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Increase in Fluorescence upon the Hydrolysis of Tyrosine Peptides: Application to Proteinase Assays

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The intrinsic fluorescence of tyrosine increases by a factor of approximately two when the carboxy group is liberated from a peptide bond by hydrolysis. The increase in fluorescence provides a novel way to monitor the hydrolysis of native tyrosine peptides that contain only proteinogenic amino acids. Thus, for example, the hydrolysis by HIV-1 proteinase of a heptapeptide viral protein fragment *gag*$^{129-135}$, Ser–Gln–Asn–Tyr–Pro–Ile–Val, was followed continuously at excitation and emission wavelengths 275 and 305 nm. The fluorescence increase is magnified by at least a factor of a thousand when a resonance energy quencher, such as *para*-nitrophenylalanine, is in the vicinity. For example, the peptide Lys–Ala–Arg–Val–Tyr–Phe(*p*-NO_2)-Glu–Ala–Nle–NH_2 [Richards *et al.* (1990) *J. Biol. Chem.* 265, 7733], widely used for spectrophotometric assays of the HIV-1 proteinase, yields a substrate:product fluorescence ratio greater than 1:1000. Tyrosine-containing substrates of pepsin and trypsin showed similar behavior. The detection limit of the present method is at least one order of magnitude lower than absorbance assays of *p*-nitrophenylalanine peptides. © 1995 Academic Press, Inc.

The natural amino acid tyrosine is strongly fluorescent. Tyrosine fluorescence has been used to monitor the biophysical properties of proteins (1–6), such as conformation (2), folding dynamics (5), or intermolecular interactions (4,6). Furthermore, Cowgill (1) has reported that tyrosine fluorescence is quenched by adjacent peptide bonds. Here we report that a simple cleavage of the tyrosyl amide bond produces an approximately twofold increase in fluorescence. This physicochemical effect was used to follow continuously the protease-catalyzed cleavage of native, structurally unaltered peptides and proteins. Conventionally, fluorogenic assays of proteinases (7–11) employ fluorophor/quencher pairs such as EDANS[2] and DABCYL, that are attached to opposite termini of the peptide substrate. Unfortunately, the steric bulk and hydrophobicity of these subunits alter the physicochemical properties of the parent peptide sequence. The EDANS/DABCYL (12) or ABZ/*p*-nitrophenylalanine (9) strategy has been used for kinetic assays of the HIV-1 proteinase (12) and other enzymes. For example, Cheng *et al.* (9) used a tyrosine–*p*-nitrophenylalanine peptide Ala–Thr–His–Gln–Val–Tyr–Phe(NO_2)-Val–Arg–Lys–Ala for absorbance assays of the HIV-1 proteinase. In order to produce a more sensitive substrate, they attached the fluorescent *o*-aminobenzoyl (anthranilyl) group at the N-terminus of this parent peptide; *p*-nitrophenylalanine acted as an internal quencher. In light of the results reported in this paper, however, chemical modification of the original peptide substrate was not necessary because tyrosine itself acts as a sufficiently strong fluorophor.

We measured the increase in tyrosine fluorescence to follow the cleavage of two different substrates for the HIV-1 proteinase: a *p*-nitrophenylalanine peptide derived from the *gag* protein, and a native HIV-1 *gag* fragment. Substrates for pepsin and trypsin, based on tyrosine fluorescence, have also been prepared and tested.

MATERIALS AND METHODS

Materials. A sample of the HIV-1 proteinase was generously donated by Dr. Paul Darke (Merck, Sharp & Dohme Research Laboratories). Pepsin and trypsin

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[2] Abbreviations used: DABCYL, 4-(4-dimethylaminophenylazo)-benzoyl; DIPCDI, diisopropyl carbodiimide; DTT, dithiothreitol; EDANS, 5-((2-aminoethyl)amino)-naphthalene-1-sulfonate; EDTA, ethylenediaminetetraacetic acid; MBHA, *p*-methylbenzhydrylamine; PAL, (5-(4-aminomethyl-3,5-dimethoxyphenoxy)-valeric acid; TFA, trifluoroacetic acid; PEG, polyethylene glycol M_r 8000–12,000.

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were of the highest purity available from Sigma Chemical Co. (St. Louis, MO). The HIV-1 proteinase substrates Lys-Ala-Arg-Val-Tyr-Phe(*p*-NO_2)-Glu-Ala-Nle-NH_2 and Ser-Gln-Asn-Tyr-Pro-Ile-Val were obtained from Bachem Bioscience Inc. (Philadelphia, PA) and Sigma Chemical Co., respectively. Peptides Ala-Tyr-Arg-Phe(*p*-NO_2)-Ala and Lys-Ala-Lys-Ala-Tyr-Phe(*p*-NO_2)-Ala-Lys-Ala-Lys-NH_2 were synthesized by standard Fmoc solid-phase methods, utilizing an MBHA support with the PAL linker for peptide amides (13). Couplings were carried out for two hours using DIPCDI as the reagent. Peptide purification was carried out on a Vydac C18 semipreparative column (25 × 240 mm), using a gradient elution with 10–80% (w/w) aqueous acetonitrile containing 0.04% trifluoroacetic acid, at a flow rate of 7.2 ml/min.

HIV protease assays. HIV-1 protease assays were conducted as described previously (14), with the following modifications: 0.1% PEG (w/v) was added as a stabilizer (18), and the assay temperature was 25°C. Fluorescence was monitored on a Hitachi F-3010 spectrophotometer with fully open excitation and emission slits (5 mm), at excitation wavelength 275 nm and emission wavelength 305 nm.

Trypsin assays. A stock solution of the substrate Ala-Tyr-Arg-Phe(*p*-NO_2)-Ala (1.0 mM) was prepared by dissolving approximately 2 mg in 500 μl of dimethyl sulfoxide. Trypsin stock solution was prepared by adding approximately 0.5 mg of crystalline enzyme to 500 μl of phosphate buffer (pH 7, 4 mM DTE, 5 mM EDTA, 1 M NaCl, 100 mM phosphate) (15). Ten microliters of the substrate solution diluted in 1 ml of acetate buffer (pH 5, 4 mM EDTA, 5 mM DTT, 100 mM acetate, 1 M NaCl) was mixed with 50 μl of the trypsin solution in temperature-equilibrated cuvette containing 1 ml phosphate buffer. Fluorescence was measured at 35°C.

Pepsin assays. A 1 mM stock solution of the substrate Lys-Ala-Lys-Ala-Tyr-Phe(*p*-NO_2)-Ala-Lys-Ala-Lys was prepared in water. A solution of pepsin (0.8 mg/ml) was prepared by dissolving the crystalline enzyme in acetate buffer, pH 5, and making successive dilutions. Final assay concentrations (in acetate buffer pH 5) were 130 nM pepsin, 200 μM substrate. Fluorescence was measured at 35°C.

RESULTS AND DISCUSSION

Trace A in Fig. 1 shows the hydrolysis, catalyzed by the HIV-1 proteinase, of the HIV-1 *gag* protein fragment **1** (Scheme 1) at the matrix/capsid Tyr^{132}–Pro^{133} juncture, giving approximately 70% increase in the signal. When *p*-nitrophenylalanine is used as a fluorescence quencher, the relative increase in fluorescence upon Tyr-Phe(*p*-NO_2) cleavage is higher than 1:1000 (Fig. 1, trace B). In this case the substrate, peptide **2** derived by Richards *et al.* (17) from the HIV-1 capsid/nucleocapsid

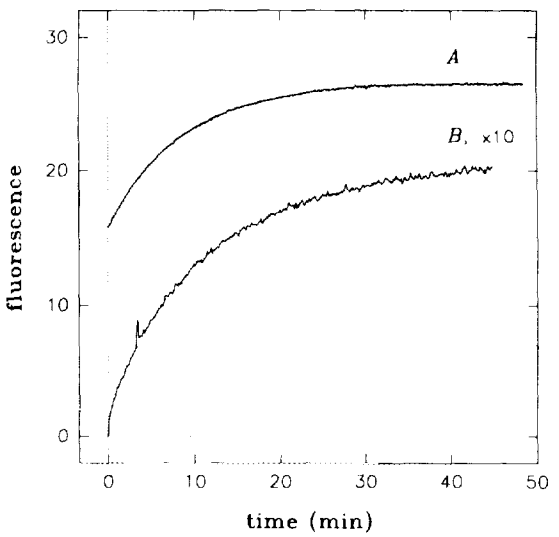

FIG. 1. Fluorescence changes during the cleavage of peptide **1** (20.0 μM, trace A) and peptide **2** (1.0 μM, trace B) by the HIV-1 proteinase. Both the abscissa and the ordinate were multiplied by a factor of ten to display trace B.

cleavage site $gag^{359-367}$, does not fluoresce at all. Under the conditions used in this study, the fluorescence signal increased linearly with substrate concentration up to 150 μM, while further increases in concentration produced a nonlinear response due to reabsorption of light. Pepsin assays by using peptide **3**, and trypsin assays with peptide **4**, yielded changes in the intrinsic fluorescence indicated in Scheme 1 (data not shown).

Several model peptides containing tyrosine at the carboxy terminus (H-Arg-Tyr-OH, H-Phe-Tyr-OH, and H-Arg-Lys-Glu-Val-Tyr-OH) were used to investigate the effect of environmental factors, such as ionic strength, or the presence of additives that stabilize the HIV-1 proteinase (e.g., polyethylene glycol (18)). In all cases, the intensity of fluorescence varied within ±20%. Very little change in fluorescence was observed when H-Lys-Ala-Arg-Val-Phe(*p*-NO_2)-Tyr-Glu-Ala-Leu-NH_2 was cleaved by the HIV-1 proteinase, leaving tyrosine at the N-terminus. The protonation state of the reaction products appears to be important in this case. Experiments with H-Arg-Tyr-OH showed that when pH decreased from 5.0 to 2.0, tyrosine fluorescence decreased by 50%. When the N-terminal amino group was fully protonated (pH 2.0), the fluorescence of H-Tyr-OMe also decreased by about 50%.

The design of the novel proteinase assay reported in this paper is based on the accumulated knowledge of environmental effects on tyrosine fluorescence (1,21). An important advantage is that the fluorescence method can utilize native (poly)peptide substrates. No structural alterations of the natural substrate (peptide **1**) were necessary to accomplish a convenient, continuous

enzyme	substrate	fluorescence increase
HIV-1 proteinase	H-Ser-Gln-Asn-Tyr-(C(=O)-NH)-Pro-Ile-Val-OH 1	1 : 1.7
HIV-1 proteinase	H-Lys-Ala-Arg-Val-Tyr-(Phe(NO_2))-Glu-Ala-Ile-NH_2 2	> 1 : 1000
pepsin	H-Lys-Ala-Lys-Ala-Tyr-(Phe(NO_2))-Ala-Lys-Ala-Lys-NH_2 3	> 1 : 1000
trypsin	H-Ala-Tyr-Arg-(Phe(NO_2))-Ala-NH_2 4	1 : 150

SCHEME 1

monitoring of the hydrolysis catalyzed by the HIV-1 proteinase.

When the peptide substrate contains an additional aromatic amino acid, acting as a resonance energy acceptor (quencher), the increase in the signal is magnified, and consequently the sensitivity of the assay increases. Because of the favorable signal amplification ratio (>1:1000), continuous fluorescence assays of retroviral and other aspartic proteinases such as porcine pepsin (peptide **3**) can now be conducted at concentrations of the substrate as low as 300–500 nM. The sensitivity of the novel tyrosine fluorescence method thus surpasses the conventional continuous ultraviolet absorbance assays, based on *para*-nitrophenylalanine substrates, which, depending on sequence, give a 13% decrease or an 8% increase in absorbance. Consequently, the absorbance assay has a lower detection limit of approximately 10 μM. The increased sensitivity of the fluorescence assay allowed us to determine the Michaelis constant for the frequently used HIV-1 proteinase substrate **2** more precisely than it was originally reported (approximately 7 μM (17)). Authors of the original report (17) used the absorbance method, which, at the substrate concentration equal to the previously estimated K_m, gives a total change of absorbance upon complete hydrolysis of 0.006 absorbance units in standard 1-cm cells. This value is at the limit of sensitivity for most research spectrophotometers. In contrast, by using the fluorescence method reported here, we were able to determine the Michaelis constant conveniently as 1.9 ± 0.3 μM. At high ionic strength (2 M NaCl), the K_m decreased even more, into the submicromolar range which is inaccessible by the absorbance method unless the nonstandard 10-cm spectrophotometric cells are used.

Tyrosine may be separated from the quencher (*p*-nitrophenylalanine) by one or more amino acids while still retaining its effect. This flexibility allows continuous fluorescence assays of proteases with diverse specificity. For example, tyrosine fluorescence was used to follow the cleavage of peptide **4** by trypsin.

The principle of monitoring the changes in tyrosine fluorescence upon peptidolysis or proteolysis is likely to find a particularly promising application in the studies of aspartic proteinases in general, and retroviral aspartic proteinases in particular. It has been established that more than 40% of all cleavages due to retroviral proteinases involve a Tyr–Pro bond (22). In fact, the decapeptide Thr–Phe–Gln–Ala–Tyr–Pro–Leu–Arg–Gly–Ala is distributed as a generic substrate for HPLC assays of retroviral proteinases. In a preliminary experiment, the cleavage of this peptide by the HIV-1 proteinase produced the expected fluorescence change, albeit at a much slower rate than peptides **1** and **2**. We use the fluorogenic substrate **2** for routine HIV proteinase assays at initial concentration of 1.0 μM. The exquisite sensitivity of the fluorescence method should prove particularly useful in kinetic studies of mutant forms of the HIV proteinase, isolated from drug-resistant strains of HIV. One may expect that the substrate affinity of the mutated proteinase could decrease significantly, producing orders of magnitude increases in K_m as well as decreases in the catalytic turnover number. The method facilitates kinetic model-discrimination studies of HIV-1 proteinase inhibition by potential anti-HIV chemotherapeutic agents, currently ongoing in this laboratory.

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REFERENCES

1. Cowgill, R. W. (1976) *in* Biochemical Fluorescence: Concepts (R. F. Chenand and H. Edelhoch, Eds.), Vol. 2, pp. 441–486, Dekker, New York.
2. Epps, D. E., Mao, B., Staples, D. J., and Sawyer, T. K. (1988) *Int. J. Peptide Protein Res.* **31,** 22–34.
3. Somogyi, B., and Lakos, Z. (1993) *J. Photochem. Photobiol. B* **18,** 3–16.
4. Eriksson, S., Norden, B., and Takahashi, M. (1993) *J. Biol. Chem.* **268,** 1805–1810.
5. Mach, H., Dong, Z., Middaugh, C. R., and Lewis, R. V. (1991) *Arch. Biochem. Biophys.* **287,** 41–47.
6. Maytus, L. (1992) *J. Photochem. Photobiol.* **12,** 323–337.
7. Yaron, A., Carmel, A., and Katchalski-Katzir, E. (1979) *Anal. Biochem.* **95,** 228–235.
8. García-Echevarriía, C., and Rich, D. H. (1992) *FEBS Lett.* **297,** 100–102.
9. Cheng, Y.-S., Yin, F. H., Foundling, S., Blomstrom, D., and Kettner, C. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9660–9664.
10. Tamburini, P. P., Dreyer, R. N., Hansen, J., Letsinger, J., Elting, J., Gore-Willse, A., Dally, R., Hanko, R., Osterman, D., Karmarck, M. E., and Yoo-Warren, H. (1990) *Anal. Biochem.* **186,** 363–368.
11. Tyagi, S., and Carter, C. (1992) *Anal. Biochem.* **200,** 143–148.
12. Mayatoshi, E. D., Wang, G. T., Kraft, G. A., and Erickson, J. (1990) *Science* **247,** 954–958.
13. Albericio, F., Kneib-Cordonier, N., Biancalana, S., Gera, L., Masada, R. I., Hundson, D., and Barany, G. (1990) *J. Org. Chem.* **55,** 3730–3743.
14. Kuzmič, P., García-Echeverría, C., and Rich, D. H. (1993) *Biochem. Biophys. Res. Commun.* **194,** 301–305.
15. Keil, B. (1970) *in* The Enzymes (P. D. Boyer, Ed.), Vol. 3, pp. 249–275, Academic Press, New York.
16. Tözsér, J., Bláha, I., Copeland, T. D., Wondrak, E. M., and Oroszlan, S. (1991) *FEBS Lett.* **281,** 77–80.
17. Richards, A. D., Phylip, L. H., Farmerie, W. G., Scarborough, P. E., Alvarez, A., Dunn, B. M. A., Hirel, P. H., Konvalinka, J., Štrop, P., Pavlíčková, L., Kostka, V., and Kay, J. (1990) *J. Biol. Chem.* **265,** 7733–7736.
18. Jordan, S. P., Zugay, J., Darke, P. L., and Kuo, L. C. (1992) *J. Biol. Chem.* **267,** 20028–20032.
19. Weber, G., and Rosenheck, K. (1964) *Biopolymers* **1,** 333–341.
20. Seidel, C., Orth, A., and Greulich, K. O. (1993) *Photochem. Photobiol.* **58,** 178–184.
21. Ross, J. B. A., Laws, W. R., Rousslang, K. W., and Wyssbrod, H. R. (1992) *in* Topics in Fluorescence Spectroscopy (J. R. Lakowicz, Ed.), Vol. 3, pp. 1–63, Plenum, New York.
22. Kotler, M., Katz, R. A., Danho, W., Leis, J., and Skalka, A. M. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4185–4189.