ABSTRACT: Activation of the proenzyme form of the malarial protease PfsUB-1 involves the autocatalytic cleavage of an Asp

- Asn bond within the motif 215 LVSADNIDIS 224. A synthetic decapeptide based on this sequence but with the N- and C-terminal residues replaced by cysteines (Ac-CVSADNIDIC-OH) was labeled with 5- or 6-isomers of iodoacetamidotetramethylrhodamine (IATR). The doubly labeled peptides have low fluorescence because of ground-state, noncovalent dimerization of the rhodamines. Cleavage of either peptide by recombinant PfsUB-1 results in dissociation of the rhodamine dimers, which abolishes the self-quenching and consequently leads to an ~30-fold increase in the fluorescence. This spectroscopic signal provides a continuous assay of proteolysis, enabling quantitative kinetic measurements to be made, and has also enabled the development of a fluorescence-based assay suitable for use in high-throughput screens for inhibitors of PfsUB-1. The structure of the rhodamine dimer in the 6-IATR-labeled peptide was shown by NMR to be a face-to-face stacking of the xanthene rings. Time-resolved fluorescence measurements suggest that the doubly labeled peptides exist in an equilibrium consisting of rhodamines involved in dimers (closed forms) and rhodamines not involved in dimers (open forms). These data also indicate that the rhodamine dimers fluoresce and that the associated lifetimes are subnanosecond.

Malaria is a major human disease for which existing drugs are relatively unsatisfactory and prone to development of resistance. In the course of a program to study malarial proteases, in part for their potential as novel drug targets (1), a fluorogenic substrate was required for the protease PfsUB-1, a subtilisin-like serine protease expressed in the merozoite stage of the human malaria parasite Plasmodium falciparum. Merozoites of P. falciparum invade and replicate within red blood cells, and invasion is known to require the activity of parasite serine proteases (1). Recombinant, enzymatically active PfsUB-1 has been produced using the baculovirus/insect cell system (2, 3). Immediately following translation, PfsUB-1 undergoes an autocatalytic activation step in which the proenzyme is cleaved at an internal Asp—Asn bond within the motif 215 LVSADNIDIS 224. An N-acetylated synthetic decapeptide (Ac-LVSADNIDIS-OH) based on this site is cleaved at the same bond by either recombinant PfsUB-1 or authentic parasite-derived enzyme (2, 3).

To utilize this peptide as a fluorogenic substrate, we were attracted to the work of other groups who had found that peptides of between 4 and 20 residues labeled at both the N- and C-termini with tetramethylrhodamine show substantial quenching of the rhodamine fluorescence. The quenching is attributed to intramolecular dimerization of the dyes, and cleavage of the sequence by a protease of appropriate specificity relieves the quenching by allowing the dye molecules to dissociate (4, 5). The dimerization, a typical property of rhodamine dyes that is normally observed at high dye concentration (e.g., refs 6–8), is promoted by the high local concentration enforced by the linking peptide. In previous work, rhodamines were attached to the peptides either through amino or thiol peptide groups. Here we replaced the N- and C-terminal residues of the decapetide by cysteines (Ac-CVSADNIDIC-OH) and alkylated both cysteine side chains with 5- or 6-IATR (Figure 1). We were pleased in early experiments to find that cleavage of the 6-IATR-labeled peptide, either specifically by PfsUB-1 or nonspecifically by Pronase, resulted in a large increase in rhodamine fluorescence. This result prompted us to make a more detailed characterization of the labeled peptide, initially

1 Abbreviations: Abz, 2-aminobenzoyl; DABCYL, 4-(4-dimethylaminophenylazo)benzoic acid; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; 6-CATR, 6-chloroacetamidotetramethylrhodamine; 5- or 6-IATR, 5- or 6-iodoacetamidotetramethylrhodamine; EDANS, 5-(2-aminoethylamino)naphthalene-1-sulfonic acid; FRET, fluorescence resonance energy transfer; NOESY, nuclear Overhauser effect spectroscopy; pepFl-5R, peptide Ac-CVSADNIDIC-OH labeled at the N- and C-terminal cysteines with 5-IATR; pepFl-6R, peptide Ac-CVSADNIDIC-OH labeled at the N- and C-terminal cysteines with 6-IATR; pHMB, p-hydroxymercuribenzoate; RP-HPLC, reversed-phase high-performance liquid chromatography; TCEP, tris(carboxyethyl)phosphine; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy.

Michael J. Blackman,*† John E. T. Corrie,‡ John C. Croney,§ Geoff Kelly,‡ John F. Eccleston,‡ and David M. Jameson§

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K., and Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii 96822-2319

Received June 27, 2002; Revised Manuscript Received August 8, 2002
DMSO-Molecular Probes (Eugene, OR); other biochemicals and detergent were from Boehringer Mannheim. TCEP was from prepared as previously described. Pronase and NP-40 de
Recombinant PfSUB-1 (synthesized in-house by standard solid-phase Fmoc chemistry. equilibrated in 30% (v/v) aqueous acetic acid] and eluted at 4.7 mL min\(^{-1}\) over 30 min with a 22.5–36% (v/v) gradient of acetonitrile in 0.1% TFA. The purified pepF1-6R was lyophilized, redissolved in DMSO (or DMSO-d\(_6\) for NMR measurements) at a concentration of 10.5 mM, and stored over desiccant in the dark at \(-20^\circ\text{C}\). This material was used for the NMR and kinetic measurements. However, analytical RP-HPLC analysis showed that it contained 10% of another rhodamine-containing species that was removed on a Vydac 4.6 mm \(\times\) 25 cm C\(_{18}\) reversed-phase column, eluted at 1 mL min\(^{-1}\) with a 4.5–58.5% (v/v) gradient of acetonitrile in 0.1% TFA over 60 min. The resulting purified product, estimated to be more than 99% pure, was used for the steady-state and time-resolved fluorescence measurements.

A similar protocol was followed for preparation of the peptide labeled with 5-IATR (called pepF1-5R).

**Specificity of PepF1-6R Cleavage by Recombinant PfSUB-1.** All concentrations were determined from the rhodamine absorption at 528 nm, based on \(\epsilon\)\(_{528}\) 52000 M\(^{-1}\) cm\(^{-1}\) \((9)\). Throughout the text, where concentrations refer to the doubly labeled peptides, they are given in terms of the peptide concentration, i.e., half that of the dye. Solutions of labeled peptide were prepared by dilution from stock DMSO solutions into protease digestion buffer [20 mM Tris-HCl, 50 mM NaCl, 12 mM CaCl\(_2\), 0.05% NP-40 (w/v), pH 7.6]. It was necessary to include NP-40 detergent in all buffers that contained the labeled peptides to avoid nonspecific adsorption to glass and plastic ware. A sample of pepF1-6R (20 \(\mu\)M in digestion buffer) was supplemented with recombinant PfSUB-1 (~40 nM protein) and incubated at 37 \(^{\circ}\text{C}\) to allow partial hydrolysis of the peptide. The sample was then fractionated by RP-HPLC fractionation on a Vydac 4.6 mm \(\times\) 25 cm C\(_{18}\) column, eluted at 1 mL min\(^{-1}\) with a 4.5–58.5% (v/v) gradient of acetonitrile in 0.1% TFA over 60 min. Eluted products were collected and analyzed by electrospray mass spectrometry as described above.

**NMR Spectroscopy.** NMR spectra of pepF1-6R were recorded at 800 MHz proton frequency on a Varian Inova spectrometer. Spectra were recorded in 10% DMSO-d\(_6\)–20 mM sodium acetate, pH 5.0 (H\(_2\)O:D\(_2\)O 9:1) at 25 \(^{\circ}\text{C}\). The low solubility in this solvent system limited the sample concentration to 50 \(\mu\)M and necessitated the use of the highest available field. Two-dimensional TOCSY (10) and NOESY (11) spectra were recorded, employing WATERGATE (12) for solvent suppression. A mixing time of 70 ms was used in the TOCSY spectrum, while separate NOESY spectra were recorded with mixing times of 200, 400, 600, and 800 ms. Quadrature detection in indirect dimensions was achieved using the States procedure (13). Spectra were referenced with respect to the residual water resonance at 4.75 ppm and were processed and analyzed using the nmrPipe/nmrDraw package (14). Molecular modeling was performed using InsightII (Accelrys Inc., Princeton, NJ). Control one-dimensional NOE spectra of 6-chloroac-
etamidotetramethylrhodamine (9) were obtained for a 2 mM solution in CDCl₃–MeOH-d₄ (7:3 v/v) at 600 MHz on a Varian Unity spectrometer.

Absorption Spectroscopy. Solutions of pepF1-5R and pepF1-6R were prepared at ~2 μM concentration by dilution from stock DMSO solutions into protease digestion buffer. Portions (4 mL) of each solution were treated with an aliquot of Pronase (16 μL of 1 mg mL⁻¹ in the same buffer) and incubated at room temperature for 30 min. Absorbance spectra of these solutions and of the corresponding labeled peptides prior to Pronase treatment were recorded (Shimadzu UV-2401PC spectrophotometer). Absorbance spectra of intact peptides were measured similarly for solutions in the range pH 5–10.

Fluorescence Measurements. Steady-state fluorescence emission spectra were recorded with an ISS PC1 spectrofluorometer (ISS Inc., Champaign, IL) using a 3 × 3 mm path-length cell. Spectra were recorded with excitation through a polarizer at 54.7° to the vertical, and emission was viewed through a vertical polarizer. These spectra were corrected for the wavelength dependence of the photomultiplier response using the ISS data correction files. Technical emission spectra were also recorded without the use of polarizers or correction for monochromator or photomultiplier responses.

Time-resolved fluorescence measurements were made using an ISS K2 multifrequency phase and modulation spectrofluorometer. The intensity of the excitation light (501 nm from a Spectra-Physics 2045 argon ion laser) was sinusoidally modulated over the range 20–250 MHz, and the phase shift and relative intensity modulation of the emission with respect to the excitation were determined (15). To circumvent polarization artifacts, the excitation light was polarized parallel to the vertical laboratory axis, and the emission was viewed through a polarizer oriented at 54.7° (16). The phase and modulation data as a function of the modulation frequency of the excitation light were analyzed as discrete exponential decays (17). Emission at wavelengths longer than 530 nm was measured through a Schott 087 long-pass filter.

Kinetic measurements of enzyme activity were made in an SLM 8000 instrument with excitation at 550 nm and emission monitored using a Wratten 22 filter. Experimental conditions are given in the legends to Figures 7 and 8.

Multiwell Fluorescence Assay. Purified recombinant PfSUB-1 at a range of concentrations in digestion buffer was dispensed in 50 μL aliquots into wells of white 96-well microtiter plates (FluoroNunc, NUNC). Wells were supplemented with either 0.5 μL of 100 mM pHMB or 0.5 μL of water; then the plates were placed on a rotary shaker and mixed at room temperature for 30 min prior to the addition of 50 μL of a solution of pepF1-6R at various concentrations in digestion buffer. Plates were sealed and incubated at 37°C for 18 h before being read on a Perkin-Elmer LS-50B luminescence spectrometer fitted with a LS50B WPR multiplate reader accessory. Measurements were performed at excitation wavelength 552 nm, slit width 4 nm, and emission wavelength 580 nm, slit width 2.5 nm. Data collection was managed using Perkin-Elmer FL WinLab software.
ences likely reflect differences in the geometry and/or the association/dissociation kinetics of the rhodamine dimers in the two intact peptides, but we have not investigated this further.

The corrected fluorescence emission spectra with excitation at 550 nm of intact and cleaved pepF1-5R and pepF1-6R together with the buffer containing NP-40 are shown in panels c and d of Figure 3, respectively. For both peptides there is a large enhancement in emission intensity upon cleavage. However, there is also a weak fluorescence signal arising from an impurity in the NP-40 with an emission maximum at 595 nm. The emission maximum of uncleaved pepF1-5R is 580 nm, and that of the cleaved peptide is 575 nm. This difference can be attributed to the larger effect of the NP-40 fluorescence on the weakly fluorescent uncleaved peptide. At 575 nm the enhancement in intensity is 18-fold, but after correction for buffer fluorescence rises to 21-fold. Integrating the emission spectrum between 555 and 650 nm gives an enhancement of 15-fold and 22-fold before and after taking the NP-40 fluorescence into account.

In the case of pepF1-6R, the emission maxima of uncleaved and cleaved peptide were at 575 and 573 nm, respectively. The enhancement at 573 nm was 23-fold before and 32-fold after correction for the NP-40 fluorescence. The equivalent values obtained by integrating the spectrum between 555 and 650 nm were 19-fold and 33-fold. Similar results were obtained for spectra uncorrected for polarization artifacts or photomultiplier spectral response. For example, pepF1-6R gave an increase of 30-fold, either at 573 nm alone or in the integrated spectrum after taking the NP-40 fluorescence into account.

Conformation of PepF1-6R. Figure 4 shows a selected region of the NOESY spectrum of intact pepF1-6R in which interactions between the rhodamines are observed. The complete NOESY spectrum, together with the 2-D TOCSY spectrum, revealed spin systems for the 10 amino acid residues. The assignments for these and the rhodamines are given in Table 1. Notably, chemical shift differences were either undetectable or very small for protons at the same positions on each rhodamine, suggesting that the two dye moieties on the labeled peptide were in similar environments. Several spectroscopic parameters indicate that the peptide does not adopt a single well-defined conformation; these were the limited chemical shift dispersion, absence of...
nonsequential NOE connectivities, and intermediate values (~6 Hz) of the $^{1}H$–$^{1}H$ scalar coupling constants (data not shown). NOE cross-peak volumes were used to derive approximate interproton distance restraints for the rhodamines, using a simple $r^{-6}$ relationship, and corrected for proton stoichiometry. Table 2 summarizes the interproton distances determined for the rhodamine component of pepF1-6R. These distances were used to model the structure of the rhodamine dimer, which is shown in Figure 5.

**Fluorescence Lifetimes.** Lifetime data were obtained on peptide samples before and after cleavage by Pronase. Peptide concentrations were ~1 μM. Phase and modulation data are shown in Figure 6 for both pepF1-6R and pepF1-5R. Table 3 shows data from the nonlinear least-squares analysis based on discrete exponential decays. Before Pronase treatment, pepF1-6R and pepF1-5R each demonstrated a long lifetime component (2.44 ns for pepF1-5R and 2.50 ns for pepF1-6R) as well as short lifetime components (~0.14 ns for pepF1-5R and ~0.076 ns for pepF1-6R). In terms of the fractional contribution to the total intensity (the $f$ terms in Table 3), these short components accounted for only 5% of the emission in both cases. However, if one assumes that...
the quantum yields and lifetimes are proportional in these systems and also that excitation at 501 nm results in relative weights of dimer to monomer in proportion to their absorption spectra, then one can calculate preexponential factors \( (R) \). These calculations indicate that the relative proportions of the molecular species associated with the short lifetime component (before Pronase treatment) were 48% (pepF1-5R) and 63% (pepF1-6R). Note that for the lifetime determinations, in contrast to the steady-state fluorescence measurements described above (Figure 3c,d), the background fluorescence from buffer containing NP-40 was less than 0.1% of the sample signal for either isomer, since excitation at 501 nm produced a significantly lower background than excitation at 550 nm. Approximately 1 h after Pronase treatment the lifetime measurements were repeated, and the 5- and 6-isomers exhibited single exponential decays of 2.43 and 2.50 ns, respectively.

**Kinetics.** Initial studies were made using the nonspecific cleavage of pepF1-6R by Pronase. Solutions (0.35 mL) of pepF1-6R ranging between 0.12 and 2.00 \( \mu \)M were incubated with Pronase (1.2 \( \mu \)g) at 20 °C, and the fluorescence intensity was recorded with time (Figure 7a). The initial rate of intensity increase was measured and converted to a rate of pepF1-6R cleavage based on knowledge of the total intensity change of the reaction and the initial concentration of pepF1-6R. The rate of cleavage of pepF1-6R was linearly dependent on [pepF1-6R] over the range measured (Figure 7b).

With the validity of this protease assay established with Pronase, similar kinetic measurements were made with PfSUB-1. In this case, pepF1-6R in digestion buffer (80 \( \mu \)L) was equilibrated at 37 °C, and recombinant PfSUB-1 (20 \( \mu \)L, containing 800 ng of purified protease) was added. A slow linear increase in fluorescence intensity was observed (Figure 8a). After sufficient data to determine that the steady-state rate of intensity increase had accumulated, Pronase (1 \( \mu \)g) was added to enable a rapid determination of the end point of cleavage. The data were converted to a rate of pepF1-6R cleavage as described above. Again, there was a linear dependence of the rate of pepF1-6R cleavage on [pepF1-6R] over the measured concentration range (Figure 8b).

**Development of a Multiwell Assay.** As well as facilitating kinetic studies, the availability of a small synthetic fluorescent protease substrate allows the development of a simple assay capable of high-throughput screening for inhibitors of the protease. To explore the potential of pepF1-6R in such an assay, trial experiments were performed to optimize the conditions for a 96-well microtiter plate assay. Figure 9 shows the results of a typical assay in which PfSUB-1 at a series of concentrations was incubated for 18 h with a standard concentration (0.1 \( \mu \)M) of pepF1-6R in the microtiter format assay. At the highest concentration of protease used in this assay (0.1 \( \mu \)g mL\(^{-1}\), approximately 2 nM protein; ref 3), an 11.8-fold increase in fluorescence was obtained over the period of incubation. The fluorescence increase was approximately proportional to the concentration of protease used and was virtually completely ablated in the presence of the PfSUB-1 inhibitor pHMB.

**DISCUSSION**

The initial motivation of our study was to develop a fluorogenic assay suitable for high-throughput screening of inhibitors of the protease PfSUB-1. However, the fluores-
rhodamine dimers in the binding site of a Bence–Jones protein, where the dye is in a protein environment and in the presence of lattice water (32), and an NMR study of rhodamine dimers in aqueous solution (33). Both studies show a stacked arrangement of the xanthene rings with the carboxy groups on the pendant phenyl rings projecting outward from the dimer core. The stacked geometry places particular protons of one rhodamine in proximity with nonisochronous protons on the partner rhodamine in the dimer, allowing determination of NOE effects and thus of distance constraints.

The interproton distances derived for pepF1-6R from NMR measurements shown in Table 2 are compared with the same interproton distances in a monomer. Table 2 also lists the relevant interproton distances for the optimized dimer model. Examination of a series of NOESY spectra recorded with different mixing times served to distinguish between direct and relayed NOE effects. Relayed effects, especially between ortho-related protons in aromatic rings, become significant at extended mixing times and explain the anomalous cross-peak seen in Figure 4 for an apparent NOE between H-7 and H-2,7' of the paired rhodamines (see footnotes to Table 2). The differences between the NOE and monomer-derived distances can be reconciled by invoking a mode of intramolecular dimerization that has a typical stacked relationship of the xanthene rings, with a ring–ring separation of ~3.5 Å, similar to that described for aqueous solutions of rhodamine homo- and heterodimers (33), and a horizontal offset of the xanthene ring centroids by ~2.1 Å (Figure 5).

Our data are incompatible with a model involving coplanar arrangement of the xanthene groups, with dimerization via an edge-to-edge interaction. In particular, observation of a significant NOE between H-4′(5′) and H-7 dictates a stacked arrangement, as the minimum H-4′(5′) to H-7 separation in an edge-on dimer would be 6.5 Å, i.e., the distance in the isolated monomer as a lower limit. The absence of any observable NOE between H-4′(5′) and H-7 in such circumstances was confirmed using 1-D steady-state NOE experiments on 6-CATR in CDCl3–MeOH-d4, under which conditions the dye is entirely monomeric (data not shown). Thus, at least in the present 6-labeled substrate, the rhodamine dimer geometry experimentally determined is best fit by a stacked model as opposed to an edge-to-edge one.

The fluorescence lifetime data indicate that in the uncleaved peptides a significant portion (~52% in pepF1-5R and ~37% in pepF1-6R) of the rhodamine moieties may remain in the unquenched (monomeric) conformation. This estimation is prone to error since (a) it is based on the assumption that the quantum yield and lifetime are proportional, which may not be the case given the large change in the absorption spectrum, (b) small errors in the lifetime of the quenched component will significantly impact on the calculation of the preexponential term, and (c) the excitation spectra of the monomeric and dimeric rhodamines likely have different maxima (24) so the fluorescence intensities based on single-wavelength excitation measurements may not accurately reflect the relative proportions of species present. Nevertheless, the data strongly suggest the presence of an equilibrium between dimer and monomer rhodamine states for both peptides. This equilibrium must be rapid on the NMR time scale since only a single set of resonances was observable for pepF1-6R. The fluorescence results further suggest that the combination of peptide flexibility and

![Graph](image-url)
intrinsic free energy of the rhodamine interaction was such that the dimer formation is more favored for the 6-isomer than for the 5-isomer. However, precise determination of the equilibrium constants for these systems is hampered because of uncertainties in the proportions of the two forms, as outlined above. Differences between the molecular arrangement within the rhodamine dimers were also evident from the fact that the resolved lifetime of the short component was lower in the case of pepF1-6R (0.076 ns) compared to pepF1-5R (0.14 ns). It is difficult to compare our lifetime results with those of Kemnitz and Yoshihara (8), who studied a series of rhodamine compounds in nonaqueous solvents. However, they did attribute a subnanosecond component to fluorescence of the dimer. The lifetime differences between pepF1-5R and -6R are paralleled by differences in the respective absorption spectra, specifically the differences in the mid-UV absorption and the different intensity ratios for the two visible peaks (518 and 549 nm) for pepF1-5R and -6R described above. The latter also suggest that the 6-isomer favors dimer formation. However, a complementary structural determination for pepF1-5R was beyond the scope of the present study.

The study by Packard et al. (4) proposed that it was desirable to incorporate “conformation-determining” regions into the peptide sequence in order to promote efficient interaction of the rhodamine monomers. However, a later study by the same group showed that incorporation of flexible linker arms generally promoted dimerization, leading to better fluorescence quenching (28). The related study by Geoghegan et al. (5) also suggested that conformation-determining regions in the peptide are not required, and our work agrees with that conclusion. Indeed, our NMR data indicate that the peptide sequence in pepF1-6R is unstructured, as would generally be expected for a small peptide. This is important, since it suggests that there is little restriction on the peptide sequence that may be used in such substrates, in turn indicating that the approach has wide application for design of fluorogenic protease substrates.

Most existing fluorogenic peptide substrates for proteases incorporate a C-terminal fluorescent group (most commonly a 7-aminocoumarin) linked in an amide bond, cleavage of which generates the fluorescence increase as the free fluorophore is released (e.g., ref 34). The utility of this class of substrates can be limited, since the presence of a bulky fluorophore directly adjacent to the scissile bond can often interfere with substrate recognition. In addition, for efficient substrate recognition, many proteases require interaction with residues on both the P and P’ sides of the scissile bond, as in their physiological protein substrate(s). The inclusion of a sequence which can interact with both the S and S’ subsites of the protease often enhances the selectivity (reflected by the $k_{cat}/K_M$ ratio) of a protease substrate. To accommodate this, a second class of fluorescent substrates is available, which use FRET to quench the fluorescence of a terminal group in the intact peptide. The general structures of the two most commonly used of these are DABCYL-(Xaa)$_n$-EDANS and Abz-(Xaa)$_n$-3-nitrotyrosine, where (Xaa)$_n$ is any amino acid sequence, and numerous other donor/acceptor pairs have also been explored (e.g., refs 35-39). In all cases the efficiency of intramolecular quenching is critically dependent on the distance between the donor and acceptor moieties. For efficient quenching in the EDANS/DABCYL system, for example, $n$ should not exceed 11 or 12; quenching efficiency at this distance is only about 10-fold (39). Our data and those of Geoghegan et al. (5) suggest that substrates based on the approach reported here are not subject to such restrictions in the length of the linking peptide; indeed, the latter authors demonstrated that an intervening sequence of up to 18 residues in length did not prevent efficient dimerization of terminal tetramethylrhodamine moieties (5). This is an important consideration when the precise S and S’ subsite requirements of a protease are unknown. Furthermore, and perhaps more important, synthesis of the doubly labeled peptide is relatively simple, due to the requirement only to incorporate an identical group at each of the two available reactive cysteine side chains; synthesis of FRET-based substrates is significantly more complex, usually requiring incorporation of the donor and acceptor groups during solid-phase synthesis (e.g., ref 36).

Synthesis of the fluorogenic peptide by labeling cysteine residues rather than N-terminal amines and/or ε-amino groups on lysine allows for the presence of lysines within the target sequence of other potential fluorogenic peptides of this general type without a need for more complex chemical strategies. The use of iodoacetamidohorodamines rather than maleimidohorodamines (5) for labeling may also be beneficial, since it does not create new stereo centers. In principle, dual labeling by a maleimide reagent of a peptide that contains two cysteines will result in four diastereoisomeric species, each of which could have different properties. At least one well-characterized example of diastereoisomers being formed upon labeling a cysteine residue with a maleimide reagent has been described (40).

Alternative approaches based on a similar principle may be taken. For example, Wei et al. used a fluorescein—rhodamine heterolabeled 13-mer peptide to study antibody binding. Fluorescence quenching in this case was mediated by a combination of ground-state and excited-state effects. Binding of the labeled peptide to a specific antibody resulted in an ~8-fold increased rhodamine fluorescence that was attributed to dissociation of intramolecular dimers (41). With a suitable bridging peptide sequence, such peptides could also be used as fluorogenic protease substrates.

At concentrations up to 5 μM there was a linear relationship between the concentration of pepF1-6R and the initial rate of its cleavage by recombinant PSUB-1, indicating that the $K_M$ for this substrate is substantially higher than 5 μM. This is consistent with previous studies of cleavage by recombinant PSUB-1 of an unlabeled peptide substrate, Ac-LVSADNIDIS-OH, where the experimentally determined $K_M$ value was ~770 μM (3). If of a similar order of magnitude, experimental determination of the $K_M$ for pepF1-6R would be very difficult, since at such concentrations quenching effects due to intermolecular dimerization might become significant and inner filter effects would come into play. Such problems are also a feature of FRET-based systems (e.g., refs 36, 38, and 39).

We have shown that pepF1-6R can be used in a microtiter plate assay suitable for scale-up to high-throughput format, thus facilitating screens of large libraries of potential inhibitors, and this has been successfully implemented (K. Ansell, B. Saxty, C. Kettleborough, and M. J. Blackman, unpublished). The results also show that interaction of the tetramethylrhodamine dye monomers in pepF1-6R is unaf-
fected between pH 5 and pH 10, allowing the substrate to be used for analyzing the pH dependence of PfSUB-1 activity over at least this range. Both pepF1-6R and pepF1-5R may also have applications for exploring the activity of PfSUB-1 in situ in the malaria merozoite, where the enzyme accumulates in secretory granules; preliminary experiments suggest that the substrates are readily membrane permeable, and cleavage of the intracellular compound may allow visualization of these organelles by fluorescence microscopy.

ACKNOWLEDGMENT

We are grateful to Peter Fletcher for synthesis of the decapeptide and to Steve Howell for mass spectrometry data. The authors also express their thanks to Walter Mangel for discussions leading up to this work.

REFERENCES
