine transducin at a concentration of 0.77 mg/ml was a kind gift from Prof. Daniel Oprian at Brandeis University. Typically a 1-μl aliquot of transducin was mixed with a known amount of labeled GAB* and denatured in 8 M urea for 45 min at 22 °C (final volume, 10 μl). Following denaturation, transducin was reduced by adding 1 μl of 1 M DTT. The reduced sulfhydryl moieties were carbamidomethylated by the addition of 1 μl of 100 mM iodoacetamide and incubated for 15 min in the dark at 22 °C. The sample was diluted to 1 M urea by addition of 100 mM Tris HCl, pH 8.0, and incubated at 37 °C for 16 h with trypsin (Promega, Madison, WI) at a 1:1 substrate:enzyme ratio (w/w). After trypsin digestion, samples were further digested by endoproteinase Lys-C (Roche Applied Science) at 1:1 substrate:enzyme ratio (w/w) for 12 h at 37 °C. The digested samples were desalted using an UltraMicroSpin™ reverse phase column (The Nest Group, Inc., Southboro, MA) according to the manufacturer's protocol. Experiments using GAB-GFP as an external standard were conducted as above in parallel with transducin. The aliquots of desalted digested samples were snap frozen and stored at −80 °C until further use.

Mass Spectrometry—An ion trap instrument (LCQ Classic, Thermo Electron, San Jose, CA) was used to obtain primary MS data for quantification and MS/MS data for qualitative peptide verification. The LC system was equipped with a peptide microtrap (Michrom BioResource) and reverse phase nanocolumn (PicoFrit BioBasic C_{18} column with 75-μm inner diameter and 15-μm tip, New Objective, Woburn, MA). Peptides were separated on reverse phase columns using gradient elution from 5% acetonitrile, 0.1% formic acid, water to 80% acetonitrile, 0.1% formic acid, water using a linear gradient from 10 to 60% for 30 min and then from 60 to 80% in 5 min. The column flow rate was 200 nl/min, and peptides were eluted directly into the mass spectrometer with applied voltage of 2.5 kV. The capillary voltage and temperature were 45 V and 150 °C, respectively. For quantification experiments, the mass spectrometer was run in MS mode only. The quantification of each peptide was determined by measurement of peak areas in extracted ion chromatograms with a mass window of ±0.3 Da. Extracted peaks were smoothed using a Gaussian filter and Genesis peak algorithm with Xcalibur® software (Thermo Electron).

RESULTS

Selection of Signature Peptides and Design of Fusion Proteins—Transducin is composed of α (GNAT1_BOVIN, Swiss-Prot accession number P04695), β (GBB1_BOVIN, Swiss-Prot accession number P62871), and γ (GBG1_BOVIN, Swiss-Prot accession number P02698) subunits having molecular masses of 40, 37, and 8 kDa, respectively (10). For identification of the proteotypic signature peptides, purified transducin was digested by trypsin followed by the sequencing of pep-

[1] The abbreviations used are: GAB, signature proteotypic peptides of γ, α, and β subunits of transducin; GFP, green fluorescent protein.

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TABLE I
Signature peptides for α, β, and γ subunits
Flanking amino acids from the native protein included in the fusion GAB* and GAB-GFP are shown in parentheses. aa, amino acids.

	Sequence (aa)	Mass	*m/z*
		Da	
α	(LSDLER)LVTPGYVPTEQDVLR(SRVKTT)	1685.9	844 (+2)
β	(ASQDGK)LIIWDSYTTNK(VHAIPL)	1352.5	677.4 (+2), 1353.5 (+1)
γ	PVINIEDLTEK(DKLKME)	1270.8	635.9 (+2), 1270 (+1)
γ_{DK}[a]	PVINIEDLTEKDK(LKME)	1512.7	505.3 (+3), 757.5 (+2)

[a] γ_{DK} denotes the partially digested signature peptide of γ subunit.

tides by MS/MS. We selected a 14-amino acid peptide for α subunit (residues 158–172), an 11-amino acid peptide for the β subunit (residues 79–89), and the first 11 amino acids from the N-terminal end of the γ subunit (Table I). Parsimony analysis defines the signature peptides of α, β, and γ subunits as distinct sequences, and their parent *m/z* did not overlap with other peptides from the complex.

We designed a 67-amino acid synthetic standard fusion protein *in silico* with the γ subunit signature peptide placed at the N terminus because it is derived from the N terminus of the native protein. The γ subunit was followed by an eight-amino acid spacer region, the α signature peptide subunit, and then the β subunit at the C terminus. The γ subunit spacer region includes the problematic KDK cleavage site that hinders tryptic proteolysis (13). Similarly the upstream and downstream regions of the α and β signature peptides incorporate six- or four-amino acid spacer regions similar to native protein. For an external standard, GAB-GFP was expressed in *E. coli* as described under "Materials and Methods." For stable isotope-labeled internal standards, GAB* was fused with a C-terminal His tag and expressed by *in vitro* translation as described under "Materials and Methods." The efficiency of incorporation of labeled amino acids was measured to be 85–90% by isotope ratio analysis of GAB*, limited by residual unlabeled lysine and arginine in the *in vitro* translation kit. Conveniently normal *E. coli* processing removed the N-terminal methionine from GAB* and GAB-GFP products, and we found no evidence of a γ signature peptide containing an N-terminal methionine. This observation is supported by the report that *E. coli* methionine aminopeptidase removes N-terminal methionine even when proline is in the P1′ position (14).

Validation of Proteolytic Digestion of Transducin and Fusion Protein Standards—Consistent peptide analyses were obtained when the transducin complex was denatured in 8 M urea at 37 °C. When transducin was digested with concentrations of trypsin increasing from 20:1 to 1:1 (substrate: trypsin, w/w), peak areas for α and β subunits plateau at 1:1 substrate:trypsin ratios, suggesting that α and β subunits were completely digested. However, even at a 1:5 substrate: trypsin concentration, the γ subunit remained incompletely digested. We performed serial digestions of transducin using trypsin followed by Lys-C for determination of digestion completeness. Transducin was digested first with trypsin at 1:1 (w/w) followed by varying concentrations of Lys-C (supplemental Fig. 2). Complete digestion of transducin γ subunit was possible only at a 1:1 (w/w) substrate:Lys-C ratio, evidenced by disappearance of MS signal for the partially digested peptide.

To demonstrate that the proteolytic characteristics of the fusion protein accurately reflected transducin properties, we followed the time course of the digestion of GAB* and transducin by trypsin and Lys-C at 1:1 substrate and enzyme ratios. As shown in Fig. 1, the temporal pattern of digestion of the γ signature peptide from GAB* and transducin exhibit parallel characteristics; identical times and enzyme concentrations are required to achieve complete digestion. The data from the α and β signature peptides are not shown because they were completely released by lower concentrations of trypsin. Table II demonstrates that the ratio of partially digested γ_{DK} peptide to completely digested γ peptide is constant in GAB* and transducin at different time intervals. Thus, the proteolytic digestion of GAB* accurately mimics that of native transducin. These results support the premise that downstream sequence may be required in signature peptide standards to emulate the digestion of full-length proteins.

Determination of Protein Stoichiometry Using Fusion Standards—In a typical experiment, a known amount of labeled GAB* was added to transducin, and the mixture was digested by the proteolytic enzymes as described. As shown in Table III for determination of the relative stoichiometry of α, β, and γ subunits of transducin, the ratios of relative peak area of signature peptides were calculated from extracted ion chromatograms normalized to corresponding labeled signature peptides. The relative molar responses of the three signature peptides are significantly different and cannot be used directly for stoichiometry determination (data not shown). Because each peptide is present in equimolar quantities in the internal standard fusion protein, normalization by ion intensities of internal signature peptide produces an accurate estimate of relative stoichiometry of different subunits.

To determine the dynamic range, consistency, and absolute concentration of transducin complex, we added different concentrations of labeled standard into the purified transducin preparation. The Fig. 2 *table inset* demonstrates that at different concentrations of GAB* the stoichiometry of the three

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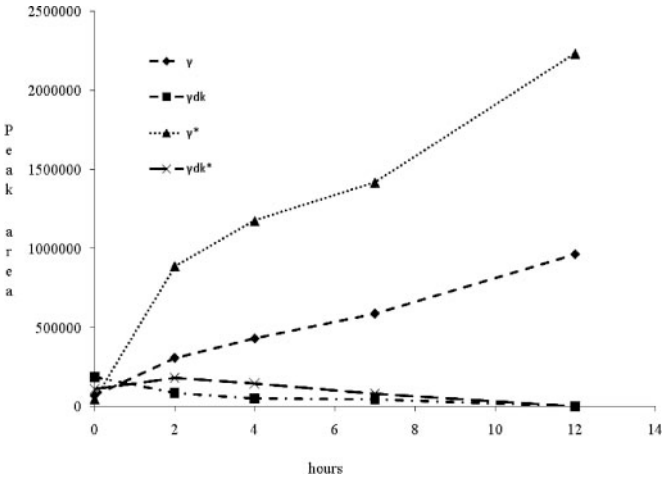

FIG. 1. **Time course of digestion of transducin and GAB* by Lys-C after trypsin digestion.** Typically sample was first digested overnight by trypsin followed by addition of Lys-C, indicated by *time 0* in the graph. After addition of Lys-C, aliquots were taken at different times and analyzed by mass spectrometry as described under "Materials and Methods." The signature peptides from the γ subunit of transducin or GAB* are denoted as γ or γ*, and the incompletely digested precursors are denoted as γ_{DK} and γ_{DK}*, respectively

subunits in transducin is remarkably similar. The average stoichiometry at five concentrations is 1:1.1:1.14 with relative S.D. of 9% (Fig. 2, *inset*). This result demonstrates that the stoichiometry of transducin can be measured accurately over at least a 5-fold range of internal standard and indicates the range, linearity, and robustness of this approach.

Alternatively we tested stoichiometric measurement using unlabeled GAB-GFP and external standardization. Transducin and external standard fusion protein GAB-GFP were digested separately, and each sample was analyzed in triplicate (same preparation) in the mass spectrometer (Table IV). Additional experiments indicated that there was higher variability in stoichiometry relative to the GAB* stable isotope internal standard methodology. A relative S.D. of 15–20% was observed when data from four runs were averaged. The higher relative S.D. from external compared with internal standards could result from sample-to-sample variation in mass spectrometry, injection, or pipette dilution errors (15).

TABLE II

The ratio of peak area of partially digested signature peptide γ_{DK} to fully digested γ in transducin and GAB at different time intervals*

Time	Transducin	GAB*
h	γ_{DK}/γ	$\gamma_{DK}^{*a}/\gamma^*$
0	2.63	2.53
2	0.27	0.2
4	0.12	0.12
7	0.07	0.06
12	0	0

[a] The "*" in the table denotes stable isotope-labeled signature peptides of γ subunit derived from standard fusion protein GAB*.

TABLE III

*Determination of stoichiometry of different subunits of transducin by the internal standard fusion protein GAB**

	Ratio of peak area of signature peptides		
	α/α^{*a}	β/β^*	γ/γ^*
TD/GAB*	0.50	0.52	0.59
TD/GAB*	0.51	0.43	0.47
TD/GAB*	0.49	0.45	0.51
TD/GAB*	0.49	0.49	0.47
TD/GAB*	0.45	0.39	0.47
Average	0.49	0.46	0.50
S.D.	0.02	0.05	0.05
Relative S.D.	4	11	10
Normalized ratio of α:β:γ		1:0.94:1.03	

[a] The "*" in the table denotes the stable isotope-labeled signature peptides of individual subunits derived from standard fusion protein GAB*.

Absolute Quantification of GAB and Transducin*—GAB-GFP was standardized with respect to GFP (Clontech) using a standard curve and then used to quantify GAB* (supplemental Fig. 3*A*). To quantify labeled GAB*, constant volumes of purified GAB* were added into serial dilutions of GAB-GFP. The proteins were digested with trypsin and Lys-C, and the peak areas of labeled peptide and unlabeled signature peptides were calculated. The ratio of labeled to unlabeled peptide were plotted to give a linear curve (R^2 value of 0.98, supple-

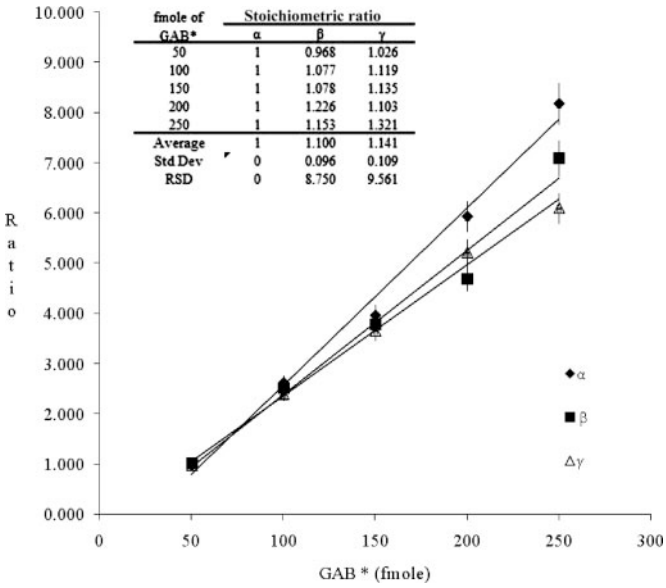

FIG. 2. **Dynamic range of stoichiometric measurements and absolute quantification of transducin by GAB*.** Varying concentrations of GAB*, accurately standardized relative to GAB-GFP (supplemental Fig. 3), were added to fixed quantities of a transducin preparation and digested by trypsin and Lys-C. The peak areas of GAB* and transducin signature peptides were calculated as described under "Materials and Methods." The *table inset* shows the stoichiometric measurements of transducin at each GAB* concentration. Peak areas for transducin were normalized with respect to GAB* added at each concentration. *Std Dev*, standard deviation; *RSD*, relative standard deviation.

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TABLE IV
Determination of stoichiometry of different subunits of transducin using the fusion protein GAB-GFP as an external standard

Run no.	Stoichiometric ratio		
	α	β	γ
1	1	0.8	1.2
2	1	0.72	1.42
3	1	0.9	1.01
4	1	1.11	1.41
Average	1.0	0.88	1.26
S.D.	0.0	0.17	0.20
Relative S.D.	0	19	15

mental Fig. 3*B*).

We measured the absolute concentration of transducin subunits in a solution at a concentration of 9.05 pmol/μl (as determined by Bradford assay) (Fig. 2). The ratio of labeled peptides from GAB* to their unlabeled transducin isotopomers is linear with R^2 value of 0.99. The concentrations of α, β, and γ subunits of purified preparation of transducin were found to be 7.8, 8.5, and 8.4 pmol/μl, respectively.

DISCUSSION

We report a combined fluorescence- and mass spectrometry-based strategy for the determination of stoichiometry and absolute quantification of a multiprotein complex. Fused GFP with concatenated signature peptides provides renewable protein standard reagents to quantify labeled internal standards and accurately estimate their molar concentration. The general concept of using quantifiable spectroscopic protein elements with fusion proteins for absolute quantification provides a route for accessible standards in mass spectrometry that can be spectrophotometrically measured with confidence. Quantitative small molecule analysis by mass spectrometry has long used stable isotope internal standards that could either be directly weighed or standardized with respect to crystalline gravimetric unlabeled standards. Protein chemistry represents a different challenge because there are few highly purified proteins suitable for gravimetric standards. Further the variability of associated molecules of solvation precludes reliable gravimetric analyses of many chemically or biosynthetically prepared peptides and proteins. The present method provides a general method that can be applied to the determination of the absolute concentration of any protein as well as its stoichiometry in a mixture. We are presently scaling this method for measuring larger sets of proteins in subcellular protein complexes. We anticipate that this method will be of general utility in clinical chemistry and biochemistry for the generation of reagents for the measurement of proteins.

Our work provides a general paradigm, having been tested with a membrane-associated hydrophobic protein complex. Transducin represents a difficult-to-digest complex containing a relatively small, highly basic subunit with limited options for signature peptides and an N-terminal proline and exhibiting a trypsin-resistant KDK motif. For accurate quantitative and stoichiometric measurements, it is mandatory to demonstrate either complete digestion or identical proteolytic characteristics of proteins and fusion protein standards (16). We achieved complete digestion by titrating the transducin complex with different amounts of proteolytic enzyme and also demonstrated the consistent and parallel digestion of transducin and GAB*, confirming their identical proteolytic microenvironments. We found that complete digestion required surprisingly high concentrations amounts of trypsin and Lys-C, suggesting that intrinsically transducin is not very amenable to proteolytic digestion. Our data show that requirements for proteolytic digestion of our fusion protein are very similar to those required for transducin. The addition of flanking sequences in fusion proteins clearly provides a method to emulate the digestion microenvironment of a full-length protein. Our data suggest that fusion proteins made from series of concatenated signature peptides with flanking sequences can be successfully used as a model to optimize the digestion conditions for larger set of proteins in complex biological mixtures.

We chose GFP as a synthetic genetically encoded spectroscopic tag because it is stable and fluoresces without any requirement of cofactors, enzymatic reactions, or substrates. However, there may be alternative protein elements that avoid some of the constraints imposed by GFP and permit the facile multiplexing of protein assays. GFP forms high molecular weight aggregates *in vitro* and *in vivo*, limiting its universal applicability and making it especially problematic for *in vitro* translation systems (17). An ideal gene-coded spectroscopic tag would enhance solubility and be produced in high efficiency *in vitro*, enabling the efficient use of stable isotope-labeled precursor amino acids. Several mutants of fluorescent proteins have been developed to overcome aggregation, exhibit different spectral properties, and slow folding rate (17). In this regard it will be interesting to test alternative monomeric red fluorescent protein as a fusion element with concatenated signature peptides (18).

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