J|A|C|S
COMMUNICATIONS

Published on Web 06/11/2003

The Puromycin Route to Assess Stereo- and Regiochemical Constraints on Peptide Bond Formation in Eukaryotic Ribosomes

Shelley R. Starck, Xin Qi, Brett N. Olsen, and Richard W. Roberts*

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125

Received February 22, 2003; E-mail: rroberts@.caltech.edu

The protein synthesis machinery can be used to incorporate unnatural amino acids into peptides,[1] proteins,[2] and molecular libraries[3] (see ref 4 for reviews). These studies indicate that the ribosome displays a broad ability to utilize residues beyond the 20 naturally occurring amino acids. Chemically misacylated tRNA fragments and tRNAs have provided one route to probe the stereo- and regiospecificity of isolated ribosomes[5] and intact translation systems.[4b,6] This approach has expanded our understanding of the range of residues incorporated by the ribosome.[7] However, entry of both β- and D-amino acids has proved challenging.[1b,2a,5] Analysis of these residues would deepen our understanding of the stereo- and regiochemical constraints of ribosome-mediated peptide bond formation.

Here, we have used a series of synthetic puromycin analogues to measure the activity of both β- and D-amino acids in an intact eukaryotic translation system. Puromycin is a small-molecule mimic of aminoacyl-tRNA (aa-tRNA), and acts as a universal translation inhibitor by entering the ribosomal A site and participating in peptide bond formation with the nascent peptidyl chain.[8] Puromycin and puromycin analogues have been very useful in exploring the activity of nucleophiles (−OH vs $-NH_2$ vs −SH) in peptide bond formation and the structural requirements for inhibition of translation.[9] Unlike aa-tRNA, puromycin is able to enter the ribosome independently, does not induce EF-Tu·GTPase activity,[10] and does not require soluble translation factors for function.[11] Puromycin and related compounds therefore provide a direct means to address ribosome-mediated peptide bond formation in the context of a fully competent translation extract.

We synthesized a series of puromycin derivatives (Figure 1) that differ in the (1) amino acid moiety, (2) amino acid stereochemistry, and (3) number of carbon units in the amino acid backbone. We then measured the activity of each compound (Figure 2) in a high dynamic-range IC_{50} potency assay using the rabbit reticulocyte protein synthesis system.[11] The naturally occurring compound, L-puromycin (**1a**), inhibits globin mRNA translation with an IC_{50} of 1.8 μM (Figure 2). Surprisingly, D-puromycin (**1b**) also inhibited translation giving an IC_{50} of 280 μM (Figure 2), a difference of 150-fold.

We reasoned that stereoselectivity should be a function of the side-chain size and geometry. To test this, we constructed compounds where the puromycin side chain was altered to bear either a bulky (L- or D-4-methyl-phenylalanine; **2a** and **2b**), or a small substituent (L- or D-alanine; **3a** and **3b**). Compound **2a** inhibits translation better than puromycin itself (IC_{50} = 1.0 μM; Figure 2C) and is the most potent compound we constructed. The D-amino acid variant (**2b**) shows much lower activity (IC_{50} = 2400 μM), is ~9-fold lower than D-puromycin (**1b**), and is 2400-fold less potent than the L-isomer. The alanine analogues (R = CH_3) show only 3-fold selectivity for the L- versus D- isomers (**3a** vs **3b**; Figure 2C). These observations argue that ribosomal stereoselectivity falls

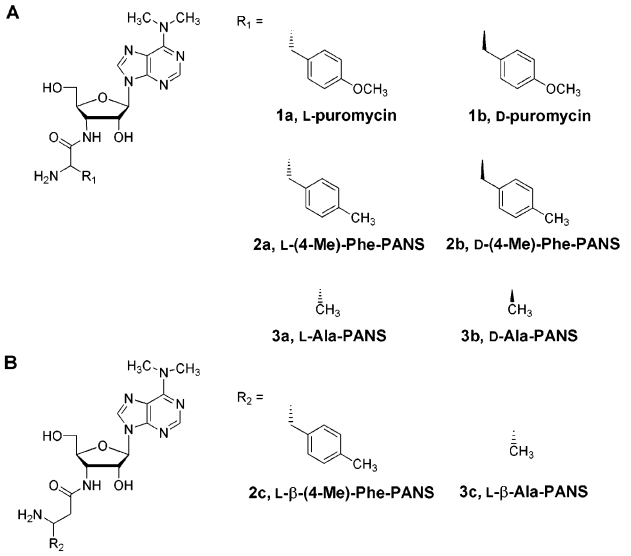

Figure 1. Puromycin analogues with (A) L- and D-α-amino acid side chains and (B) L-β-amino acid side chains.

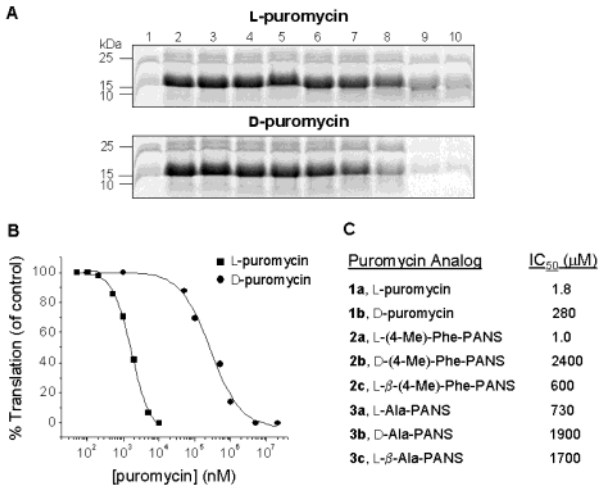

Figure 2. IC_{50} determination for puromycin and puromycin analogues, **1a**−**3c**. (A) Tricine-SDS-PAGE analysis of [^{35}S]Met-globin translation reactions in the presence of L-puromycin (**1a**) and D-puromycin (**1b**): Lane 1, no template; lane 2, globin alone; lanes 3−10, concentrations from 50 nM to 10 mM for **1a** and from 100 nM to 20 mM for **1b**. (B) Percent globin translation relative to the globin only control for L-puromycin (**1a**) and D-puromycin (**1b**). (C) IC_{50} values for puromycin analogues **1a**−**3c**.

over a broad range and is primarily dictated by the size and geometry of the pendant side chain. Within the L-amino acid series (**1a**, **2a**, and **3a**), marked variation is also seen based solely on side chain identity. Larger, hydrophobic side chains provide improved function, consistent with previous observations.[9c−d] In the D-amino acid series, the 4-O-methyltyrosine derivative (**1b**)

10.1021/ja034817e CCC: $25.00

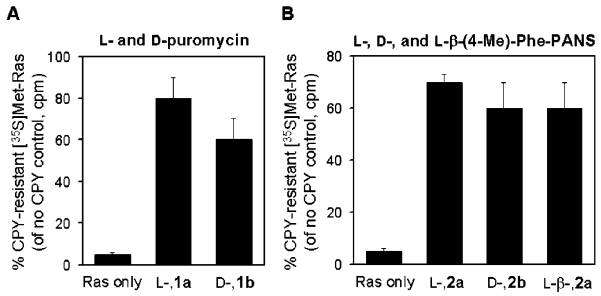

Figure 3. Carboxypeptidase Y (CPY) analysis of protein−puromycin products.[11] TCA precipitation of [^{35}S]Met-protein (Ras) from translation reactions after CPY treatment containing (A) Ras only, L-puromycin (**1a**) at 2 μM, and D-puromycin (**1b**) at 500 μM and (B) Ras only, L-(4-Me)-Phe-PANS (**2a**) at 1 μM, D-(4-Me)-Phe-PANS (**2b**) at 1500 μM, and L-β-(4-Me)-Phe-PANS (**2c**) at 1000 μM. Data represent the mean ± standard error for at least three independent experiments.

functions the best overall, and has ~3-fold better activity than the natural L-alanine variant (**3a**).

We next examined puromycin derivatives bearing β-amino acids. β-Amino acids have been previously incorporated at low levels using nonsense suppression techniques.[1c,2a] In our experiments, both L-β-(4-Me)-Phe-PANS (**2c**) and L-β-Ala-PANS (**3c**) were able to fully inhibit translation (IC_{50} = 600 and 1700 μM, respectively).

Finally, we wished to confirm that our puromycin analogues participated in peptide bond formation within the ribosome. Incorporation of puromycin blocks the C terminus, rendering the protein carboxypeptidase resistant.[8] Previous work in our laboratory demonstrated that covalent puromycin incorporation is most efficient near the IC_{50} value.[11] Consistent with this observation, protein synthesis performed in the presence of our puromycin derivatives near the IC_{50} resulted in a 12- to 16-fold increase in carboxypeptidase Y (CPY)-resistant protein compared with the no-drug control (Figure 3, A and B). All derivatives also produce truncated protein fragments, consistent with entry and attachment both internally and at the end of the template (Supporting Information, Figure S1).[11]

The structural basis for stereoselectivity in rabbit ribosomes cannot be addressed presently, as there are no high-resolution structures available. However, modeling D-puromycin (**1b**) into the active site of the *Haloarcula marismortui* 50S subunit[12] is consistent with the idea that steric effects play a role in chiral discrimination. In the atomic resolution structure, U2620 (U2585 *E. coli*) is the closest nucleotide to the D-side chain (see model in Supporting Information). Also, while many of the ribosome active-site nucleotides are highly conserved, the fact that critical residues can be mutated,[13] implies that construction of ribosomes with altered stereo- and regiospecificity may be possible.

Our data lead us to conclude that L-, D-, and β-amino acids can participate in ribosome-mediated peptide bond formation when constructed as analogues of puromycin. This route allows us to rank both natural and unnatural residues as substrates in a physiologically complete protein-synthesizing system. Analysis using intact systems is important as reconstituted or purified systems that are incapable of synthesizing proteins can produce markedly different results.[4b,11,14] The data here provide one metric of the chiral and regiospecificity of mammalian ribosomes. We are hopeful that this approach, along with other information such as the ability to optimize tRNA affinity for elongation factor Tu (EF-Tu)[15] (EF1A in eukaryotes), will facilitate the incorporation of desirable but recalcitrant unnatural residues into peptides and proteins.

Acknowledgment. This work was supported by NIH Grant (R01 60416) to R.W.R. and by NIH training Grant GM07616 (S.R.S.).

Supporting Information Available: Tricine-SDS-PAGE analysis of protein−puromycin products (Figure S1); model of D-puromycin−ribosome complex (Figure S2); experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Heckler, T. G.; Zama, Y.; Naka, T.; Hecht, S. M. *J. Biol. Chem*. **1983**, *258*, 4492−4495. (b) Bain, J. D.; Glabe, C. G.; Dix, T. A.; Chamberlin, A. R. *J. Am. Chem. Soc*. **1989**, *111*, 8013−8014. (c) Bain, J. D.; Wacker, D. A.; Kuo, E. E.; Chamberlin, A. R. *Tetrahedron* **1991**, *47*, 2389−2400.

(2) (a) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182−188. (b) 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.

(3) (a) Li, S.; Millward, S.; Roberts, R. W. *J. Am. Chem. Soc*. **2002**, *124*, 9972−9973. (b) Takahashi, T. T.; Austin, R. J.; Roberts, R. W. *Trends Biochem. Sci*. **2003**, *28*, 159−165.

(4) (a) Ellman, J.; Mendel, D.; Anthony-Cahill, S.; Noren, C. J.; Schultz, P. G. *Methods Enzymol.* **1992**, *202*, 301−336. (b) Hecht, S. M. *Acc. Chem. Res*. **1992**, *25*, 545−552. (b) Cornish, V. W.; Mendel, D.; Schultz, P. G. *Angew. Chem., Int. Ed. Engl*. **1995**, *34*, 621−633. (c) Gillmore, M. A.; Steward, L. E.; Chamberlin, A. R. *Top. Curr. Chem*. **1999**, *202*, 77−99. (d) van Hest, J. C. M.; Tirrell, D. A. *Chem. Commun.* **2001**, *19*, 1897−1904.

(5) (a) Yamane, T.; Miller, D. L.; Hopfield, J. J. *Biochemistry* **1981**, *20*, 7059−7064. (b) Chládek, S.; Sprinzl, M. *Angew. Chem., Int. Ed. Engl*. **1985**, *24*, 371−391. (c) Heckler, T. G.; Roesser, J. R.; Xu, C.; Chang, P.-I.; Hecht, S. M. *Biochemistry* **1988**, *27*, 7254−7262. (d) Roesser, J. R.; Xu, C.; Payne, R. C.; Surratt, C. K.; Hecht, S. M. *Biochemistry* **1989**, *28*, 5185−5195.

(6) Mendel, D.; Ellman, J.; Schultz, P. G. *J. Am. Chem. Soc*. **1993**, *115*, 4359−4360.

(7) (a) Killian, J. A.; Van Cleve, M. D.; Shayo, Y. F.; Hecht, S. M. *J. Am. Chem. Soc*. **1998**, *120*, 3032−3042. (b) Koh, J. T.; Cornish, V. W.; Schultz, P. G. *Biochemistry* **1997**, *36*, 11314−11322. (c) Eisenhauer, B. M.; Hecht, S. M. *Biochemistry* **2002**, *41*, 11472−11478. (d) England, P. M.; Zhang, Y.; Dougherty, D. A.; Lester, H. A. *Cell* **1999**, *96*, 89−98.

(8) Nathans, D. *Proc. Natl. Acad. Sci. U.S.A.* **1964**, *51*, 585−592.

(9) (a) Fahnestock, S.; Neumann, H.; Shashoua, V.; Rich, A. *Biochemistry* **1970**, *9*, 2477−2483. (b) Gooch, J.; Hawtrey, A. O. *Biochem. J*. **1975**, *149*, 209−220. (c) Nathans, D.; Neidle, A. *Nature* **1963**, *197*, 1076−1077. (d) Harris, R. J.; Hanlon, J. E.; Symons, R. H. *Biochim. Biophys. Acta* **1971**, *240*, 244−262.

(10) Campuzano, S.; Modolell, J. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 905−909.

(11) Starck, S. R.; Roberts, R. W. *RNA* **2002**, *8*, 890−903.

(12) Nissen, P.; Hansen, J.; Ban, N.; Moore, P. B.; Steitz, T. A. *Science* **2000**, *289*, 920−930.

(13) Polacek, N.; Gaynor, M.; Yassin, A.; Mankin, A. S. *Nature* **2001**, *411*, 498−501.

(14) (a) Krayevsky, A. A.; Kukhanova, M. K. *Prog. Nucleic Acids Res*. **1979**, *23*, 2−51. (b) Bhuta, P.; Kumar, G.; Chládek, S. *Biochim. Biophys. Acta* **1982**, *696*, 208−211.

(15) (a) LaRiviere, F. J.; Wolfson, A. D.; Uhlenbeck, O. C. *Science* **2001**, *294*, 165−168. (b) Asahara, H.; Uhlenbeck, O. C. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 3499−3504.

JA034817E