Abstract

Puromycin is a protein synthesis inhibitor that acts as a structural analog of an aminoacyl-tRNA. The ribosome mistakenly inserts puromycin in place of aminoacyl-tRNA resulting in truncated proteins containing the drug at their C-terminus. Herein, the puromycin reaction is re-examined in a cell-free eukaryotic translation extract and in live cells. The framework for puromycin reactivity in terms of potency, product distribution, and mechanism is studied *in vitro*. These insights are used to develop a series of fluorescent puromycin-based reagents to detect protein expression in living cells that does not require transfection, radiolabeling, or the prior choice of a candidate gene. Further, puromycin is used to examine the stereo- and regiochemical nature of protein synthesis both *in vitro* and *in vivo*. These data indicate that the ribosome tolerates a broader range of substrates than previously recognized, including D-amino acids. Overall, these studies yield a series of insights about protein synthesis and the ribosome, including mechanistic observations, the development of reagents to explore the proteome, and a theory about the evolution of amino acid homochirality.