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Characteristics of a Subcellular System from *Cellvibrio gilvus* for the Incorporation of Amino Acids into Protein (32635)

THONG-SUNG KO AND LEWIS B. BARNETT (Introduced by R. W. Engel)
Department of Biochemistry, Virginia Polytechnic Institute, Blacksburg, Virginia 24061

The bacterium *Cellvibrio gilvus* can be induced to synthesize extracellular cellulases—four enzymes which are all specific for both the second and the third glucosidic bonds from the nonreducing end of the cellulose molecule (1)—when grown in a medium containing cellulose. Investigations of the location of these cellulases within the cell have revealed that the membrane bound ribosomes contain an unusually high enzymic activity (2). In order to study the location of cellulase synthesis by this organism, it was necessary to prepare and characterize a cell-free amino-acid incorporating system from it.

This report details the properties of such an amino acid incorporating system, giving the effects of ribosome concentration, pH, temperature, pH 5 fraction, and magnesium ions. Furthermore, the effects of transfer RNA, polyuridylic acid, antibiotics, polyamines, deoxyribonuclease and ribonuclease are presented. In the present investigation, we have not attempted to characterize the proteins which were synthesized during the course of amino acid incorporation.

Materials and Methods.[1] *Cell-free preparations. Cellvibrio gilvus,* strain 11, obtained from Dr. K. W. King, was grown at 30°C with strong aeration in the culture medium of Hulcher and King (3) with the following modifications: 0.25 gm of $(NH_4)_2SO_4$ was substituted for an equal weight of $NaNO_3$ per liter; 2 drops of antifoam (Nalco 71-D5, Nalco Chemical Co., Chicago, Ill.) were added per liter; the pH was adjusted to 6.6 with HCl. Cell growth was measured using a Bausch and Lomb Spectronic 20 colorimeter with 0.5 inch test tubes at 540 mμ. Cells were harvested during early log phase of growth, when the absorbancy was between 0.3 and 0.4. The wet weight yield of cells was about 0.8 gm/liter.

Cells were isolated by centrifugation in a refrigerated centrifuge and all following steps were performed in the cold. After washing the cells three times with 0.02 *M* Tris–HCl buffer, pH 7.8, containing 0.009 *M* $Mg(Ac)_2$, 0.07 *M* KCl, 0.006 *M* β-mercaptoethanol (Matheson, Coleman, and Bell), referred to hereafter as "buffer", the cells were suspended in buffer (3 gm of packed cells per 10 ml of buffer) and sonicated for 45 sec at maximum output on a Model DF 101 Raytheon Sonic Oscillator, Waltham, Mass. The resulting material was centrifuged twice at 27,000 *g* for 15 min and the supernatant was spun for 1 hour at 100,000 *g*. The top three-fourths of resulting supernatant were removed by aspiration, brought to pH 5 by the dropwise addition of 1 *N* acetic acid and the resulting precipitate was collected by centrifugation and dissolved in buffer, which was brought to pH 7.8, and used in the cell-free system. This is referred to as the pH 5 fraction. The 100,000 *g* precipitate, which is referred to as the "ribosomes," was washed with buffer, recentrifuged and suspended in buffer for immediate use in the cell-free system.

Preparation of transfer-ribonucleic acid

[1] Tris, tris(hydroxymethyl)aminomethane; poly-U, polyuridylic acid; tRNA, transfer ribonucleic acid; ATP, adenosine 5′triphosphate; GTP, guanosine 5′-triphosphate; UTP, uridine 5′-triphosphate; CTP, cytidine 5′-triphosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; TCA, trichloroacetic acid; $Mg(Ac)_2$, magnesium acetate.

TABLE I. Characteristics of Leucine-^{14}C Incorporation into Protein by a Cell-Free System from *C. gilvus*.

System		Leucine-^{14}C incorporated per mg ribosomal protein ($\mu\mu$moles)
Complete system (30 min)		31
	(0 min)	0
Minus:	ribosomes	3
	pH 5 fraction	2
	ATP system	4
	GTP	13
	NH_4Cl	23
Plus:	chloramphenicol (100 μg)	11
	puromycin (100 μg)	1
	actinomycin D (100 μg)	30
	deoxyribonuclease (100 μg)	29
	ribonuclease (20 μg)	0
	spermidine (0.5 μmole)	31
	spermine (0.5 μmole)	35
	putrescine (0.5 μmole)	25

from C. gilvus. The tRNA was isolated from *C. gilvus*, grown and washed as above, by the procedure of Nirenberg and Matthaei (4).

Amino acid incorporation experiments. One ml of the complete reaction system contained the following components (in μmoles unless otherwise specified): 20 Tris-HCl, pH 7.8, 9 $Mg(Ac)_2$, 70 KCl, 6 β-mercaptoethanol, 2 ATP, 0.2 GTP, 100 NH_4Cl, 0.0018 DL-leucine-^{14}C (74,000 cpm Volk Radiochemical Co.) or 0.02 DL-phenylalanine-^{14}C (50,000 cpm New England Nuclear Radioactive Chemicals), 5 phosphoenolpyruvate, (Boehringer Biochemicals, New York), 10 μg pyruvate kinase (E.C. 2.7.1.40), 0.3–0.7 mg ribosomal protein, and 0.5–1 mg pH 5-fraction protein. For phenylalanine-^{14}C incorporation studies, 0.4 mg of polyuridylic acid and 0.6 mg of tRNA were added to each tube unless otherwise indicated. One ml of the reaction mixture was incubated at 30° for 30 min and then 1 ml of 10% trichloroacetic acid was added to stop the reaction. The precipitate was washed by the method of Siekevitz (5), dissolved in formic acid, plated on aluminum planchets and counted with a gas-flow counter. Correction was not made for self-absorption. Incorporation is expressed as $\mu\mu$moles of amino acid incorporated into protein per mg of ribosomal protein.

Protein estimation. Protein was estimated by the method of Lowry *et al.* (6), using dialyzed bovine serum albumin (Nutritional Biochemicals Corp.) as a standard.

Materials. ATP, GTP, CTP, and UTP were purchased from P-L Biochemicals, Milwaukee. Ribonuclease and deoxyribonuclease were products of Worthington Biochemicals Co., Freehold, N. J. Puromycin was obtained from Nutritional Biochemicals Corp., whereas chloramphenicol came from Parke, Davis and Co., Detroit. Actinomycin-D was a gift from Merck, Sharp and Dohme, West Point, Pa. Polyuridylic acid was obtained from Miles Chemical Co., Elkhart, Ind. Putrescine dihydrochloride was obtained from Mann Research Laboratories, New York, whereas spermine tetrahydrochloride and spermidine trihydrochloride were from the California Corporation for Biochemical Research, Los Angeles.

Results and Discussion. Characteristics of the amino acid incorporating system. The general characteristics of the incorporation of leucine-^{14}C by the cell-free system from *C. gilvus* are shown in Table I. The system is dependent on ribosomes, on the pH 5 fraction and on an energy source. Of the three antibiotics tested, puromycin was inhibitory to the greatest extent (97%), whereas chloramphenicol and actinomycin D were inhibitory to the extent of 65 and 3%, respectively. Deoxyribonuclease had little effect on

TABLE II. Characteristics of the Phenylalanine-^{14}C Incorporation into Protein by a Cell-Free System from *C. gilvus*.

System	Phenylalanine-^{14}C incorporated per mg ribosomal protein ($\mu\mu$moles)
Complete system	359
Minus: ribosomes	86
pH 5 fraction	62
tRNA	123
poly-U	27
$Mg(Ac)_2$	24

amino acid incorporation, whereas ribonuclease almost completely abolished this activity. For optimal incorporation GTP and NH_4Cl were required.

The dependence of the amino-acid incorporating system on ribosomes, pH 5 fraction (or supernatant) and an energy source along with the effect of ribonuclease and deoxyribonuclease is similar to the systems isolated from various organisms, such as *Escherichia coli* (7), *Bacillus cereus* (8), a diploid yeast, *Saccharomyces fragilis, Saccharomyces dobzanskii* (9), *Euglena gracilis* (10), and rabbit reticulocytes (11).

With respect to the effect of antibiotics, all of the above systems were inhibited by puromycin. However, chloramphenicol was inhibitory to amino-acid incorporation by yeast (9), *B. cereus* (8), *E. coli* (7), and *C. gilvus,* whereas there was no effect on *E. gracilis* (10) and reticulocytes (11). The effect of actinomycin D on amino-acid incorporations has also been found to be somewhat species dependent, for it was observed to have no inhibitory effect on any system except heart muscle (12).

The effect of polyamines on amino-acid incorporation also varies considerably with the source of the cell-free system. Stimulation was observed in the case of *E. coli* (7), *B. cereus* (8), yeast (9); there was no effect on *E. gracilis* (10), and inhibition was observed with reticulocytes (11). Martin and Ames observed with a *Salmonella typhimurium* system that the effect of polyamines depend upon the concentration of magnesium ion in the solution (13). The *C. gilvus* system responded quite erratically to the polyamines tested; it was slightly stimulated by spermine, slightly inhibited by putrescine, and showed no significant response to spermidine. These observations were reproducible and no definite explanations for them can be offered.

In Table II are shown the characteristics of the incorporation of phenylalanine-^{14}C by the cell-free amino-acid incorporating system from *C. gilvus*. From these data, *C. gilvus* can be added to the long list of organisms from which a cell-free system can be obtained that has poly-U stimulated polyphenylalanine synthesis. This phenomenon was first observed by Matthaei and Nirenberg (7). Poly-U stimulation was observed to vary linearly with concentration up to a value of 0.4 mg polyuridylic acid. Although tRNA had no effect on leucine incorporation into protein, it was observed to stimulate threefold the poly-U dependent phenylalanine incorporation.

Optimum conditions for amino acid incorporation. In order to optimize the conditions for amino-acid incorporation into hot trichloroacetic acid insoluble material, experiments were performed in which one environmental parameter was varied at a time. The kinetics of the system revealed that incorporation was linear with time up to 30 min, at which time there was no additional change. When the effects of temperature and of pH on incorporation were studied, both were found to give bell-shaped curves with maxima at 30° and at a pH of 8.0. The concentrations of both magnesium acetate and the ATP system were found to be very critical for maximum incorporation. In the case of magnesium acetate, the optimum concentration was 0.012 *M*, whereas in the case of the ATP system, the optimum level was 0.15 ml of the ATP-system solution. This solution contained (per 1.5 ml) 3.4 μmoles of ATP; 0.34 μmoles each of GTP, CTP, and UTP; 8.4 μmoles of phosphoenolpyruvate; and 15 μg of pyruvate kinase.

Other characteristics of the cell-free C. gilvus *system.* The incorporation of phenylalanine-^{14}C into hot TCA insoluble material was observed to vary linearly: (a) with pH 5-fraction protein to a concentration of 2 mg protein per ml, at which point incorporation leveled off; (b) with ribosomal concentration to a concentration of 0.5 mg ribosomal protein per ml and inhibition was observed above 2

mg protein per ml; (c) with poly-U to a concentration of 0.4 mg poly-U per ml at which point there was a slight inhibition.

Summary. A cell-free system which was active with respect to amino acid-^{14}C incorporation into hot TCA insoluble material was derived from *Cellvibrio gilvus* and characterized. This amino-acid incorporation was typical in its requirement for ribosomes, pH 5 fraction, ATP, and magnesium ions, and in its inhibition by ribonuclease and puromycin. Deoxyribonuclease and actinomycin D had little effect, whereas chloramphenicol caused 65% inhibition. The optimal condition for incorporation was 30 min incubation at 30° and at pH 8.0. The addition of an exogenous source of a polyamine (spermidine, spermine, or putrescine) had little effect on this system. Poly-U dependent phenylalanine incorporation was observed with this system and found to be stimulated by an exogenous source of tRNA, although tRNA had no stimulatory effect on leucine incorporation.

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Inhibition of Intestinal Cholesterogenesis *in Vitro** (32636)

M. N. CAYEN AND D. DVORNIK (with the assistance of Mrs. Marsha Black)
Department of Biochemistry, Ayerst Research Laboratories, Montreal, Quebec, Canada

Most mammalian tissues are capable of synthesizing cholesterol *in situ* (1) and it is possible that this cholesterogenic capacity is a general property of growing cells (2). The liver has long been regarded as the principal endogenous source of the circulating cholesterol pool. Recently (3), it has been found that, in man, a significant portion of endogenous cholesterol arises in extrahepatic tissues. While it has been known for some time that the intestine is highly active in synthesizing cholesterol (4–7), its contribution to the pool of circulating cholesterol in man has been established only recently (8). Subsequently, several studies have been reported on the localization and control of intestinal cholesterogenesis in the rat (9, 10).

It is believed that intestinal synthesis of cholesterol follows a pathway similar to that found in the liver. Such a similarity depends upon the presence of similar enzyme systems in both organs. A convenient method of establishing the presence of these enzymes is based on the use of agents known to inhibit enzymatic reactions involved in the biosynthesis of cholesterol (*e.g.*, 11). In the present report, the effect was studied of several inhibitors of hepatic cholesterogenesis on the incorporation of acetate-[2-^{14}C] and DL-mevalonate-[^{3}H] into cholesterol by sections of everted rat ilea. The agents used were AY-9944 (12), 22,25-diazacholestanol[1] (13),

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