POLY r-U DIRECTED AMBIGUOUS AMINO ACID INCORPORATION IN THE RABBIT RETICULOCYTE SYSTEM

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Received August 12, 1969

Summary

In a cell-free protein synthesizing system obtained from rabbit reticulocytes, poly r-U stimulated ambiguous incorporation of radioactively labelled leucine, serine, and valine only when unlabelled phenylalanine was added to the incubation mixture.

In the bacterial protein synthesizing system, poly r-U directs the incorporation of leucine, isoleucine and to a lesser extent serine, tyrosine, and valine, in addition to the usual incorporation of phenylalanine. Several factors including Mg^{++}, pH and aminoglycosidic antibiotics control the extent of this ambiguous incorporation (1-6). Poly r-U directed ambiguous amino acid incorporation has also been observed in the mammalian systems (7-9).

In this paper, we present evidence that in the rabbit reticulocyte system, poly r-U directed ambiguous amino acid incorporation is dependent on added phenylalanine. Amino acid incorporation was studied using washed reticulocyte ribosomes prepared as previously described (10). Such ribosomal preparations are essentially free of endogenous amino acids and tRNA's, but retain all the enzymic activities necessary for protein synthesis, and efficiently incorporate amino acids in response to added polyribonucleotide messengers (11).

Materials and Methods

Washed, twice pelleted, reticulocyte ribosomes used in these experiments were prepared from anemic rabbit blood by the procedure described previously (10). Poly r-U was purchased from Miles Laboratory. The absorption spectra of the material in solution was characteristic of poly r-U and indicated that

no other nucleoside was present. Other materials used in these experiments were the same as before; amino acid incorporation was assayed by the procedure described previously for homopolyribonucleotide messengers (11).

Results

Under standard amino acid incorporation conditions (8 mM Mg^{++}), poly r-U stimulated the incorporation of only phenylalanine; no significant incorporation of any other amino acid was observed. However, when unlabelled phenylalanine was added to the incubation mixture, poly r-U stimulated significant incorporation of (^{14}C) labelled leucine, serine, and valine. Under these

TABLE I

(^{14}C) Amino Acid Incorporation Directed by Poly r-U

Amino Acid	(^{14}C) Amino Acid Incorporated (μμmoles/ml)			
	-Unlabelled Phenylalanine		+Unlabelled Phenylalanine	
	-Poly r-U	+Poly r-U	-Poly r-U	+Poly r-U
Asp	10	8	7	8
Cys	30	30	30	34
Gly	9	9	8	8
Ileu	5	7	6	7
Leu	25	27	24	59
Phe	37	1,761	--	--
Pro	16	14	14	14
Ser	18	24	24	46
Threo	20	17	19	18
Tyro	12	10	11	9
Val	24	22	25	40

Standard amino acid incorporation conditions (11) were used. Radioactive amino acids were tested separately in the absence of other unlabelled amino acids, except where, as indicated, 60 mμ moles of unlabelled phenylalanine was added per ml of reaction mixture. Incubation period was 2 hours.

conditions no significant incorporation of isoleucine or tyrosine could be detected. The results of amino acid incorporation in the presence and absence of unlabelled phenylalanine are shown in Table I.

The time course of (^{14}C) phenylalanine incorporation with and without unlabelled leucine, and (^{14}C) leucine incorporation with and without unlabelled phenylalanine is shown in Fig. 1. There was slight stimulation of phenylalanine incorporation in the presence of unlabelled leucine, but (^{14}C) leucine incorporation was completely dependent on added unlabelled phenylalanine during the whole course of the reaction. The ratio of leucine to phenylalanine incorporation was approximately 0.02 and remained constant.

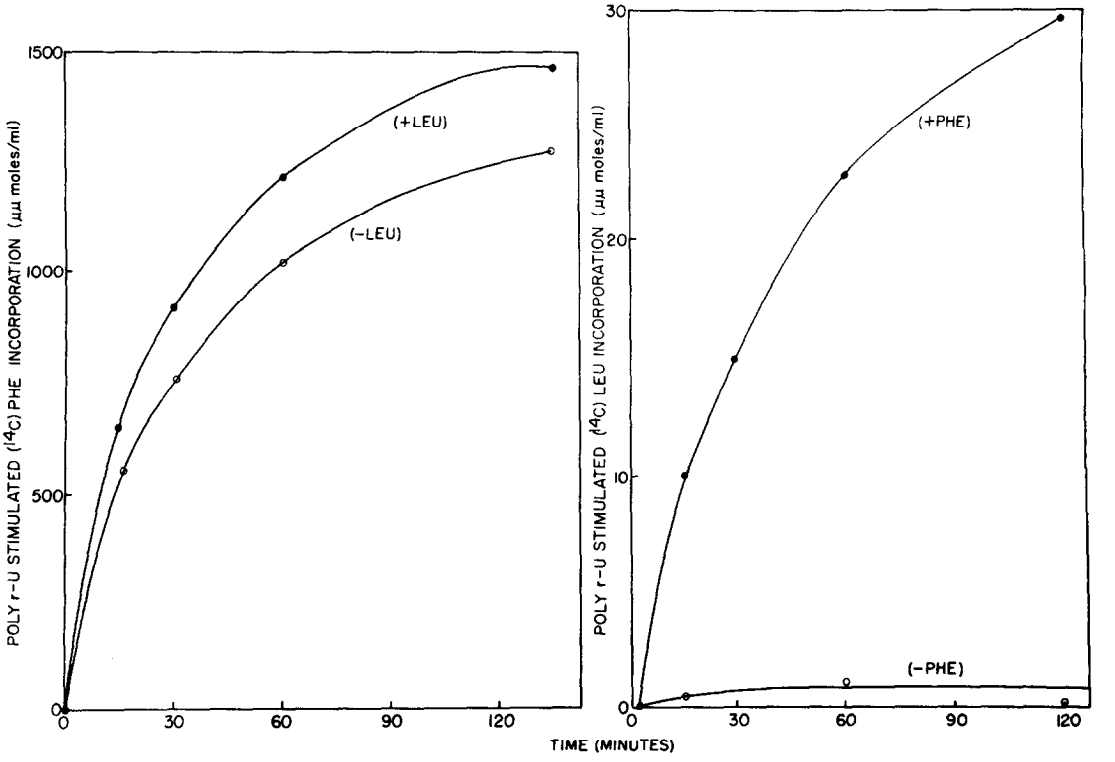

Fig. 1. Time course of Poly r-U directed (^{14}C) amino acid incorporation in the rabbit reticulocyte system. Standard amino acid incorporation conditions were used, except where, as indicated, 60 mμ moles of unlabelled amino acid per ml of the reaction mixture was added. Stimulation of the amino acid incorporation refers to the total incorporation of the amino acid in the presence of poly r-U minus incorporation of the same amino acid in the absence of poly r-U.

This suggests that under the conditions of the experiment, a copolypeptide of leucine and phenylalanine was synthesized.

The Mg^{++} requirement for phenylalanine and leucine incorporation is shown in Fig. 2. Phenylalanine incorporation under the assay conditions was maximum at 6 mM Mg^{++}, and 90 percent of the maximum leucine incorporation was also observed at the same Mg^{++} concentration. Optimum Mg^{++} concentration for leucine incorporation was 8 mM. No (^{14}C) leucine incorporation was observed at any Mg^{++} concentration in the absence of unlabelled phenyalanine.

The effect of 100,000 x g supernatant enzyme fraction on poly r-U stimulated phenylalanine and leucine incorporation was studied and the results of a

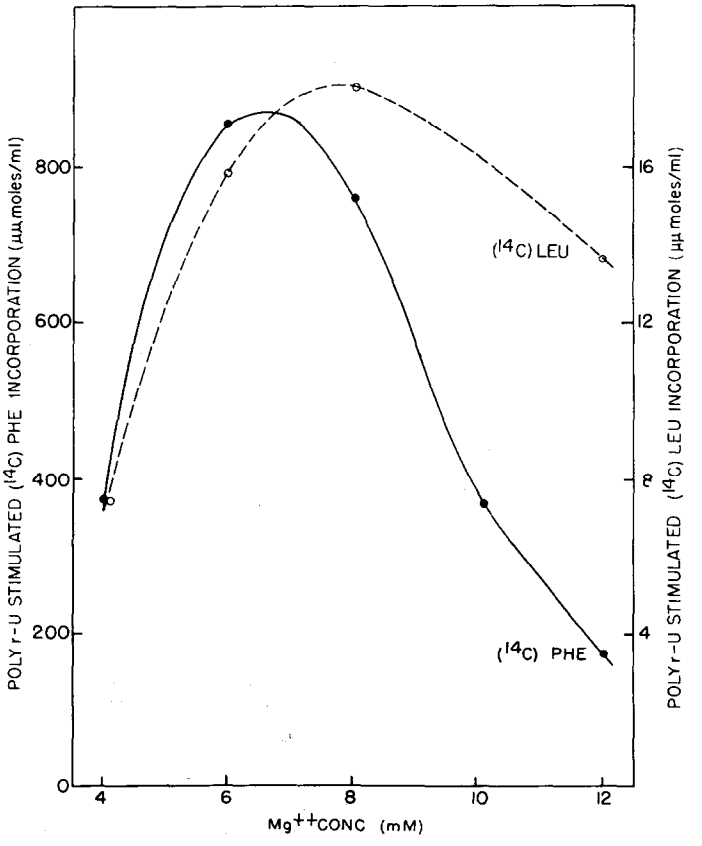

Fig. 2. Effect of magnesium ion concentration on (^{14}C) amino acid incorporation. Standard amino acid incorporation conditions were used; (^{14}C) leucine incorporation was studied in the presence of unlabelled phenylalanine. Incubation period was 1 hour.

typical experiment are shown in Table II. The supernatant fraction was dialyzed for 6 hours against 4 liters of a solution containing 0.01 M Tris-HCl at pH 7.5, 0.005 M β-mercaproethanol and 0.005 M $MgCl_2$ before use. As can be seen, poly r-U stimulated leucine incorporation was completely dependent on added phenylalanine. Control experiments in which either poly r-U or unlabelled phenylalanine or both (not shown in Table II) were omitted, gave no significant differences in (^{14}C) leucine incorporation. However, while addition of supernatant enzymes had little effect on Mg^{++} requirement for (^{14}C) phenylalanine incorporation, the optimum requirement for Mg^{++} for leucine incorporation was shifted to 12 mM. It is conceivable that in the absence of supernatant enzymes, the ribosomal conformation is more flexible and miscoding can occur at lower

TABLE II

Poly r-U Directed Phenylalanine and Leucine Incorporation in the Presence of 100,000 x g Supernatant

Mg^{++} (M)	Poly r-U	(^{14}C) Amino Acid Incorporated (μμmoles/ml)					Ambiguity Leu/Phe x 100
		Phenylalanine		Leucine			
			net	-unlabelled Phe	+unlabelled Phe	net	
0.004	-	20			47		
	+	1,390	1,370	54	76	29	2
0.008	-	36			80		
	+	1,760	1,724	87	115	35	2
0.012	-	37			69		
	+	490	453	85	134	65	14
0.016	-	31			45		
	+	148	117	56	54	9	8

Experimental conditions were the same as described in Table I; approximately 0.5 mg of dialyzed 100,000 x g supernatant protein was added per ml of the reaction mixture.

Mg^{++} concentration, whereas in the presence of supernatant proteins, the ribosomes assume a more native conformation and mismatching of the codon and anticodon bases requires a greater Mg^{++} concentration.

Discussion

Data presented in this paper clearly establish the requirement of phenylalanine for poly r-U directed ambiguous amino acid incorporation in the rabbit reticulocyte system. This observation may explain the conflicting reports from different laboratories on poly r-U directed ambiguous amino acid incorporation in the rabbit reticulocyte system. Friedman et.al. (8) studied poly r-U directed leucine incorporation in the absence of added phenylalanine and observed only poor leucine incorporation. On the other hand, Lamfrom and Grunberg-Manago (9) added nineteen unlabelled amino acids and 150,000 x g reticulocyte supernatant in the incorporation experiments and reported significant leucine incorporation in the presence of poly r-U. The characteristics of poly r-U directed leucine incorporation reported by these authors are in general agreement with our results of leucine incorporation in the presence of dialyzed supernatant enzymes and unlabelled phenylalanine (Table II).

The characteristics of poly r-U directed ambiguous amino acid incorporation described here are clearly different from the characteristics in other cases of ambiguity in the rabbit reticulocyte system recently described by Gupta (11). In all reported cases, the ambiguous amino acid incorporation was inhibited in the presence of the unlabelled amino acid normally coded by the ambiguous codon. The dependence on phenylalanine for ambiguous amino acid incorporation may be due to poor recognition of UUU triplet codon by other amino acyl t-RNA's and phenylalanine oligopeptide synthesis is necessary to make bridges between randomly incorporated ambiguous amino acid residues.

The dependence of poly r-U directed ambiguous amino acid incorporation on added phenylalanine has not been demonstrated in the bacterial system,

though So and Davie (6) reported that in the E. coli amino acid incorporating system, addition of unlabelled phenylalanine increased poly r-U directed leucine incorporation. It is possible that in the E. coli system, the supernatant enzyme fraction, used in amino acid incorporation experiments, contains sufficient endogenous phenylalanine to give the observed leucine incorporation. Also, it is clear that in the E. coli system, several ribosomal factors (4, 12, 13) control the ambiguous amino acid incorporation. The nature of the ambiguity induced by these factors may be different.

Acknowledgements

This investigation was supported by Grant GU-2054 from the National Science Foundation and Grant WOO-4114-99A from the United States Public Health Service, administered through the University of Nebraska Research Council.

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