AMINO ACID INCORPORATION INTO PROTEIN BY EXTRACTS OF *E. COLI*

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SUMMARY

The incorporation of amino acids into protein in extracts of *E. coli* is described under conditions which show a dependence on the addition of ATP and an ATP generating system, GTP, Mg^{++}, KCl, and an amino acid mixture. The site of incorporation is the bacterial ribonucleoprotein particle. The addition of the non-sedimentable 105,000 $\times g$ supernatant solution stimulates this incorporation. Large bacterial fragments are not required for the incorporation. Ribonuclease and chloramphenicol inhibit it.

In order to permit meaningful interpretation of the cell-free capacity of the system, it has been found essential that the whole cell contamination of these preparations be no greater than $1 \cdot 10^5$ viable cells per ml at the start of a 15-min incubation period. The importance of determining the degree of contamination of bacterial extracts by whole cells in studies on the proteosynthetic mechanism is emphasized.

INTRODUCTION

Studies on the details of amino acid incorporation into protein have led to the postulation of the following sequence of reactions: (a) amino acid activation; (b) transfer of the amino acid to a low molecular weight "soluble" RNA; (c) amino acid incorporation into the protein of the ribosome (ribonucleoprotein particle). Reactions (a) and (b) have been reported in both mammalian and bacterial cell-free systems[1-4]. In addition, TISSIÈRES AND WATSON[5] have shown that the ribosomes of *E. coli* have many physico-chemical properties in common with ribosomes of rat liver described by PETERMANN *et al.*[6]. These similarities suggested that amino acid incorporation of the type described, using cell-free fractions of rat liver[7,8] might also occur in bacteria. There are a number of reports of bacterial amino acid incorporation systems, *cf.* SIMKIN[9]. This communication describes the conditions required for amino acid incorporation into extracts of *E. coli*. The results show that the components of this system are similar to those of mammalian systems.

Abbreviations used: RNA, ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; ATP, adenosine triphosphate; GTP, guanosine triphosphate; PEP, phosphoenol pyruvate; PEP kinase, pyruvate kinase (obtained from C. F. Boehringer und Sohne, Mannheim, Germany); CTP, cytosine triphosphate; and UTP, uridine triphosphate. All nucleotides were obtained from the Sigma Company.

MATERIALS AND METHODS

E. coli (K-12), kindly supplied by Dr. L. GORINI, was grown with moderate aeration at 37° on either the minimal medium of DAVIS AND MINGIOLI[10] (omitting the citrate) or on a complete medium which contained peptone. The bacteria were harvested shortly after the population had entered the log phase. The yield varied between 1 and 4 g wet wt./15 l of medium.

Bacterial extracts were prepared by grinding washed whole cells with Alumina (Alcoa A-301, 325 mesh). The alumina and cells (3:1) were homogeneously mixed in an ice cold mortar. To the white, paste-like material a volume of extracting solvent was added equal to the wet weight of cells. The solvent contained: Tris buffer (0.1 *M*, pH 7.9); $MgCl_2$ ($7 \cdot 10^{-3}$ *M*); and KCl ($6 \cdot 10^{-2}$ *M*). The mixture of solvent, cells and alumina was vigorously ground for 5 min. Precipitable material was removed by centrifugation (10 min, 10,000 × *g*). The precipitate was re-extracted two more times as before, except that 2 volumes of extracting solvent were used each time; the supernatants of all centrifugations were then combined and used immediately.

L-[1-^{14}C]leucine was synthesized[11] and kindly furnished by Dr. Robert B. Loftfield. The leucine was diluted to a specific activity of $1.8 \cdot 10^6$ counts/min/μmole. The precipitated protein samples were counted on a Nuclear gas flow counter with a Micromil end window. The counting efficiency was approx. 30%. The washing, plating, and counting procedures described by SIEKEVITZ[12] were used. Leucine incorporation described in this paper was stable to hot trichloroacetic acid extraction.

To determine if the incorporated leucine was fixed in α-peptide linkage, [^{14}C]protein was hydrolyzed by a modification of the method described by ZAMECNIK AND KELLER[7]. Following hydrolysis the protein was dried, dissolved in a minimal amount of water and chromatographed on paper in a solvent consisting of *n*-butanol–water–acetic acid (5:4:1). Ninhydrin positive material and areas of radioactivity (determined by a Nuclear-Chicago model C-100-A Actigraph II) were compared at intervals during the course of hydrolysis, Partial hydrolysis (2 *N* HCl, 3 h, 115°) revealed that the radioactivity had been distributed among four discrete, ninhydrin positive areas. The R_F's were: 0.85, 0.62, 0.36, 0.00. The R_F of free leucine was 0.60, and unhydrolyzed protein was stationary in this solvent. The release of free radioactive leucine increased with the time of hydrolysis. Complete hydrolysis (6 *N* HCl, 24 h, 115°) showed that radioactivity could be located only in the area of free leucine. These results are consistent with the interpretation that, following partial hydrolysis, radioactive leucine is present in peptides which are further degraded to release free leucine on complete acid hydrolysis.

EXPERIMENTAL

Short-term incorporation studies (15 min) using whole cells showed that tracer amounts of leucine are rapidly incorporated into protein without exogenous additions. When cultures of *E. coli* (K-12) are serially diluted and each dilution is incubated with the same tracer amount of [^{14}C]leucine, viable cells are still present at the point where leucine incorporation is insignificant. Fig. 1 shows that, when the number of viable cells is $1 \cdot 10^5$ per ml or less, the amount of labeled protein is not above background. In four experiments conducted in this manner, the range of the inflection point was from $5 \cdot 10^4$ to $1 \cdot 10^6$, with an average value of $3 \cdot 10^5$ viable cells per ml. Extracts prepared as

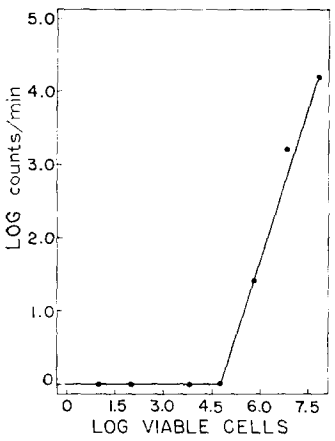

Fig. 1. Whole cell incorporation of L-[1-^{14}C]leucine. A suspension of whole, viable cells ($1 \cdot 10^8$ per ml) was serially diluted in 10-fold steps. 0.2 ml of each dilution was incubated at 37° for 15 min. In addition to the whole cell suspension the complete system contained: Tris ($1 \cdot 10^{-2}$ *M*, pH 7.8), L-[1-^{14}C]leucine (0.1 μmole, containing $1.8 \cdot 10^5$ counts/min), $MgCl_2$ (7 μmoles), KCl (60 μmoles), final volume 1 ml. Viable cell counts made by the pour plate method (in triplicate). After incubation, 4 mg of carrier protein were added to each flask. Samples which had a count rate between 1 and 0 were plotted as 1.

TABLE I

REQUIREMENTS FOR AMINO ACID INCORPORATION

The complete system contained: $MgCl_2$, $7 \cdot 10^{-3}$ *M*; KCl, $6 \cdot 10^{-2}$ *M*; ATP, 1 μmole; GTP, 0.25 μmoles; PEP, 5 μmoles; PEP kinase, 50 μg protein; 18 L-amino acids, 0.2 μmoles each; the mixture of ROBERTS *et al.*[20] minus diaminopimelic acid was used; Tris, $1 \cdot 10^{-2}$ *M*, pH 7.9; L-[1-^{14}C]leucine, 0.1 μmole, $1.8 \cdot 10^5$ counts/min; 4 mg of crude 10,000 × *g* supernatant of *E. coli* extract. 15 min incubation at 37° with occasional shaking. Final volume 1.0 ml, final pH 7.8.

Incorporation system	*Number of experiments*	*Percentage of "complete" specific activity**
Complete		100*
— Mg^{++}	3	0**
— KCl	6	41 ± 14
— ATP	4	64 ± 17
— GTP	4	76 ± 4
— PEP, PEP kinase	4	47 ± 2
— ATP, GTP, PEP, PEP kinase	3	0.2 ± 0.3
— Amino acids	4	38–80**
+ Ribonuclease (0.01 μg/ml)	1	40
+ Ribonuclease (1.0 μg/ml)	3	4 ± 0.6
+ Chloramphenicol (30 μg/ml)	3	31
+ Chloramphenicol (100 μg/ml)	2	8

* The average specific activity for the "complete" system was 480 counts/min/mg of protein ($2.6 \cdot 10^{-4}$ μM of [1-^{14}C]leucine incorporated/mg of protein). The standard deviation indicates the variation of effect observed in each of the experiments. It is not the variation of duplicate samples.

** See text for details.

described above rarely contain more than $1 \cdot 10^5$ viable whole cells per ml. ROGERS AND NOVELLI[13] report that the efficiency of ornithine transcarbamylase synthesis in protoplasts is increased 7-fold by the addition of bacterial extracts. In contrast to their protoplast preparation, the efficiency of amino acid incorporation into whole cells is not increased by the presence of the cellular milieu during short time incubations.

Incorporation studies in extracts which do not contain a significant number of whole cell contaminants show one striking difference from whole cell incorporation studies. For maximal incorporation, certain additions are required, as shown in Table I. The absence of magnesium completely inhibits the incorporation. The cells

are routinely ground, therefore, with alumina in the presence of magnesium. The crude bacterial extract shows the same requirement as liver homogenates for ATP, GTP and the energy generating system[7,8]. Removal of any of these additives reduces the incorporation. If all of these components are withheld, the incorporation level is close to zero. There is no stimulation of incorporation by addition of CTP, UTP, or the four common deoxyribonucleotides.

A mixture of 18 L-amino acids stimulates the incorporation of leucine into protein. No stimulation of leucine incorporation is obtained when only valine and isoleucine are substituted for the 18 amino acid mixture. The stimulation by amino acids is variable and is related to the composition of the medium used for the growth of the intact cells prior to rupture. Maximal stimulation (50–80 %) is observed using extracts of bacteria grown on the complete medium. Extracts of cells grown on minimal medium are stimulated by amino acids (20–40 %) only after a preliminary incubation, presumably to deplete the extract of residual free amino acids.

Low concentrations of ribonuclease (0.01 μg/ml) (Worthington) significantly inhibit the incorporation. Neither *E. coli* soluble RNA (a gift of Dr. A. TISSIÈRES) nor yeast soluble RNA were capable of reactivating the inhibited system. Chloramphenicol (30 μg/ml) also causes marked inhibition of leucine incorporation in the bacterial extract system. This concentration of chloramphenicol also inhibits protein synthesis of whole cells.

TABLE II

INCORPORATING ABILITY OF VARIOUS CELL FRACTIONS

Each of the fractions was incubated for 15 min at 37° in the complete system described in Table I. Specific activity is defined as counts/min/mg of protein, corrected for self absorption. In the case of Fraction 1, this would be equivalent to $4.09 \cdot 10^{-4}$ μM of leucine/mg of protein.

Fraction No.	*Fraction description*	*Total counts/min*	*Specific activity*
1	10,000 × *g* (10 min) supernatant	2870	757
2	30,000 × *g* (20 min) pellet*	0	0
3	30,000 × *g* (20 min) supernatant	2795	777
4	100,000 × *g* (2 h) pellet*	714	340
5	100,000 × *g* (2 h) supernatant	3	3
6	Fractions 2 + 5 (1:1)	14	6
7	Fractions 4 + 5 (1:1)	1734	468
8	Fractions 4 + 5 (1:2)	3000	527
9	Fractions 4 + 5 (1:3)	3419	444

* Pellets were resuspended in Tris buffer (0.01 *M*, pH 7.8) and diluted to the volume of the original 10,000 × *g* supernatant solution.

Utilizing the optimal conditions described in Table I, the amino acid incorporation system can be fractioned by centrifugation as shown in Table II. After high speed centrifugation, there is no incorporation of amino acid by the supernatant fraction alone. When this supernatant solution is added to the fraction sedimenting at 100,000 × *g* (2 h), roughly 70 % of the crude extract incorporation is recovered.

DISCUSSION

These results point out the desirability of assessing the degree of whole cell contamination in each experiment in which incorporation of amino acids into protein in

extracts of bacterial systems is being studied. It is necessary in the present system, where a 15-min incubation is employed, to reduce the whole cell contamination to a level of $1 \cdot 10^5$ cell/ml or less, in order to obtain meaningful incorporation values for the cell-free fractions. The risks of whole cell artifact are increased in the presence of an incorporation medium rich in amino acids and nucleotide cofactors, and become increasingly grave with longer incubation times. As shown, contamination can be determined by cell count or by comparing the incorporation of paired flasks in the presence and absence of ATP, GTP, PEP and PEP kinase, since only the incorporation into bacterial extracts requires these additions.

The growth phase of the cells is also important. For maximal incorporation, the cells must be in the earliest stage of log phase growth. Experimentally it has been observed that yields in excess of 4 g of cells (wet wt./16 l of media) give extracts which have specific incorporating activities less than $6 \cdot 10^{-5}$ μmole of leucine/mg of protein.

The present system closely resembles the rat liver cell-free system previously described[7,8,14], in that both require sedimentable and non-sedimentable fractions for maximal incorporation, both are stimulated by ATP, GTP, and a nucleoside triphosphate generating system, and optimal concentrations of each of these additives are comparable. In addition, the kinetics of incorporation are similar (rapid initial incorporation followed by a plateau-ing after 15-30 min incubation). Recently TISSIÈRES AND WATSON found that the magnesium concentration is a critical factor for the stability of the bacterial ribosomes[5], an observation parallel to the findings of PETERMANN *et al.*[6] in animal systems. As shown here, maximal incorporation occurs at the magnesium concentration optimal for stabilizing the 70-S particles described by TISSIÈRES AND WATSON.

The incorporation described herein differs from the *in vitro* bacterial systems of BELJANSKI AND OCHOA[15], SPIEGELMAN[16], GALE AND FOLKES[17] and HUNTER *et al.*[18] in that a low speed precipitable membrane fraction is not required. An additional distinction from the mucopeptide synthesis of MANDELSTAM AND ROGERS[19] is that the present system is chloramphenicol-sensitive.

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FURTHER STUDIES ON CYSTATHIONINE SYNTHETASE-SERINE DEAMINASE OF RAT LIVER

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SUMMARY

1. The purity of rat liver cystathionine synthetase-serine deaminase was more than doubled over that previously reported through the use of zone electrophoresis. The ratio of the two enzymic activities of the more purified preparation remained unchanged.

2. Enzymic activity was slightly inhibited by certain pyridoxine derivatives and strongly inhibited by carbonyl reagents, sulfhydryl reagents, various metal chelating agents, and by a number of heavy metal ions. Mg^{++} and Mn^{++} slightly stimulated the serine deaminase activity.

3. Study of the substrate specificity of the enzyme showed that only L-serine and L-threonine were deaminated and only homocysteine was utilized for synthesis of a sulfur-containing diamino acid with L-serine. L-cysteine strongly inhibited cystathionine synthesis.

INTRODUCTION

A procedure for the partial purification of cystathionine synthetase-serine deaminase from rat liver and some of the properties of this enzyme were reported earlier[1]. The

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