acid composition. In the early stages of fractionation these amino acid conjugates appeared to have an absorption maximum at 268 mμ, but in later chromatographic steps the ninhydrin positive components separated from the u.v. absorbing components. Since this work was initiated several laboratories[7–9] have reported the presence of nucleotide–peptide complexes in microorganisms. The results described above strongly suggest the occurrence of similar compounds in mammalian tissue. Because of the low yields obtained, however, it has not yet been possible to establish conclusively the chemical nature of the complexes.

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BIOSYNTHETIC INCORPORATION OF FLUOROPHENYLALANINE INTO CRYSTALLINE PROTEINS*

MARTHA VAUGHAN AND DANIEL STEINBERG

Section on Metabolism, Laboratory of Cellular Physiology and Metabolism, National Heart Institute, National Institutes of Health, Bethesda, Md. (U.S.A.)

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SUMMARY

1. [^{3}H]OFP and [^{3}H]PFP are incorporated into ovalbumin and into lysozyme, as well as into a mixed tissue protein fraction by minced hen's oviduct incubated *in vitro*. In these purified proteins, the analogue is present as an integral part of the molecule and has been found to be distributed among several peptides separated after partial degradation of the respective proteins.

2. The significance of these and of similar studies demonstrating the incorporation of amino acid analogues into otherwise apparently complete and normal proteins is discussed.

* Preliminary reports of these studies have been published[1,2].

The abbreviations used are: PFP, *p*-fluorophenylalanine; OFP, *o*-fluorophenylalanine; TCA, trichloroacetic acid.

INTRODUCTION

The incorporation of *p*-flurophenylalanine, an amino acid not known to occur in nature, into bacterial protein has been demonstrated in several laboratories[1–5]. In these, as in most similar studies with amino acid analogues, the protein examined has been a mixture, in most cases the total TCA-precipitable protein of the bacteria or tissues studied. It is possible that the analogue contained in this material is present only in incomplete protein molecules, the biosynthesis of which was halted by the introduction of the unnatural amino acid. It is apparent from studies with bacterial mutants that some amino acid analogues can be very effectively utilized in the biosynthesis of functional proteins[6] and are therefore almost certainly incorporated into complete proteins. Isolation and characterization of proteins containing the analogue would be the necessary direct and conclusive evidence for utilization of any given amino acid analogue in protein synthesis. The studies described below were undertaken to determine whether PFP and OFP could be incorporated into purified lysozyme and ovalbumin by hen's oviduct incubated *in vitro*.

METHODS

Preparation of [^{3}H]OFP and [^{3}H]PFP

OFP and PFP were labeled with tritium by the WILZBACH[7] technique*. After removal of labile tritium, samples for use in incorporation experiments were subjected to chromatography on 1 × 20 cm columns of Dowex 50 (200–400 mesh) employing 0.2 *M* citrate buffer, pH 5.0, as the eluant. The two analogues behaved similarly on the column, emerging almost exactly at the effluent volume characteristic for phenylalanine. Approximately 50 % of the radioactivity placed on the column emerged with the front, but this peak of radioactivity contained no ninhydrin reactive material. The peak of ninhydrin reactive material, representing 80–90 % recovery of amino acid, coincided with a second peak of radioactivity. The material in this peak was pooled and desalted. The specific radioactivity of the chromatographically purified OFP was $2.8 \cdot 10^5$ counts/min/μmole. This specific radioactivity was unchanged by treatment with 6 *N* HCl under the same conditions used in later experiments to hydrolyze protein. After this acid treatment OFP was recovered from paper chromatograms developed with Solvent I (*tert*-butanol–methyl ethyl ketone–water, (2:2:1)). PFP purified by column chromatography had a specific radioactivity of $4.5 \cdot 10^5$ counts/min/μmole. PFP appears to be well separated from phenylalanine, which has a lower R_F, by chromatography on paper with Solvent I [3]. When, however, the labeled analogue was chromatographed under these conditions about 10 % of the radioactivity was found in the phenylalanine area. When the PFP band from such a chromatogram was eluted and re-chromatographed in the same solvent, radioactivity in the phenylalanine area again amounted to about 10 % of that in the PFP region. Similar data were obtained using [^{3}H]PFP which had been subjected to "acid hydrolysis". It was concluded that the appearance of radioactivity in phenylalanine area was due to "tailing" of PFP.

* Exposure of the OFP to tritium gas was kindly carried out by Dr. K. E. WILZBACH. PFP was tritiated by the New England Nuclear Company.

Incubation of tissue and isolation of proteins

Minced hen's oviduct was incubated for 4 h at 37° in Krebs bicarbonate buffer containing [^{3}H]PFP or OFP, following which the tissue was homogenized in its incubation medium. The homogenate was centrifuged for 20 min in a Servall angle-head centrifuge at 4°. The sedimented material was washed twice with cold 5 % TCA, once with 5 % TCA at 90° for 15 min, twice with ethanol–ether (1:1), and twice with ether. This material is referred to as mixed tissue protein.

The supernatant fraction of the homogenate was made 40 % saturated with ammonium sulfate (pH 5.0) and the protein which precipitated was removed. Ovalbumin was crystallized from the remaining solution by adjusting the pH to 4.5 and adding ammonium sulfate to approx. 45 % saturation. It was recrystallized to constant specific radioactivity. Ovalbumin prepared in this way from four different incubation flasks (two containing [3-^{14}C] phenylalanine and two containing [^{3}H]OFP), when subjected to electrophoresis (sodium phosphate buffer, pH 6.8; ionic strength, 0.1) showed the two characteristic ovalbumin peaks, A_1 and A_2, but no others.

Lysozyme was then recovered from the supernatant fraction remaining after the first crystallization of ovalbumin as discussed above. Carrier lysozyme was added to the supernatant fraction, which was then dialyzed to remove ammonium sulfate and lyophilized. The lyophilized material was dissolved in water and lysozyme was crystallized from it by adjusting the pH to 9.5 and adding NaCl to a final concentration of 5 %. The crystalline lysozyme was chromatographed on IRC-50, according to the method of GONCALVES *et al.*[8]. In some experiments the lyophilized material was taken up in buffer and was chromatographed on a column of IRC-50 without further preliminary purification. The material in the lysozyme peak was dialyzed and the enzyme was crystallized. The enzymic specific activity of lysozyme isolated by these techniques was assayed with *Micrococcus lysodeikticus*[9] and was identical with that of the crystalline lysozyme used as carrier. Because of the use of carrier enzyme and because of the relatively limited incorporation rates observed in this system, only a minute fraction of the recovered lysozyme molecules contained analogue. Consequently it is not possible to determine whether or not those molecules containing analogue retained full enzymic activity.

Degradation of lysozyme and ovalbumin

Proteins were hydrolyzed for 18 h in 6 *N* hydrochloric acid in an autoclave at 15 lb. pressure. The hydrolysate, after removal of most of the acid, was placed on a column of Dowex 50-H$^+$ and the amino acids were eluted with 2 *N* ammonium hydroxide. PFP and OFP were separated from this effluent by chromatography on Whatman No. 3 paper using Solvent I.

Oxidized ovalbumin was prepared by dissolving 30 mg of 5 times crystallized ovalbumin, which had been dialyzed and lyophilized, in 0.8 ml 88 % formic acid. 0.2 ml 30 % H_2O_2 was added and the solution was allowed to stand for 1.5 h. After dilution with 5 ml of water and concentration *in vacuo* the oxidized ovalbumin was recovered by precipitation with acetone, washed with acetone and dried. It was digested with 0.5 mg chymotrypsin at pH 7.5 for 18 h at room temperature. Lysozyme, denatured by heating at 105° for 1 h, was digested similarly with chymotrypsin. The aromatic peptides from the digest of lysozyme were adsorbed on activated charcoal and eluted with a mixture of acetic acid and ethyl acetate[10].

10 mg of 5 times crystallized ovalbumin were dissolved in 0.2 ml 50% sodium bicarbonate solution and, after the addition of 0.4 ml of 10% (w/v) solution of fluorodinitrobenzene, the mixture was shaken for 2 h at room temperature. The dinitrophenyl-derivative of ovalbumin, which precipitated during the course of the reaction, was washed 3 times with water, 3 times with ethanol and 3 times with ether. After acid hydrolysis of this material and extraction with ether, the free amino acids were recovered by elution with 2 *N* NH_4OH from a column of Dowex 50.

High voltage electrophoresis on paper was employed for the separation of peptides in some experiments, using an apparatus of the type described by MICHL[11] with pyridine–acetate buffer, pH 3.7.

Assay of ^{14}C and ^{3}H radioactivity

Samples of dry protein or amino acid containing either ^{14}C or ^{3}H were dissolved in 1 *M* hyamine (*p*-di-isobutylcresoxymethoxyethyldimethylbenzyl ammonium hydroxide, Rohm and Haas Co., Philadelphia, Pennsylvania) in methanol in 5 dram glass vials, following which 15 ml of a solution of diphenyloxazole in toluene (6 g/l) was added. Radioactivity was assayed in a Tri-Carb Liquid Scintillation Spectrometer, using appropriate voltage and window settings for each isotope. Corrections were made for quenching by the use of an internal standard. Details of these procedures have been previously described[12].

RESULTS AND DISCUSSION

As shown in Tables I and II, both OFP and PFP appear to be incorporated into crystalline ovalbumin and into the mixed tissue protein fraction. With two of the OFP experiments parallel experiments were carried out using a portion of the same oviduct incubated with 5 mg [3-^{14}C]phenylalanine. The relative incorporation into ovalbumin and into the mixed tissue protein was the same for phenylalanine and for the analogue in these two experiments (Table I). These results suggest that the mechanism for ovalbumin synthesis is neither more nor less selective against the analogue than are the systems for production of the bulk of the oviduct proteins. They do not, of course, exclude the possibility that individual proteins are synthesized which contain relatively greater or lesser amounts of fluorophenylalanine than are incorporated into the ovalbumin.

In all of these studies the ovalbumin was recrystallized until the specific radioactivity was constant. For example, in the case of one sample of ovalbumin isolated

TABLE I

INCORPORATION OF ^{3}H-OFP INTO OVIDUCT PROTEINS

Expt.	*Protein fraction*	*Protein specific activity (counts/min/mg)*	
		^{3}H-OFP	*^{14}C-phenylalanine*
O-1	Ovalbumin	111	15,739
	Mixed tissue protein	104	15,512
O-2	Ovalbumin	48	3,328
	Mixed tissue protein	155	12,265

TABLE II

INCORPORATION OF ^{3}H-PFP INTO OVIDUCT PROTEINS

Expt.		*Concentration of PFP (mM)*	*Incubation time (h)*	*Protein specific activity (counts/min/mg)*
P-1	Ovalbumin	1.1	4	406
	Mixed protein			803
P-2	Ovalbumin	2.0	3	544
	Mixed protein			850

after incubation of minced oviduct with tritiated PFP specific radioactivities measured after the third, fourth and fifth recrystallizations were, respectively, 448, 399 and 413 counts/min/mg.

It has been reported that under certain conditions amino acid adenylates can be non-enzymically incorporated into protein[13,14]. This appears to be due to acylation of free amino groups on the protein by the activated amino acid. In order to rule out incorporation by such a mechanism in the present studies a sample of ovalbumin from experiment P-1 was treated with fluorodinitrobenzene. After acid hydrolysis and removal of the dinitrophenyl-amino acids essentially all of the original radioactivity was recovered in the free amino acid fraction, indicating that PFP was incorporated into the fabric of the ovalbumin molecules in a manner such that it was not free to react with fluorodinitrobenzene. In order to show that the incorporated analogue was distributed throughout the protein molecule, another sample of this ovalbumin preparation was oxidized and digested with chymotrypsin. The digest was subjected to chromatography on paper using a solvent mixture of *n*-butanol–acetic acid–water (4:2:1) and a series of arbitrary bands, numbered 1 to 10 from the origin to the solvent front, was eluted. As shown in Table III, radioactivity was present in varying amounts in several areas, indicating the presence of the analogue in a number of peptide fragments.

TABLE III

DISTRIBUTION OF ^{3}H IN PEPTIDES PRODUCED BY CHYMOTRYPTIC DIGESTION OF OVALBUMIN AND OF LYSOZYME

Ovalbumin P-1		*Lysozyme O-1*	
Chromatographic band	*counts/min ± S.D.**	*Electrophoretic band*	*counts/min ± S.D.**
1	23 ± 5.1	C	15.7 ± 1.77
2	18 ± 5.0	D	40.1 ± 1.99
3	126 ± 6.4	F	20.7 ± 1.82
4	30 ± 5.0	G	5.7 ± 1.68
5	47 ± 5.7	H	7.6 ± 1.70
6	39 ± 5.5	J	362 ± 3.83
7	63 ± 6.0		
8	34 ± 5.2		
9	70 ± 6.1		
10	44 ± 5.4		

* S.D. = Standard deviation.

In order to demonstrate that the tritium contained in ovalbumin actually represented incorporated analogue, a sample from Expt. P-1 (Table II) was hydrolyzed in 6 *N* HCl and the amino acid mixture was chromatographed on paper. The PFP band accounted for the major fraction of the radioactivity of the protein. In similar fashion samples of ovalbumin from Expts. 0–1 and 0–2 (Table I) were hydrolyzed and OFP was isolated by chromatography on Dowex 50. Tritium in this fraction accounted for 80% of the radioactivity found in the original ovalbumin samples. These studies indicate that very little, if any, defluoridation occurred during incubation of the labeled analogues with oviduct mince. This is consonant with the results of MUNIER AND COHEN[4] who found, using *E. coli* grown in the presence of ^{14}C-PFP, no evidence of conversion of PFP to phenylalanine. ARMSTRONG AND LEWIS[15] reported the urinary excretion of inorganic fluoride in rats fed PFP, but the fluoride may have been derived, not directly from the PFP molecule, but from some previously partially degraded product of the amino acid analogue.

Both OFP and PFP were also incorporated into lysozyme by the minced oviduct. A sample of crystalline, chromatographically pure lysozyme from P-1 was hydrolyzed with acid. That the protein radioactivity represented PFP was shown by paper chromatography of an acid hydrolysate using Solvent I. Approximately 80% of the incorporated radioactivity was found in the PFP band.

Lysozyme from Expt. 0–1 was denatured and digested with chymotrypsin. From a fraction containing aromatic peptides prepared by adsorption on charcoal, eleven ninhydrin staining bands designated A through K (anode to cathode) were separated by high voltage electrophoresis on paper. In six of these peptides phenylalanine could be identified chromatographically after acid hydrolysis. Since lysozyme contains only three residues of phenylalanine per mole of protein, it is clear that, either the digestion was incomplete or that there was hydrolysis of bonds involving non-aromatic residues. The OFP-phenylalanine area was eluted and, in all 6 peptides, was found to contain radioactivity, but the amounts varied widely (Table III). Since the yields of the different peptides may also have varied widely, this observation cannot be interpreted as a result of non-uniformity of labeling.

OFP and PFP have been shown to be incorporated into proteins which are indistinguishable, by the methods employed, from normal ovalbumin or lysozyme. It has been established that tritium present in these proteins is entirely or almost entirely representative of analogue, and that PFP is incorporated into ovalbumin in such a manner that it is not free to react with fluorodinitrobenzene. Enzymic digestion of lysozyme and of ovalbumin produced several fragments from each protein that contained radioactivity. The demonstration that an amino acid analogue can be incorporated into what are otherwise apparently normal ovalbumin and lysozyme molecules indicates that the introduction of an unnatural amino acid into a protein molecule during the process of synthesis does not necessarily prevent completion of that molecule. Several years ago BLACK AND KLEIBER[16] isolated [^{14}C]norleucine from milk casein after feeding the analogue to cows. This was the first demonstration of the incorporation of an unnatural amino acid into a single purified protein, although proof of incorporation into the peptide chain was not presented. Recently [^{14}C]PFP has been found in several crystalline enzymes isolated from the muscle of a rabbit fed this amino acid[17]. A methionine requiring mutant of *B. subtilis* growing in the presence of ethionine produces enzymically active α-amylase in which one-third of

the methionine is replaced by ethionine[18]. This is the most extensive replacement of an amino acid by its analogue which has been demonstrated in a single pure protein, although in the case of bacterial auxotrophic mutants growing on an analogue of the amino acid they require it may be assumed that there is essentially complete replacement of the amino acid with its analogue in the proteins which are formed[6].

From all of these observations it may be inferred that the process of protein biosynthesis is not absolutely specific. On the other hand, it seems probable that under normal environmental conditions with only normal amino acids present at normal concentrations a cell is probably capable of repeatedly reproducing a given protein without error. Although the present methods for determination of amino acid sequence in protein do not exclude the possibility that a small fraction of molecules differ in sequence from the majority, thus far errors have been observed only when the capacity of the protein synthesizing system to discriminate is severely challenged, as it is in analogue studies. For example, PFP, which has been shown by MUNIER AND COHEN[4], to replace phenylalanine in the proteins of *E. coli*, is structurally more similar to phenylalanine than is phenylalanine to tyrosine, its closest relative in the family of naturally occurring amino acids. The atomic diameters of the hydrogen and fluoride substituents are very similar (H, 2.4 Å; F, 2.7 Å) while the hydroxyl group is considerably larger (approx. 5 Å). Because amino acid analogues can be incorporated into proteins it does not necessarily follow that protein synthesis lacks specificity under normal environmental conditions[19]. On the other hand it becomes important to recognize that the question of specificity must be posed in a quantitative rather than a qualitative sense.

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