RAPID DEGRADATION OF PUROMYCYL PEPTIDES IN HEPATOMA CELLS AND RETICULOCYTES

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1. Introduction

Puromycin inhibits protein synthesis by substituting for an amino-acyl tRNA on ribosomes, causing release of growing polypeptide chains [1, 2]. It has been suggested that these released peptides accumulate within the cell, causing secondary effects on RNA synthesis, [3] carbohydrate metabolism [4] and respiration [5]. However, early studies with reticulocytes favoured the alternative idea that proteolytic enzymes might degrade most of the polypeptides released by puromycin [6, 7]. More recent studies have shown that in *E. coli* puromycyl peptides are rapidly degraded [8], possibly after their transient accumulation into intracellular granules [9]. This communication provides evidence for a mechanism in both reticulocytes and hepatoma cells which very rapidly degrades puromycyl peptides. This degradative ability is completely lost in cell-free systems, implying that it may require some structure normally removed from these preparations.

2. Materials and methods

Rat hepatoma cells (HTC; source Dr. G. M. Tomkins) were grown in suspension culture in Swims S77 medium containing 10% calf serum [10]. Amino acid incorporation into protein at 37°C was measured using radioactive leucine at concentrations shown in the figure legends. Replicate 1 ml aliquots containing 3.5–4.0 × 10^5 cells were removed and the cells spun down, washed in saline and lysed in 0.5 ml 0.5% w/v Na lauryl sulphate followed by addition of 1 ml 1 N NaOH and incubation for 20 min at 37°C. 1.5 ml 25% w/v trichloroacetic acid was added, and the precipitates collected onto glass fibre filters for scintillation counting.

The HTC S10 was prepared from 5 × 10^8 cells which were washed, and then homogenised in twice their packed volume of buffer (10 mM KCl, 1.5 mM $(CH_3COO)_2Mg$, 20 mM Tris; pH 7.5). 1/10 volume of 10 × concentrated solution A (Solution A – 125 mM KCl, 4 mM $(CH_3COO)_2Mg$, 6 mM mercaptoethanol, 25 mM Tris; pH 7.5) was added and the homogenate centrifuged for 10 min at 10 000 *g* (4°C). The lipid-containing layer was discarded and the remainder passed through Sephadex G25 in solution A. Incubations were as given by Balkow and Korner [14], but with 125 mM KCl and 4 mM $(CH_3COO)_2Mg$, in a volume of 1 ml. Additions of radioactivity were as in the figure legends. Aliquots of 30–50 μl were dispensed into 0.5 ml 0.3 N NaOH, incubated at 37°C for 20 min and precipitated with 3 ml 10% w/v CCl_3COOH.

Rabbit reticulocytes were prepared as described [11]. Incubations were with 0.25 ml of packed cells diluted to 1 ml in standard medium [12]. Aliquots of 50 μl were precipitated with 3 ml 0.5 M perchloric acid, dissolved in 1 ml of 1 N NaOH, incubated at 37°C for 20 min, and then reprecipitated with 1 ml 2 M CCl_3COOH. Reticulocyte lysates prepared by the method of Lamfrom and Knopf [13], were incubated according to Balkow and Korner [14] with 25 μM haem and 1 μCi/ml [^{14}C] leucine. Incubation volumes were of 1 ml, and aliquots of 50 μl were treated as for the HTC S10.

3. Results and discussion

HTC cells incorporated [^{14}C]leucine into protein linearly for 30 min or more. However, if 0.2–1 mM puromycin is added after 5 min there is an immediate loss of 14–20% of the CCl_3COOH precipitable radioactivity, which is virtually complete within one minute (fig. 1). The most likely explanation for this is a very rapid degradation of some of the acid precipitable material, presumably peptides released by puromycin. A similar experiment using reticulocytes shows an inhibition of incorporation of [^{14}C] leucine after puromycin addition, but only a small loss of CCl_3COOH precipitable counts (fig. 2a). This difference may result from a lower permeability of reticulocytes to puromycin, giving a lower intracellular concentration of inhibitor than in HTC cells. This would allow amino acid incorporation to continue at a reduced rate and to balance the loss of radioactivity due to degradation of puromycyl peptides. If this is so, then reducing the amount of puromycin added to HTC cells should give a result similar to that obtained with reticulocytes in fig. 2a and this is shown to be the case in fig. 1b. Fig. 2b further confirms that the difference between the two cell types may be only in permeability to the inhibitor. Reticulocytes were incubated with 0.05 mM puromycin (inhibiting amino acid incorporation by about 50%). After 5 min 0.1 mM cycloheximide was added to inhibit protein synthesis completely (cycloheximide alone gives no loss of CCl_3COOH precipitable counts). There was an immediate loss of radioactivity from the acid precipitable fraction which amounted to some 33% of the total counts incorporated at 5 min, indicating that reticulocytes are just as efficient as HTC cells in removing puromycyl peptides.

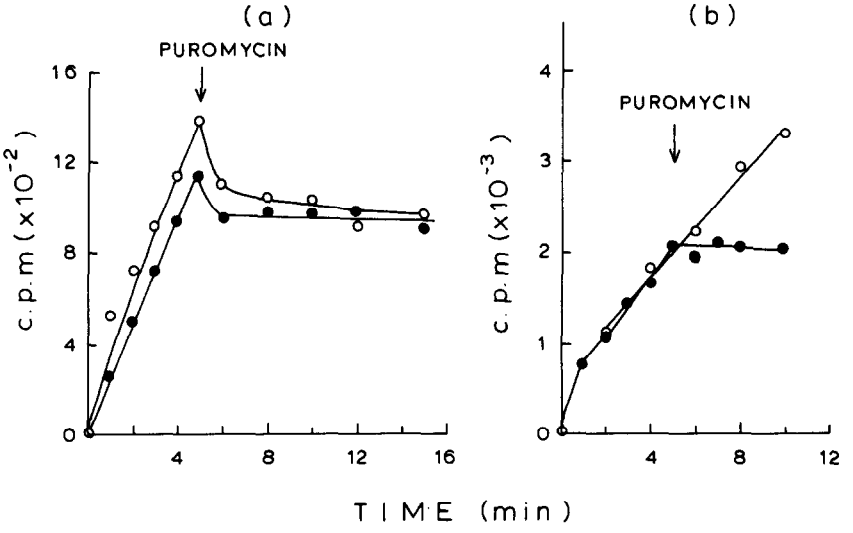

Fig. 1. Effect of puromycin on the incorporation of radioactive leucine into protein by intact HTC cells. (a) HTC cells incubated with 0.5 μCi/ml [^{14}C] leucine (50 μCi/ml; 342 mCi/mmol) (o——o) 1 mM puromycin dihydrochloride (Serva Feinbiochemica) added after 5 mins. (Similar results were obtained with 0.2 mM puromycin). (•——•) 1 mM puromycin and 1 mM leucine (unlabelled added at 5 mins. (b) Incubation with 0.5 μCi/ml [^{3}H] leucine. (1 Ci/ml; 50 Ci/mmol): (•——•) 0.1 mM puromycin added; (o——o) Control – no additions.

That it is the puromycyl peptides which are degraded was confirmed by the use of [^{3}H] puromycin. When reticulocytes were incubated with [^{3}H] puromycin (diluted with cold puromycin to give a final concentration of 0.05 mM) for 15 min, and then cycloheximide added, there was an immediate loss of CCl_3COOH precipitable radioactivity (fig. 2c). Similar results were obtained with HTC cells (not shown). However, in neither case are all the acid

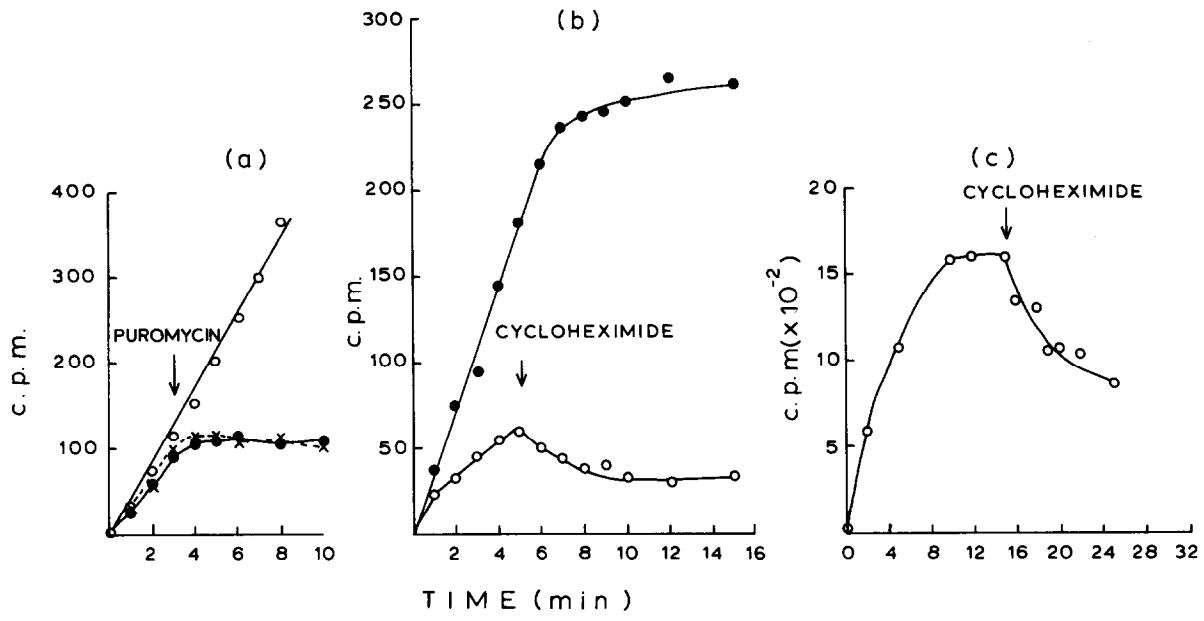

Fig. 2. Effect of puromycin on [^{14}C] leucine incorporation by intact rabbit reticulocytes and degradation of [^{3}H] puromycyl peptides. (a) Incubations with 1 μCi/ml [^{14}C] leucine. (o——o) Control reticulocytes incubated with no further additions. (•——•) 0.2 mM puromycin added at 3 min; (X- - - -X) 0.2 mM puromycin and 5 mM unlabelled leucine added at 3 min. (b) Incubations with 1 μCi/ml [^{14}C] leucine. (•——•) Control reticulocytes. (o——o) Reticulocytes incubated in the presence of 0.05 mM puromycin. 0.1 mM cycloheximide added after 5 min. (c) (o——o) Time course of incorporation of [^{3}H] puromycin dihydrochloride (0.4 mCi/ml; 3.7 Ci/mmol, Radiochemical Centre, Amersham) into intact rabbit reticulocytes. Incubation was with 5 μCi/ml [^{3}H] puromycin made up to a final conen. of 0.05 mM with non-radioactive puromycin. 0.1 mM cycloheximide was added at 5 min.

precipitable counts lost, suggesting that some puromycyl peptides (perhaps those above a certain length) can fold into a conformation which is resistant to proteolysis).

There have been reports that cell-free systems optimised for protein synthesis are unable to degrade proteins which normally have a short half life in intact cells [15]. To see whether this is true also of puromycyl peptides, we repeated our experiments in lysates of HTC cells and reticulocytes. No CCl_3COOH precipitable radioactivity was lost on addition of puromycin to an HTC S10 (fig. 3a) or a reticulocyte lysate (unpublished results). Moreover, incorporation of [^{3}H] puromycin, or of [^{14}C] leucine plus 0.05 mM puromycin, followed by addition of 0.1 mM cycloheximide gave no loss of CCl_3COOH precipitable counts (fig. 3b and unpublished results). Therefore it has been concluded that there is no degradation of puromycyl peptides in either of these two systems.

It has been suggested [6] that in reticulocytes only internally initiated peptide chains released by puromycin are degraded by proteolytic enzymes, while released peptides initiated from the correct N-terminus are stable because they have a three-dimensional structure resistant to proteolysis. This hypothesis was based on experiments in which excess cold amino acids were added with puromycin (0.2 mM) to reticulocytes previously incubated with [^{14}C] amino acids, so that radioactivity in released peptides was confined to those already initiated at the correct N-terminus. When this procedure was adopted under our conditions of incubation in reticulocytes there was very little difference in the results (fig. 2a). However, with HTC cells there was some reduction in the loss of CCl_3COOH precipitable radioactivity following addition of puromycin (fig. 1a). This suggests that while some of the degraded

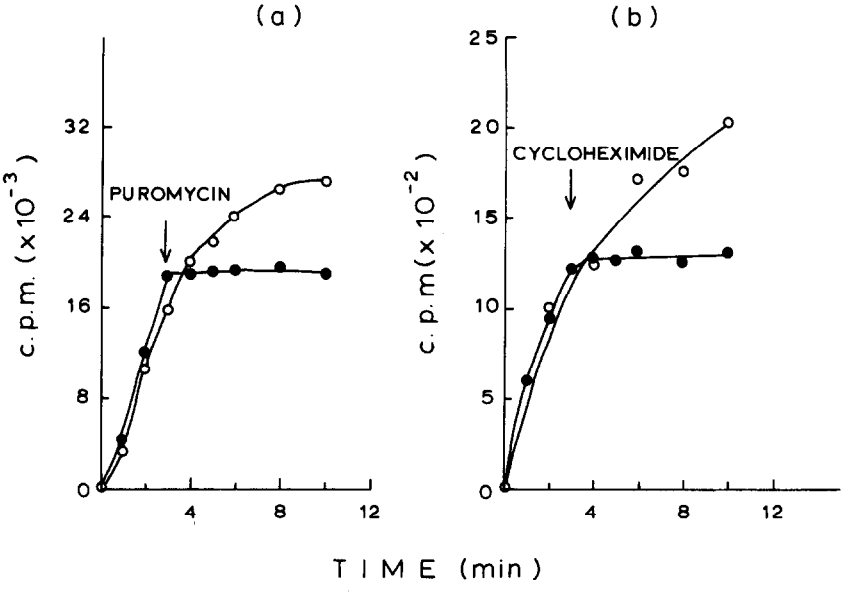

Fig. 3. Effect of adding puromycin to an HTC S10 and the stability of [^{3}H] puromycyl peptides in this system. (a) With 1 μCi/ml [^{14}C] leucine in the incubation medium: (o——o) Control – no additions; (•——•) 1 mM puromycin added at 3 min. (b) 5 μCi/ml [^{3}H] puromycin (1.4 μM) added to incubation medium: (o——o) Control; (•——•) 0.1 mM cycloheximide added at 3 min.

peptides may be internally initiated, others do have a correct N-terminal sequence.

The present findings provide strong evidence for the existence of a cellular mechanism for the degradation of peptides containing puromycin. This is indicative of a more general process for eliminating proteins which have changes in their tertiary structure making them more susceptible to proteolysis. It has been shown in both *E. coli* [6, 19] and in reticulocytes [16] that proteins containing amino acid analogues are also selectively broken down. The idea that structural changes are recognized is also supported by the finding [17] that conditions which increase susceptibility of proteins to proteolysis in *E. coli* enhance their sensitivity to well-characterised endopeptidases in vitro. Natural 'abnormal' proteins could be those produced by errors in transcription or translation, or those which become denatured randomly with age.

Cell-free protein synthesizing systems of both reticulocytes and HTC cells are unable to degrade puromycyl peptides. During preparation nuclei, mitochondria and other organelles, as well as large membrane fragments are removed, leaving mainly cytosol and the small amount of microsomes present in these cells. Measurement of proteolytic activity in various sub-cellular fractions in rat liver [18] has indicated that the cytosol has very low activity. Perhaps the proteolytic enzymes concerned are in the lysosomes or are found associated with large membranes fragments.

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