J|A|C|S
COMMUNICATIONS

Published on Web 07/27/2002

Fluorescence Resonance Energy Transfer between Unnatural Amino Acids in a Structurally Modified Dihydrofolate Reductase

Raymond D. Anderson, III, Jia Zhou, and Sidney M. Hecht*

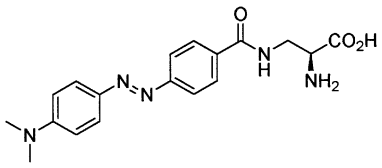
Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia 22904

Received April 25, 2002

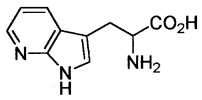
The use of misacylated suppressor transfer RNAs has permitted the elaboration of proteins containing nonnatural amino acids at single, predetermined positions.[1] Most of these studies have involved readthrough of the UAG codon. Recent experiments from the Sisido laboratory demonstrating the use of four-base codons in in vitro protein biosynthesizing systems[2] have provided a strategy for the introduction of multiple nonnatural amino acids into a single protein, although the realization of this strategy is limited both by the overall readthrough efficiency of multiple suppressor tRNAs and by the occasional apparent misfolding of nascent proteins containing modified amino acids.[3]

Presently we demonstrate (i) the synthesis of dihydrofolate reductase having a fusion peptide at its N terminus that contains a fluorescence acceptor and donor flanking an HIV-1 protease cleavage site, (ii) energy transfer between the fluorescence donor and acceptor, and (iii) stoichiometric cleavage of the peptide by HIV-1 protease that can be monitored in real time by fluorescence resonance energy transfer (FRET), with an increase in fluorescence emission intensity quantitatively identical with that anticipated from protein cleavage.

Dabcyl-diaminopropionic acid derivative **1** and 7-azatryptophan (**2**) were chosen for incorporation into the DHFR fusion peptide.



N^{β}-dabcyl-1,2-diaminopropionic acid **(1)**
fluorescence acceptor



7-azatryptophan **(2)**
fluorescence donor

The choice of 7-azatryptophan as fluorescence donor reflects its efficient incorporation into proteins in vitro,[4] as well as its ability to undergo selective excitation in water in the presence of tryptophan.[5,6] Dabcyl-1,2-diaminopropionic acid was selected because it has a broad visible absorption spectrum[7] that overlaps with the 7-azaTrp emission spectrum. In addition, dabcyl-1,2-diaminopropionic acid is nonfluorescent, thereby facilitating the detection of 7-azaTrp.[7]

The nature of the modified protein elaborated for study is illustrated in Figure 1. The mRNA encoding the protein included

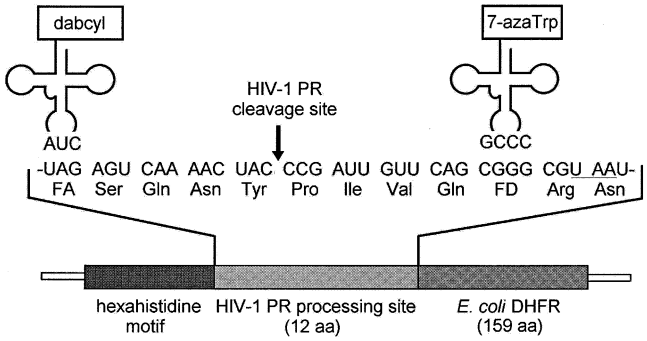


Figure 1. Modified *E. coli* DHFR fusion protein elaboration for study. The encoding nucleotide sequence and derived amino acid sequence of the DHFR fusion peptide are shown. FA, fluorescence acceptor; FD, fluorescence donor.

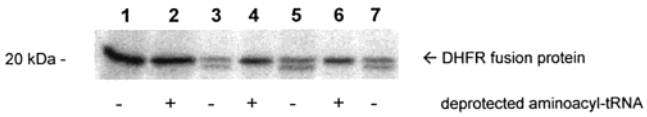


Figure 2. Autoradiogram of a 15% SDS-polyacrylamide gel illustrating the in vitro synthesis of DHFR fusion proteins. Protein synthesis was carried out in vitro in the presence of ^{35}S-methionine, mRNA for "wild-type" DHFR fusion protein (lane 1), mRNA containing a CGGG stop codon (lanes 2 and 3), mRNA containing a UAG stop codon (lanes 4 and 5), or mRNA containing both UAG and CGGG stop codons (lanes 6 and 7). Lane 1, no suppressor tRNA; lane 2, 7-azatryptophanyl-tRNA$_{CCCG}$; lane 3, NVOC 7-azatryptophanyl-tRNA$_{CCCG}$; lane 4, dabcyl-diaminopropionyl-tRNA$_{CUA}$; lane 5 pentenoylated dabcyl-diaminopropionyl-tRNA$_{CUA}$; lane 6, 7-azatryptophanyl-tRNA$_{CCCG}$ and dabcyl-diaminopropionyl-tRNA$_{CUA}$; lane 7, NVOC 7-azatryptophanyl-tRNA$_{CUA}$ and pentenoylated dabcyl-diaminopropionyl-tRNA$_{CUA}$. The faint bands that comigrated with full length protein in lanes 3, 5, and 7 are believed to have resulted from nonsense codon readthrough by noncognate tRNAs.

a UAG codon (to be decoded by dabcyl-diaminopropionyl-tRNA$_{CUA}$) and a four-base CGGG codon[2] (to be decoded by 7-azatryptophanyl-tRNA$_{CCCG}$).[8] As shown, the derived protein contained an HIV-1 protease cleavage site situated between the fluorescence donor and acceptor. Also elaborated were the same proteins containing valines in lieu of dabcyl-1,2-diaminopropionic acid or 7-azatryptophan.

The DHFR fusion proteins were synthesized in an *Escherichia coli* S-30 protein-synthesizing system containing diminished levels of release factor 1.[9] As shown in Figure 2, the presence of deprotected dabcyl-diaminopropionyl-tRNA$_{CUA}$ and deprotected 7-azatryptophanyl-tRNA$_{CCCG}$ were necessary for the synthesis of full length protein, indicating that dabcyl-1,2-diaminopropionic acid and 7-azatryptophan must be incorporated into the DHFR fusion protein at the sites of the UAG and CGGG codons, respectively.[10] Phosphorimager quantitation of the ^{35}S-labeled protein indicated that the yield of the fully modified DHFR fusion protein was 13% that of the "wild-type" fusion protein.[11]

* To whom correspondence should be addressed. E-mail: sidhecht@virginia.edu.

10.1021/ja0205939 CCC: $22.00

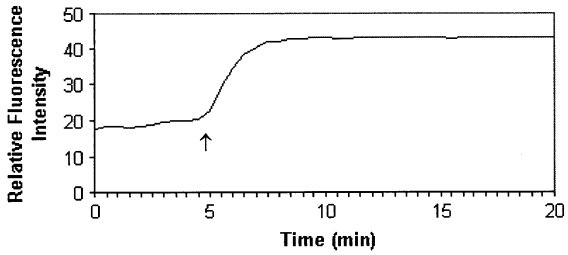


Figure 3. Hydrolytic cleavage of DHFR fusion protein containing **1** and **2** by HIV-1 protease at 23 °C over a period of 20 min as monitored by steady-state fluorescence. A typical assay contained 30 ng (1.5 nM) DHFR fusion protein. Arrow indicates the time of addition of 50 ng of HIV-1 protease to a final concentration of 1.7 pM.

The DHFR fusion proteins were purified on Ni NTA−agarose,[12] and the amount of protein was determined on the basis of quantification of incorporated ^{35}S-methionine. Excitation of the intact DHFR fusion protein containing **1** and **2** produced emission somewhat greater than that produced by excitation of wild-type DHFR or DHFR fusion protein lacking any modified amino acid (not shown); the background fluorescence in wild-type DHFR was due to the five tryptophan residues present. In contrast, treatment of the purified DHFR fusion protein containing **1** and **2** with HIV-1 protease[13] resulted in a significant increase in light production upon subsequent irradiation[14] (Figure 3). These observations indicated efficient energy transfer between the fluorescence donor and acceptor in the fusion peptide.[15] That this increase had resulted from cleavage at the protease cleavage site,[7] and consequent physical separation of the fluorescence donor and acceptor, was verified by polyacrylamide gel electrophoretic analysis of the HIV-1 protease-treated DHFR fusion proteins (Figure 1, Supporting Information). The cleaved proteins were also shown to bind to methotrexate−agarose and to convert dihydrofolate to tetrahyrofolate with the same efficiency as authentic DHFR (Table 1, Supporting Information). Further, a solution of the intact DHFR fusion protein to which a stoichiometric amount of free 7-azatryptophan had been added produced the same fluorescence intensity following excitation as the HIV-1 protease-treated fusion protein.

These experiments demonstrate the elaboration of a fusion protein containing fluorescence donor and acceptor amino acids. They illustrate energy transfer between the incorporated probes, as well as the feasibility of monitoring the (proteolytic) separation of the donor and acceptor in real time by the decrease in fluorescence energy transfer. It seems reasonable to anticipate that suitably placed reporter groups may also be able to monitor related events such as alteration of protein conformation or protein denaturation.

Acknowledgment. We thank Dr. James Demas and Zachary J. Fuller for assistance with fluorescence measurements. This work was supported at the University of Virginia by Research Grant CA77359 from the National Cancer Institute.

Supporting Information Available: PAGE analysis of DHFR fusion protein cleavage by HIV-1 protease and analysis of the properties of the cleaved fusion protein (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182−188. (b) Ellman, J. A.; Mendel, D.; Schultz, P. G. *Science* **1992**, *255*, 197−200. (c) Mendel, D.; Ellman, J. A.; Chang, Z.; Veenstra, D. L.; Kollman, P. A.; Schultz, P. G. *Science* **1993**, *256*, 1798−1802. (d) Mendel, D.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1993**, *115*, 4359−4360. (e) Nowak, M. W.; Kearney, P. C.; Sampson, J. R.; Saks, M. E.; Labarca, C. G.; Silverman, S. K.; Zhong, W.; Thorson, J.; Abelson, J. N.; Davidson, N.; Schultz, P. G.; Dougherty, D. A.; Lester, H. A. *Science* **1995**, *268*, 439−442. (f) Mamaev, S. V.; Laikhter, A. L.; Arslan, T.; Hecht, S. M. *J. Am. Chem. Soc.* **1996**, *118*, 7243−7244. (g) Saks, M. E.; Sampson, J. R.; Nowak, M. W.; Kearney, P. C.; Du, F.; Abelson, J. N.; Lester, H. A.; Dougherty, D. A. *J. Biol. Chem.* **1996**, *271*, 23169−23175. (h) Steward, L. E.; Collins, C. S.; Gilmore, M. A.; Carlson, J. E.; Ross, J. B. A.; Chamberlin, A. R. *J. Am. Chem. Soc.* **1997**, *119*, 6−11. (i) Arslan, T.; Mamaev, S. V.; Mamaeva, N. V.; Hecht, S. M. *J. Am. Chem. Soc.* **1997**, *119*, 10877−10887. (j) England, P. M.; Lester, H. A.; Davison, N.; Dougherty, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 11025−11030. (k) Karginov, V. A.; Mamaev, S. V.; An, H.; Van Cleve, M. D.; Hecht, S. M.; Komatsoulis, G. A.; Abelson, J. N. *J. Am. Chem. Soc.* **1997**, *119*, 8166−8176. (l) Short, G. F., III; Lodder, M.; Laikhter, A. L.; Arslan, T.; Hecht, S. M. *J. Am. Chem. Soc.* **1999**, *121*, 478−479. (m) Wang, B.; Lodder, M.; Zhou, J.; Baird, T. T., Jr.; Brown, K. C.; Craik, C. S.; Hecht, S. M. *J. Am. Chem. Soc.* **2000**, *122*, 7402−7403. (n) Wang, B.; Brown, K. C.; Lodder, M.; Craik, C. S.; Hecht, S. M. *Biochemistry* **2002**, *41*, 2805−2813.

(2) (a) Hohsaka T.; Ashizuka Y.; Sasaki, H.; Murakami, H.; Sisido M. *J. Am. Chem. Soc.* **1999**, *121*, 12194−12195. (b) Hohsaka, T.; Ashizuka, Y.; Taira, H.; Murakami, H.; Sisido, M. *Biochemistry* **2001**, *40*, 11060−11064.

(3) (a) Murakami, H.; Hohsaka, T.; Ashizuka, Y.; Hashimoto, K.; Sisido, M. *Biomacromolecules* **2000**, *1*, 118−125. (b) Muranaka, N.; Hohsaka, T.; Sisido, M. *FEBS Lett.* **2002**, *510*, 10−12.

(4) (a) Cornish, V. W.; Benson, D. R.; Altenbach, C. A.; Hideg, K.; Hubbell, W. L.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2910−2914. (b) Steward, L. E.; Collins, C. S.; Gilmore, M. A.; Gilmore, M. A.; Carlson, J. E.; Ross, J. B. A.; Chamberlin, A. R. *J. Am. Chem. Soc.* **1997**, *119*, 6−11. (c) Hohsaka, T.; Kajihara, D.; Ashizuka, Y.; Murakami, H.; Sisido, M. *J. Am. Chem. Soc.* **1999**, *121*, 34−40.

(5) Negerie, M.; Bellefeuille, S. M.; Whitham, S.; Petrich, J. W.; Thornburg, R. W. *J. Am. Chem. Soc.* **1990**, *112*, 7419−7421.

(6) In water 7-azatryptophan exhibits absorption and emission maxima at 288 and 410 nm, respectively, while tryptophan exhibits the respective maxima at 280 and 398 nm.

(7) Matayoshi, E. D.; Wang, G. T.; Krafft, G. A.; Erickson, J. *Science* **1990**, *247*, 954−958.

(8) Translation of the intended CGGG codon as CGG would result in termination of translation at the (underlined) downstream UAA codon.

(9) Short, G. F., III; Golovine, S. Y.; Hecht, S. M. *Biochemistry* **1999**, *38*, 8808−8819.

(10) N^{α}-pentenoylated dabcyl-diaminopropionyl-tRNA$_{CUA}$ (Lodder, M.; Golovine, S.; Laikhter, A. L.; Karginov, V. A.; Hecht, S. M. *J. Org. Chem.* **1998**, *63*, 794−803) and N^{α}-NVOC 7-azatryptophanyl-tRNA$_{CCCG}$ (Robertson, S. A.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2722−2729) were prepared in analogy with previous reports.

(11) This was the yield anticipated on the basis of 24% readthrough by dabcyl-diaminopropionyl-tRNA$_{CUA}$ of a construct containing only the UAG codon, and 51% readthrough by 7-azatryptophanyl-tRNA$_{CCCG}$ of a construct containing only the CGGG codon.

(12) Janknecht, R.; de Martynoff, G.; Lou, J.; Hipskind, R. A.; Nordheim, A.; Stunnenberg, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8972−8976.

(13) Short, G. F., III; Laikhter, A. L.; Lodder, M. L.; Shayo, Y.; Arslan, T.; Hecht, S. M. *Biochemistry* **2000**, *39*, 8768−8781.

(14) Spectral measurements were taken with a Spex Fluorolog 2+2 Spectrofluorimeter with excitation and emission wavelengths of 288 and 410 nm, respectively.

(15) The maximum distance between the fluorescence acceptor and donor has been estimated as 29 Å, and efficient energy transfer between dyes in the same peptide has been documented previously.[7]

JA0205939