PROTEIN BIOSYNTHESIS BY A CELL-FREE BACTERIAL SYSTEM

III. DETERMINATION OF NEW PEPTIDE BONDS; REQUIREMENT FOR THE "AMINO ACID INCORPORATION ENZYME" IN PROTEIN BIOSYNTHESIS

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SUMMARY

The incorporation of [^{14}C] amino acids into protein by *Alcaligenes faecalis* fragments has been demonstrated. Using [^{14}C]valine, it was found that most of the incorporated amino acid was within the peptide chains. *Alcaligenes* fragments have been partially solubilized by treatment with perfluoro octanoate without affecting the incorporation activity. The soluble fraction obtained after this treatment can be replaced by purified "amino acid incorporation enzymes".

INTRODUCTION

In two previous papers BELJANSKI AND OCHOA have reported that a particulate preparation of *Alcaligenes faecalis* is able to incorporate various [^{14}C] amino acids into protein[1,2]. It was also shown that under appropriate conditions, a highly purified enzymic preparation obtained from crude extracts of this organism stimulates the incorporation of amino acids when added to a particulate preparation. The purified enzyme preparation had no ability to promote the exchange of labeled pyrophosphate with ATP in the presence of amino acids, yet it completely replaced the "pH 5.0 enzymes" in the incorporation of amino acids into protein of rat liver microsomes[2]. This new enzymic preparation was referred to as the "amino acid incorporation enzyme".

In this paper, evidence is presented to show that the incorporated [^{14}C] amino acids are in true peptide bonds. We further wish to report results indicating that the same particulate preparation appears to be free of so called "amino acid activating enzymes" and contains the "amino acid incorporation enzyme". This latter system is able to catalyse the Mg^{++} dependent exchange of four labeled diphosphonucleosides

Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP adenosine monophosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; UTP, uridine triphosphate; UDP, uridine diphosphate; CTP, cytidine triphosphate; CDP, cytidine diphosphate; PP, pyrophosphate; Tris, tris (hydroxymethyl) aminomethane; FDNB, 2,4-fluoro-dinitrobenzene.

with their homologous triphosphonucleosides[3]. The exchange reactions seem to be related to the incorporation of amino acids into peptide bond.

MATERIALS AND METHODS

The preparation of *Alcaligenes faecalis* (8750) fragments and the determination of the incorporation of [^{14}C] amino acids into protein have been described previously[1].

Solubilisation of Alcaligenes *particles*

Partial solubilisation of the particulate preparation has now been achieved with sodium perfluoro-octanoate (Minnesota Mining and Manufacturing Co., St Paul 6, Minn., U.S.A.). 1 ml of the particulate preparation in Tris buffer, 0.05 M, pH 7.3, containing 10–15 mg of protein was mixed with 2 mg of perfluoro-octanoate dissolved in 0.1 ml of distilled water. After standing at room temperature for 25 min, the lysed preparation (L) was centrifuged at 30,000 $\times$ g for 25 min in the cold giving a pellet (P 30) and a yellow-brown supernatant (S 30) which contained about 50 % of the initial protein (ratio 280/260 = 1.7). The pellet (P 30) was resuspended in the same volume of Tris buffer as the original mixture (ratio 280/260 = 1.0).

Peptide bond determination

In order to show the position of the labeled amino acid in the newly formed protein, a large amount of labeled protein was obtained by incubation of the particulate preparation containing 20 mg of protein with uniformally labeled [^{14}C]valine (0.04 μM, 500,000 counts/min) and a mixture of 18 L-amino acids as previously described[1].

Effect of carboxypeptidase: The incorporation of [^{14}C]valine into the C-terminal position of peptide chains was tested with crystalline carboxypeptidase[4] (Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.). The conditions of incubation are described in the legend to Table IV. After stopping the reaction with 5 ml of 10 % trichloroacetic acid (TCA), the precipitated protein was collected by centrifugation, washed twice with cold TCA, dried with acetone and ether and the radioactivity measured. Any decrease in radioactivity of the protein after treatment with carboxypeptidase would indicate the occurrence of incorporated [^{14}C]valine in the C-terminal position of peptide chains. Carboxypeptidase activity was tested beforehand with native and denatured bovine serum albumin. Protein was measured by the biuret reaction.

Treatment with fluoro-dinitrobenzene: The possible presence of [^{14}C]valine in the N-terminal position of peptide chains was tested with 2,4-fluoro-dinitrobenzene (FDNB)[5]. 10 mg of protein labeled with [^{14}C]valine (1300 counts/min/mg) were mixed with 10 mg of $NaHCO_3$ in 1 ml of water and 2 ml of an ethanolic solution of FDNB, (5 % v/v). The mixture was vigorously shaken at room temperature for 2 h. A few drops of concentrated HCl were then added and the protein was washed several times with ether. The protein was finally hydrolysed with 6 N HCl for 16 h at 110°. The DNP-amino acids were separated from free amino acids by extraction with ether. The radioactivity of dried samples of the ether extract and the water solution was determined.

Partial hydrolysis of labeled protein: The incorporation of [^{14}C]valine within peptide chains was demonstrated by partial hydrolysis of the protein with HCl and

isolation and analysis of labeled peptides. 20 mg of labeled protein (1360 counts/min/mg) were dissolved in 1 ml of concentrated HCl and allowed to stand for 4 days at 37°. The HCl was removed by evaporation in the presence of distilled water. Aliquots of the hydrolysate were chromatographed on Whatman No. 2 paper in two dimensions. The separated peptides were detected by radioautograph. Three of the distinct radioactive materials resulting from the partial hydrolysis of the protein were eluted and were each hydrolysed with 6 N HCl for 24 h at 110°. After removal of HCl by evaporation each sample was chromatographed. The chromatogram was sprayed with ninhydrin and then exposed to film.

ATP–PP exchange: The exchange of ATP with [^{32}P]pyrophosphate was measured as previously described[1].

Exchange of labeled di- with unlabeled triphosphonucleosides: The method for measurement of exchange of labeled diphosphonucleosides with unlabeled triphosphonucleosides is described in the following paper[6]. All unlabeled di- and triphosphonucleosides were purchased from Sigma Chemical Company (St Louis Ill., U.S.A.).

Myokinase activity: Myokinase activity of the particulate preparation of *Alcaligenes faecalis* was tested by incubation of [^{14}C]ADP with Mg^{++} ions and subsequent analysis for appearance of labeled ATP and AMP. Essentially no myokinase activity was detected.

RESULTS

Amino acid incorporation into protein by particulate preparation of Alcaligenes faecalis

A number of [^{14}C]labeled amino acids were tested for their ability to be incorporated into protein by *Alcaligenes faecalis* particulate preparations. As shown in Table I all of the L- or DL-amino acids tested were incorporated into protein. It is interesting to note that the addition of unlabeled D-leucine, and D-valine did not depress the incorporation of their respective labeled L-enantiomorphs; on the other hand addition

TABLE I

INCORPORATION OF ^{14}C LABELED AMINO ACIDS INTO PROTEIN BY *Alcaligenes faecalis* PARTICULATE PREPARATION

The samples contained: Tris buffer, pH 7.3, 50 µmoles; $MgCl_2$, 5.0 µmoles; K_2HPO_4, 5.0 µmoles; 0.2 µmole of labeled (120,000 counts/min/µmole) and unlabeled amino acid; unwashed *Alcaligenes* particles with 300–400 µg of protein[1]. Final volume, 1.0 ml; incubation in air, 1 h at 34°. Addition of ATP had little stimulating effect. The system seems to depend on oxidative phosphorylation for generation of the ATP[1].

Added amino acids		*Labeled amino acids incorporated mµ M/mg protein N/h*
Labeled	*Unlabeled*	
Glycine (0 time)		0
Glycine		44
L-valine		18
L-valine	D-valine	18
DL-leucine		24
DL-leucine	D-leucine	24
DL-phenylalanine		26
DL-phenylalanine	D-phenylalanine	19
DL-glutamic acid		12
DL-glutamic acid	D-glutamic acid	5

of D-phenylalanine and D-glutamic acid did depress the incorporation of the labeled amino acids (Table I). Similar results have been obtained with perfluoro-octanoate treated preparations.

Chloramphenicol effect: Since chloramphenicol is generally considered to be an inhibitor of protein biosynthesis[7-9] but not of cell wall synthesis in bacteria[10], the effect of this antibiotic in incorporation of labeled amino acids into protein by the *Alcaligenes faecalis* particulate preparation was tested. Addition of chloramphenicol at different concentrations to the incubation mixture without preincubation of particles with antibiotic resulted in strong inhibition of amino acid incorporation, Table III.

Peptide bond formation: Previous observations have suggested strongly the formation of true peptide bonds when amino acids are incorporated into hot TCA insoluble material by *Alcaligenes faecalis* particles[1,2]. In the present studies the labeled protein obtained after incubation with [^{14}C]valine was treated with carboxypeptidase and with FDNB and was also partially hydrolysed with hydrochloric acid to obtain labeled peptides. Treatment of the labeled protein with carboxypeptidase

TABLE II

INCORPORATION OF [^{14}C]GLYCINE INTO PROTEIN OF *Alcaligenes faecalis* PARTICULATE PREPARATION UNTREATED AND TREATED WITH PERFLUORO-OCTANOATE

Conditions as in Table I: with 300 µg of protein of washed particles[1]; supernatant (S 30) with 150 µg of protein; pellet (P 30) with 150 µg of protein; "amino acid incorporation enzyme" (specific activity 20) 20 µg of protein.

Preparation	*[^{14}C]glycine incorporated mµ M/mg of protein N/h*
Untreated (0 time)	0
Untreated	20
Treated (L)	25
Pellet (P 30)	4
Supernatant (S 30)	0
Pellet (P 30) + supernatant (S 30)	30
Pellet (P 30) + heated supernatant (S 30)	10
Pellet (P 30) + "amino acid incorporation enzyme"	26

TABLE III

EFFECT OF CHLORAMPHENICOL ON INCORPORATION OF [^{14}C] AMINO ACIDS INTO PROTEIN OF *Alcaligenes faecalis* PARTICULATE PREPARATION

Conditions as in Table I: unwashed *Alcaligenes* particles[1] with 350 µg of protein; chloramphenicol 50 µg and 100 µg/ml.

Added labeled amino acids	*Labeled amino acid incorporated mµ M/mg of protein N/h*		
	Chloramphenicol		
	No addition	*Addition 50 µg*	*Addition 100 µg*
Glycine	55	8	5
DL-leucine	13	1.5	0.4
DL-glutamic acid	13	2.5	2.1

had no significant effect on the radioactivity of the TCA insoluble material (Table IV). Although this is not absolute proof, the negative effect obtained with carboxypeptidase suggests that [^{14}C]valine was not present in any significant quantity in the C-terminal position of peptide chains.

Table V shows that when the N-terminal position of the incorporated amino acid was determined with the DPN-method[5] about 15 % of the [^{14}C]valine reappeared as DNP-valine. This indicates that although the labeled amino acid was incorporated primarily within the peptide chains, a significant quantity of the valine was present at the N-terminal position.

TABLE IV

RADIOACTIVITY OF LABELED PROTEIN WITH [^{14}C]VALINE OF *Alcaligenes* PARTICLES TREATED WITH CARBOXYPEPTIDASE

The samples contained: phosphate buffer, pH 7.4, 0.5 *M*; 7.6 mg of [^{14}C]protein (1350 counts/min/mg); 1350 µg of carboxypeptidase in 0.03 ml of 13 % LiCl; final volume 0.64 ml; Temperature, 38°.

Incubation time (hours)	*Counts/min*	*Protein mg*	*Counts/min/mg protein*
0	1400	1.05	1350
1	1646	1.25	1320
7	1230	1.00	1230

TABLE V

N-TERMINAL POSITION OF [^{14}C]VALINE IN PROTEIN OF *Alcaligenes* PARTICULATE PREPARATION

Conditions as described in MATERIALS AND METHODS.

Preparation	*Total counts/min*
Labeled protein with [^{14}C]valine	13,500
DNP [^{14}C] amino acid	2,000
Free [^{14}C] amino acid	11,550

Partial hydrolysis with HCl and chromatography of the hydrolysate revealed the existence of a number of radioactive residues (Fig. 1). After elution of the spot corresponding to position "A", hydrolysis and chromatography of the eluted material revealed the existence of six amino acids by the ninhydrin test, only one of which was radioactive on exposure to X ray film. The ninhydrin positive, radioactive spot corresponded to that expected for valine (Fig. 2). This procedure was also carried out

Isolation of peptides labeled with [^{14}C]valine from partially hydrolysed protein of *Alcaligenes particles*.

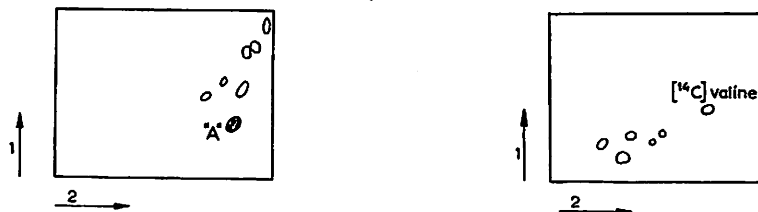

Fig. 1. Chromatogram. Peptide spots printed on X ray film.

Fig. 2. Chromatogram. Amino acids from hydrolysed spot "A" from Fig. 1.

Solvent 1, *n*-butanol–acetic acid–water (250:60:250); solvent 2, phenol–water (4:1).

with similar results with two other labeled peptides. These observations demonstrate that [^{14}C]valine was to a great extent incorporated into peptide bonds within the peptide chains.

Dependency of incorporation of amino acids into Alcaligenes *fragments on the content of the "amino acid incorporation enzyme"*

Previous results have shown that a particulate preparation of *Alcaligenes faecalis* incorporates ^{14}C amino acids into protein. However, we were unable to detect the presence of "amino acid activating enzymes" in this preparation[1]. In the present study pretreatment with perfluoro-octanoate was used to partially solubilise the particulate preparation. As shown in Table VI the treated preparation does not show any detectable pyrophosphate–ATP exchange activity. The supernatant obtained at 30,000 × *g* of the treated preparation contains the "amino acid incorporation enzyme" activity. This fraction also is capable of catalysing a rapid exchange of all four di- with triphosphonucleosides (Table VI).

TABLE VI

PRESENCE OF THE "AMINO ACID INCORPORATION ENZYME" IN THE *Alcaligenes faecalis* PARTICULATE PREPARATION UNTREATED AND TREATED WITH PERFLUORO-OCTANOATE

Conditions as described in the following paper[6]: with 220 µg of protein of untreated and treated (L) *Alcaligenes faecalis* particulate preparation (washed particles[1]; 115 µg of supernatant (S 30) and 100 µg of pellet (P 30)). Incubation time 30 min. Calculations according to DE MOSS AND NOVELLI[22].

Preparation	*Rate of exchange: µmoles/mg protein/h*				
	ATP–ADP	*GTP–GDP*	*UTP–UDP*	*CTP–CDP*	*ATP–^{32}P^{32}P*
Untreated	0.75	0.85	0.90	0.60	0.00
Treated (L)	1.90	1.75	1.65	1.14	0.00
Supernatant (S 30)	3.20	3.40	2.95	2.05	0.00
Pellet (P 30)	0.40	0.70	0.75	0.60	0.00

When the supernatant solution was used under the conditions described in the legend to Table II, no [^{14}C] amino acids were incorporated into protein. The pellet (P 30) obtained after perfluoro-octanoate treatment still retained some incorporation activity. Combination of the two fractions resulted in an incorporation into protein which was equal to the incorporation of amino acids exhibited by the *Alcaligenes faecalis* preparation treated with perfluoro-octanoate without centrifugation.

It was found that the addition of purified "amino acid incorporation enzyme" to the pellet (P 30) instead of the supernatant fraction (S 30) resulted in a stimulation of amino acid incorporation into protein which is equivalent to that obtained with untreated fragments (Table II). This purified fraction which completely replaced the supernatant solution contains no amino acid activating enzymes[1].

When the 40 % ammonium sulfate fraction[1] which is a fraction rich in amino acid activating enzymes was added to the perfluoro-octanoate pellet (P 30) in the presence of ATP no stimulation of the incorporation of amino acids was observed.

DISCUSSION

In our previous paper we have described a particulate preparation of *Alcaligenes faecalis* which is capable of active incorporation of amino acids into protein although the amino acid dependent exchange of PP with ATP was not detectable[1]. In further

study of this same material presented here, it is more definitely shown that the "amino acid activating enzymes" are absent in the system, since the perfluoro-octanoate solubilized particles do not show, in detectable manner, the ability to activate the amino acids by ATP. This is particularly interesting in view of the suggestion by HOAGLAND and his colleagues[11] and others[12–15] that the carboxyl activation of amino acids is the first step in protein biosynthesis. On the other hand, the same partially solubilised material is able to incorporate [^{14}C] amino acids into peptide bonds. Similar observations were made by COHN[18] and ZALTA[19] with microsomal systems from animal tissues. Recently, NISMAN AND FUKUHARA have described the properties of a "soluble fraction" called "amino acid polymerase" isolated from cell membrane fragments of *E. coli*. This preparation actively incorporates amino acids into protein in the absence of amino acid activating enzymes[20]. The above results suggest that the incorporation of amino acids into protein does not necessarely involve preliminary formation of amino acyl adenylates.

It is particularly interesting to note that the "amino acid incorporation enzyme" which is able to replace the requirement for the solubilized fraction (S 30) of *Alcaligenes* fragments shows the ability to exchange phosphate between all four di- and triphosphonucleosides. This suggests that the latter activity may be directly or indirectly involved in protein biosynthesis. It is also of interest to recall the experiments of SNOKE AND BLOCH[21] on glutathione formation where it was shown that a highly purified enzyme which catalysed the formation of glutathione, also catalysed the exchange of orthophosphate from ATP to ADP.

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