Cell-Free Amino Acid-Incorporating System from *Pseudomonas indigofera*

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ABSTRACT

SHIIO, TSURU (Washington State University, Pullman), AND BRUCE A. McFADDEN. Cell-free amino acid-incorporating system from *Pseudomonas indigofera*. J. Bacteriol. 90:978-983. 1965.—A cell-free preparation from *Pseudomonas indigofera* incorporated C¹⁴-phenylalanine and C¹⁴-leucine into a product which was insoluble in hot trichloroacetic acid. The phenylalanine incorporation process, which had a temperature optimum of 30 C and a pH optimum of 7.6, had many characteristics of protein synthesis. The process depended upon both "ribosomes" and supernatant fraction from centrifugation at 105,000 × *g*. Incorporation required adenosine triphosphate, apparently depended upon guanosine triphosphate, and was inhibited by chloramphenicol, puromycin, actinomycin, ribonuclease, and deoxyribonuclease. Leucine incorporation was also studied and had many similar characteristics. C¹⁴-phenylalanine uptake was stimulated by sRNA or polyuridylic acid, and together these substances had a synergistic effect upon stimulation. The incorporation of C¹⁴-phenylalanine into a product which was precipitated by antiserum to crystalline isocitrate lyase was also observed.

The properties of several microbial extracts which incorporate amino acid(s) into protein have been described (for example, see Gale, 1962; Fruton, 1963). Because such incorporation is a reflection of an exceedingly complex process, it is often difficult to demonstrate reproducibly. To date, there has apparently been no description of an amino acid-incorporating system for members of the genus *Pseudomonas*. The present communication describes the preparation, properties, and stability of a cell-free incorporation system from *P. indigofera*, which was developed as a result of our interest in the synthesis of isocitrate lyase, one of its major "soluble" proteins. Also described is the incorporation of C¹⁴-phenylalanine by extracts, into a product which is precipitated by antiserum to isocitrate lyase.

MATERIALS AND METHODS

Special materials. Special reagents were supplied as follows: L-amino acids, tricyclohexylammonium phosphoenolpyruvate (PEP), and polyuridylic acid (poly-U) by Calbiochem; bovine albumin and puromycin dihydrochloride by Nutritional Biochemicals Corp., Cleveland, Ohio; adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP), and uridine triphosphate (UTP) by Pabst Laboratories, Milwaukee, Wis.; chloramphenicol by Parke Davis and Co., Detroit, Mich.; uniformly labeled phenylalanine and leucine by Schwarz BioResearch Inc., Orangeburg, N.Y.; deoxy-

ribonuclease, PEP kinase, and ribonuclease by Sigma Chemical Co., St. Louis, Mo.; and rabbit antbovine albumin serum (Merthiolated, 1:10,000) by The Sylvania Co., Millburn, N.J. We gratefully acknowledge the gift of actinomycin D from G. E. Boxer of Merck and Co., Inc., Rahway, N.J.

Cells. *P. indigofera* M1 was cultured as described earlier (Shiio, Shiio, and McFadden, 1965) in a Biogen (American Sterilizer Co., Erie, Pa.) or aeration bottle, and was harvested by continuous centrifugation during the late exponential phase (Klett reading of about 240 with a red filter). Cells were washed once with standard buffer which contained the following components at pH 7.7: 0.01 M tris (hydroxymethyl) aminomethane (Tris), 0.01 M MgCl₂, 0.1 M KCl, and 0.06 M β-mercaptoethanol. After centrifugation, the washed-cell mass was frozen until used.

Cell-free preparations. Frozen cells (6 g) were suspended in 15 ml of standard buffer and disrupted by passage once through a French pressure cell (ca. 6,000 psi) cooled to 2 C. The resulting suspension was centrifuged for 20 min at 12,100 × *g* to remove cells and large particles and to yield supernatant S₁. S₁ was then centrifuged at 23,500 × *g* for 30 min to yield supernatant S₂, which was centrifuged at 105,000 × *g* for 90 to 120 min yielding a pellet and supernatant S₃. The pellet was washed with standard buffer and centrifuged for 90 min at 105,000 × *g* to yield a ribosomal sediment, P₁₀₅. All centrifugations were conducted at ca. 0 C. To remove components of low molecular weight, S₃ was either passed through a Sephadex

G-25 (coarse) column equilibrated with standard buffer to yield S_{105} -s or, in some cases, dialyzed overnight at 3 C against 100 volumes of standard buffer to yield S_{105} -d. P_{105} and S_{105} preparations were usually either used immediately or stored in liquid N_2 .

Incorporation of C^{14} -phenylalanine into protein. The complete reaction mixture of 0.5 ml contained the following at pH 7.7: Tris, 50 μ moles; $MgCl_2$, 5 μ moles; KCl, 50 μ moles; ATP, 0.3 μ mole; PEP, 0.25 μ mole; pyruvate kinase, 20 μ g; CTP, GTP, and UTP, 0.05 μ mole each; a mixture containing 2.5 to 0.025 μ moles of 17 natural L-amino acids (about 0.15 to 0.0015 μ mole each and lacking cysteine, hydroxyproline, and phenylalanine), β -mercaptoethanol, 5 μ moles; P_{105} protein, about 1 mg; S_{105} protein, 0.2 to 0.3 mg; and C^{14} -L-phenylalanine (specific activity, 140–165 mc/mmole), 0.25 μ c or C^{14} -L-leucine (specific activity, 7.14 mc/mmole), 0.5 μ c. Ordinarily P_{105} and S_{105} were added to the incubation mixture and then the reaction was initiated by addition of C^{14} -amino acid. After incubation for 40 min at 30 C (unless otherwise specified), the reaction mixture was chilled in ice. Samples of 0.10 ml were then transferred to filter-paper discs. The separation of labeled protein from other components and radioactivity measurements were conducted by the procedure of Mans and Novelli (1961). Radioactivity was ordinarily assayed with an automatic Packard Tricarb liquid scintillation spectrometer. Activity is expressed as counts per minute of C^{14} incorporated per milligram of protein. It was impossible to determine precisely the counting efficiency without an internal protein standard, which was not readily available. However, Mans and Novelli (1961) estimated that the counting efficiency was about 55%. On this basis, the best incorporations observed in the present work under the standard assay conditions were ca. 50 μ moles per mg of P_{105} plus S_{105} protein. Protein was determined in P_{105} and S_{105} fractions by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The concentration of ribonucleic acid (RNA) was calculated by assuming that 1.0 mg/ml has an absorbancy at 260 $m\mu$ of 24.

For tests of inhibition, reaction mixture lacking only the C^{14} -amino acid was preincubated for 10 min at the temperature of the subsequent incubation. Then the C^{14} -amino acid was added, and the incubation and workup were carried out in the usual way.

Preparation of amino acyl transfer RNA from *P. indigofera*. Soluble RNA (sRNA) was prepared from S_{105} of *P. indigofera* by the method of Bishop, Roche, and Nisman (1964). Attached amino acids were then removed (Von Ehrenstein and Lipmann, 1961). When dissolved in 0.01 N KOH, the final preparation had a ratio of 2 for absorbance at 260 $m\mu$ to absorbance at 280 $m\mu$ and a ratio of 2.1 for absorbance at 260 $m\mu$ to absorbance at 230 $m\mu$.

Preparation of rabbit anti-isocitrate lyase serum. Antiserum against isocitrate lyase was prepared by use of rabbits into which a saline solution of

crystalline isocitrate lyase from *P. indigofera* (Shiio et al., 1965) was injected intravenously. For example, one male albino rabbit (4.5 kg) was injected at 3-day intervals with 2-, 2-, and 6-mg doses of the enzyme. At 13 days after the first injection, blood was taken from the ear, the immune response was tested, and a final booster dose of 10 mg was administered. After an additional week, another blood sample was withdrawn, and 54 ml of sera were pooled. The sera precipitated isocitrate lyase, and the neutralization point was determined by adding various amounts of antiserum to a known amount of the enzyme. No cross-reaction was observed with bovine serum albumin.

RESULTS

Characteristics of amino acid incorporation. Incorporation of C^{14} -amino acid into the protein fraction depended upon the presence of P_{105} and S_{105} . The dependence of incorporation on various components of the reaction mixture and the effect of inhibitors are reported in Table 1. Although data from experiment 2 suggested that omission of the ATP-generating system decreased incorporation, generally absence of the generating system had little effect if ATP was provided. Therefore, in some experiments, which are specified, PEP and pyruvate kinase were omitted from the standard incubation mixture. As can be seen, the absence of UTP, CTP, and GTP decreased incorporation, but the omission of UTP and CTP had little effect. The effect of the amino acid mixture was variable. At higher concentrations varying degrees of inhibition were observed, an observation of unknown significance but in accord with one made for a yeast-incorporating system which had also been Sephadex-treated or dialyzed (Bretthauer et al., 1963). Incorporation was quite sensitive to ribonuclease or deoxyribonuclease and was also inhibited by actinomycin D, chloramphenicol, and puromycin. There was no stimulatory effect of reduced glutathione (Eisenstadt and Brawerman, 1964) at ca. 10^{-3} M or of NH_4^+ when used instead of K^+ (Spyrides, 1964). Other amino acids may be incorporated into protein, as the data in Table 2 demonstrate. As is evident, characteristics of leucine incorporation were similar to those of phenylalanine.

The time course of incorporation is shown in Table 3.

Figures 1 and 2 show pH and temperature dependence, respectively, of incorporation.

When poly-U was added to the complete reaction mixture, the incorporation of C^{14} -phenylalanine into the material insoluble in hot 5% trichloroacetic acid was increased (Table 4). The degree of stimulation varied in fresh preparations and was inversely related to the length of storage

TABLE 1. *Characteristics of C¹⁴-phenylalanine incorporation into protein*

Expt	System*	C ¹⁴ -phenylalanine incorporated†
1	Complete	1,840
	Minus P ₁₀₅	392
	Minus S ₁₀₅ -d	635
2	Complete	261
	Minus ATP and generator	48
	Minus ATP generator	90
	Minus CTP, GTP, UTP	84
	Minus CTP, UTP	330
3	Complete	482
	Plus puromycin (100 µg)	130
	Plus chloramphenicol (100 µg)	100
4	Complete	8,600
	Minus P ₁₀₅	830
	Minus amino acid mixture	7,150
	Plus 2.5 µmoles of each amino acid	8,000
	Plus ribonuclease (10 µg)	685
	Plus deoxyribonuclease (100 µg)	1,010
	Plus actinomycin D (100 µg)	2,400
	Plus puromycin (100 µg)	875

* The complete systems were as described in Materials and Methods. In experiments 1 and 4, 0.025 µmole of amino acid mixture was used, and 2.5 µmoles were used in experiments 2 and 3.

† Expressed as counts per minute per milligram of protein. Specific incorporations are not directly comparable because counting efficiencies in experiments 1 and 4 were about twice as high as those for 2 and 3. In the latter experiments, a different liquid scintillation counter was used for radioactivity assay. Furthermore, incubations were carried out at 35 C for 30 min in experiments 1, 2, and 3, but at 30 C for 40 min in experiment 4.

of the cell-free system. Poly-U resulted in only a slight stimulation of leucine incorporation, although the data are not shown. As shown in Table 4, transfer RNA also enhanced incorporation of C¹⁴-phenylalanine and in combination with poly-U produced a very striking increase in incorporation.

As can be seen in Table 5, actinomycin D did not inhibit the increment of incorporation stimulated by poly-U; both chloramphenicol and puromycin inhibited this stimulation. Messenger RNA (mRNA) may not have been synthesized in the course of experiment 1 (Table 5), since control incorporation was insensitive to ribonuclease. This may account for failure to observe chlor-

TABLE 2. *Characteristics of C¹⁴-leucine incorporation into protein*

System*	C ¹⁴ -leucine incorporated†
Complete (0.25 µmole of amino acid).....	160
Complete (0.025 µmole of amino acid).....	145
Minus amino acids.....	172
Minus P ₁₀₅	8
Minus S ₁₀₅ -d.....	91
Plus ribonuclease (10 µg).....	13
Plus deoxyribonuclease (10 µg).....	73
Plus puromycin (50 µg).....	38

* Conditions are described in Materials and Methods. The incubation was conducted for 30 min at 30 C.

† Expressed as counts per minute per milligram of protein.

TABLE 3. *Time course of incorporation*

Incubation time	C ¹⁴ -phenylalanine incorporated*
<i>min</i>	
0	152
10	655
20	1,080
30	1,060
40	1,850
60	2,080

* Expressed as counts per minute per milligram of protein. Conditions were as described in Materials and Methods. The temperature of incubation was 35 C, and 2.5 µmoles of amino acids were used.

amphenicol inhibition (Jardetsky and Julian, 1964) in this particular experiment.

Effect of storage on the particles (P₁₀₅) and supernatant (S₁₀₅). The effects of storage in liquid N₂ (−196 C) were tested (Fig. 3). About 50% of the incorporating activity was lost in 2 weeks. Storage in liquid N₂ was far superior to storage at 1 C, which resulted in 50% inactivation after only 2 days. Preliminary experiments have suggested that storage of P₁₀₅ at −196 C and of S₁₀₅ at 1 C preserves all activity for up to 1 week.

Immunological assays for isocitrate lyase formed in vitro. An attempt was made to determine whether the observed incorporation of labeled amino acid into protein was associated with a corresponding incorporation into enzyme protein. As a probe for incorporation into isocitrate lyase, this enzyme was precipitated with specific antiserum, and radioactivity of the precipitate was assayed (Table 6). Radioactivity in the enzyme-antienzyme precipitate was a maximum of 2% of

FIG. 1. Effect of temperature upon 30-min phenylalanine incorporation into protein. An amino acid mixture containing 0.025 μ mole of amino acids and S_{105} -s was employed. All other conditions were as described in Materials and Methods.

FIG. 2. Effect of pH upon 30-min incorporation of phenylalanine into protein. The pH of the incubation mixture was adjusted with HCl, and KCl was added as necessary to insure a constant concentration of Cl^- . A mixture containing 0.025 μ mole of amino acids and S_{105} -d was used. The temperature during incorporation was 35°C. All other conditions were as described in Materials and Methods.

the total radioactivity in "soluble" protein. About 1.5% of the total radioactivity was precipitated, however, when the foreign protein bovine albumin was precipitated by its antiserum in the presence of the radioactive "soluble" protein.

DISCUSSION

In general, the characteristics of incorporation of labeled amino acid into protein by "ribosomes"

TABLE 4. Stimulatory effect of sRNA and poly-U on incorporation

Expt*	System	C^{14} -phenylalanine incorporated†
1	Complete	1,000
	Plus sRNA	1,525
	Plus poly-U	11,600
	Plus sRNA and poly-U	19,000
2	Complete	2,376
	Plus sRNA	4,125
	Plus poly-U	17,045

* In both experiments, incubation was carried out for 40 min at 35°C with S_{105} -s and 0.025 μ mole of amino acids. The rest of the components were as described in Materials and Methods except that ATP generator was omitted in experiment 2. sRNA and poly-U were used in the following amounts, respectively: experiment 1, 45 and 60 μ g; experiment 2, 123 and 60 μ g.

† Expressed as counts per minute per milligram of protein.

TABLE 5. Relationship between inhibitors and poly-U stimulation

System*	C^{14} -phenylalanine incorporated†
<i>Experiment 1</i>	
(a) Complete†	1,170
(b) Plus poly-U	7,500
(c) Plus chloramphenicol	1,520
(d) Plus poly-U and chloramphenicol	3,800
<i>Experiment 2</i>	
(a) Complete	8,600
(b) Plus poly-U	11,300
(c) Plus puromycin	875
(d) Plus poly-U and puromycin	730
<i>Experiment 3</i>	
(a) Complete	8,600
(b) Plus poly-U	11,300
(c) Plus actinomycin D	2,400
(d) Plus poly-U and actinomycin D	7,000

* In all experiments, incubations were carried out for 40 min at 30°C with 0.025 μ mole of amino acids under standard conditions with S_{105} -s. Levels of inhibitors and poly-U employed were 100 and 60 μ g, respectively. The percentage of poly-U-stimulated incorporation in the presence of test compound $[(d - c)/(b - a) \times 100]$ was 36 for chloramphenicol, 0 for puromycin, and 170 for actinomycin D.

† Expressed as counts per minute per milligram of protein.

‡ Incorporation by this system was unaffected by ribonuclease.

FIG. 3. Effect of storage of cell-free preparation at -196°C . P_{105} and S_{105} -s were combined and stored in liquid N_2 . Periodically, they were assayed for incorporation activity at 30°C for 40 min by the standard procedure, except that the incubation mixture was supplemented with about $45\text{ }\mu\text{g}$ of sRNA and lacked ATP generator. The mixture containing $0.025\text{ }\mu\text{mole}$ of amino acids was used.

(P_{105}) plus supernatant (S_{105}) from *P. indigofera* are similar to those for extracts from several other bacteria (Gale, 1962; Fruton, 1963). It is dependent upon ATP and GTP, is stimulated by sRNA, and is inhibited by chloramphenicol, puromycin, and actinomycin D. Incorporation of phenylalanine into product(s) insoluble in hot trichloroacetic acid is stimulated by poly-U, whereas leucine incorporation is only slightly stimulated by poly U. The system is also inhibited by ribonuclease and deoxyribonuclease, although the latter observation is of somewhat questionable significance because of the possibility that the commercial deoxyribonuclease employed was contaminated with ribonuclease. The inhibition by actinomycin D implies that in some experiments, at least, mRNA is manufactured.

Work in a number of laboratories suggests that actinomycin acts by interfering with transcription of DNA. Its failure in the present experiments to inhibit in vitro incorporation stimulated by artificial mRNA, i.e., poly-U, is in accord with this mode of action. Inhibition of the poly-U-stimulated incorporation by chloramphenicol or puromycin is also in accord with their postulated modes of action (see, for example, Jardetsky and Julian, 1964; Yarmolinsky and De Le Haba, 1959).

The incorporation of label into a product precipitated by anti-isocitrate lyase serum merits comment. Although the incorporations represent about 2% of the radioactivity found in "soluble" protein, it is about 1.5% for controls in which bovine albumin is precipitated by antbovine albumin serum in the presence of radioactive "soluble" protein. The incorporation of 1.5% may

TABLE 6. Immunological assay for incorporation of C^{14} -phenylalanine into isocitrate lyase*

Expt	System	C^{14} -phenylalanine incorporated/1.5 ml of reaction mixture
		count/min
1	Protein fraction of reaction mixture	7,082
	Protein fraction of supernatant fluid	6,179
	Precipitate from isocitrate lyase-antiserum reaction	136
	Precipitate from bovine albumin-antiserum reaction	106
2	Protein fraction of reaction mixture	10,356
	Protein fraction of supernatant fluid	10,370
	Precipitate from isocitrate lyase-antiserum reaction	158
	Precipitate from bovine albumin-antiserum reaction	108

* Incubations were carried out in the standard way at 30°C for 40 min in a total volume of 4.0 ml. A mixture of $0.20\text{ }\mu\text{mole}$ of amino acids was used, and the reaction mixture was supplemented with $360\text{ }\mu\text{g}$ of sRNA. Concentrations of other components were as described in Materials and Methods, but ATP generator was omitted. After incorporation, a sample of the incubation mixture was removed for incorporation assay by the standard technique. The remainder was stored overnight at -20°C and then centrifuged at $105,000\times g$; a sample of the supernatant fraction was removed for incorporation assay by the standard technique. To 1.5 ml of supernatant fraction, 0.5 ml of crystalline isocitrate lyase ($100\text{ }\mu\text{g}$) and 1.0 ml of anti-isocitrate lyase serum (a slight excess) were added. For a control, 0.5 ml of bovine albumin ($100\text{ }\mu\text{g}$) and 0.44 ml of rabbit antbovine albumin were added to 1.5 ml of the supernatant fluid; the volume was adjusted to 3.0 ml with 0.85% NaCl. Both tubes were then stored overnight at 1°C . Each mixture was then centrifuged at $2,000\times g$, and the precipitates were washed twice with 3 ml of 0.85% NaCl and then dissolved in 0.5 ml of 5% NH_4OH . Radioactivity was assayed in the usual way by placing 0.4 or 0.45 ml of the solution on a disc.

reflect nonspecific coprecipitation. If so, a precise control for this phenomenon would be difficult. Nevertheless, in immunoassays for in vitro protein synthesis, the inclusion of controls of this type has been generally overlooked.

If a net of 0.5% of the total incorporation is into isocitrate lyase, and de novo synthesis is assumed, an estimate of the increase in enzyme can be made from its known amino acid composition (Shiio et al., 1965). This increase would be

equivalent to ca. 10^{-5} enzyme units per ml from an initial value of ca. 1.8 units per ml. Our chemical assay would not detect such a small increase. It is clear from these estimates that the detection by chemical methods of an increase in enzyme in cell-free preparations may only reflect the terminal stage of protein synthesis, as has been suggested by Whiteley and Oishi (1963). In any case, characteristics of the apparent cell-free synthesis of isocitrate lyase are being investigated.

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