PHENANTHRENE BOUND TO A PROTEIN BY BIOSYNTHESIS*

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Received April 9, 1971

SUMMARY

Phenanthrene was bound to β-galactosidase by protein biosynthesis in a system of E. coli incubated with labeled dihydrohydroxyphenanthrylcysteine (DHP-cysteine). The amino acid analogue penetrated the cells but less readily than the natural amino acids. In the presence of an inducer, the hydrocarbon-cysteine conjugate was incorporated into protein, principally β-galactosidase. The enzyme was isolated in purified form, radioactive peptides were isolated from the tryptic digest, and DHP-cysteine was recovered from Pronase digests of the peptides. This demonstrates unequivocally that the newly synthesized protein carried a phenanthrene group attached to the cysteine sulfur by a sulfide linkage.

Direct binding of polycyclic hydrocarbons to proteins in metabolizing systems is well established (1). Since hydrocarbons are not reactive with proteins, a metabolic intermediate must be involved. Such an intermediate logically reacts with amino acids also. The products may constitute amino acid analogues, particularly S-substituted cysteines. This report provides further evidence that a hydrocarbon such as phenanthrene may be bound to proteins via biosynthetic pathways involving intermediary formation of an amino acid analogue. S-(9,10-dihydro-9-hydroxy-10-phenanthryl)-L-cysteine (DHP-cysteine) which is a metabolic product produced by the action of phenanthrene in vivo (2) is incorporated by E. coli into β-galactosidase. This represents a pathway alternative to direct binding of the hydrocarbon to tissue proteins.

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*This investigation was supported in part by USPHS Research Grant CA-01228 from the National Cancer Institute.

MATERIALS AND METHODS

Cystine-^{35}S and protamine sulfate were obtained from Schwarz/Mann. Phenanthrene was tritiated by the Wilzbach method by Amersham/Searle. S(-9,10-dihydro-9-hydroxy-10-phenanthryl)-L-cysteine-^{35}S and S-(9,10-dihydro-9-hydroxy-10-phenanthryl-^{3}H)-L-cysteine were prepared as described by Bucovaz et al (3). E. coli B was grown in glycerol-salts medium (4) and harvested in the late log phase after 16 hours. The saline-washed cells (1-2 g wet weight) were resuspended in 100 ml of unsupplemented medium and incubations were carried out as described in Table I in the presence of cysteine-^{35}S, leucine-^{14}C, or DHP-cysteine labeled with ^{35}S or ^{3}H. After 3 hours the cells were harvested by centrifugation and were washed with saline and then disrupted by sonification. Cellular debris was removed by centrifugation and 0.4 volume of neutralized 1% protamine sulfate solution was slowly added to the supernatant liquors. The protamine precipitate was removed after 2 hours at 4°. β-Galactosidase was isolated according to the method of Craven et al (5). Homogeneity was demonstrated by electrophoresis on 5% acrylamide gel which was stained for both enzyme activity and protein content.

A tryptic digest of the purified enzyme was placed on a 150 x 2.5 cm Sephadex G-25 column which was eluted with 0.2 M NH_4HCO_3. The elution fractions were assayed for radioactivity and optical density at 280 nm. Appropriate fractions were pooled and lyophilized. The radioactive peptide fractions were digested with Pronase for 72 hrs. in 0.06M NH_4HCO_3 at 37°. The digests were lyophilized and DHP-cysteine was extracted from the residue with dry methanol. The compound was identified on paper chromatograms developed with butanol, acetic acid, water (2:1:1)., Radioactivity of the eluted material was determined by

TABLE I

PENETRATION OF E. COLI BY DHP-CYSTEINE

E. coli B was grown in 2000 ml of aerated glycerol-salts medium and harvested in the late log phase after 16 hrs. Saline washed cells (1-2 g wet weight) were resuspended in 100 ml of medium per flask and preincubated for 45 min at 37°. The medium was then supplemented with 2 ml of glycerol, 450 mg of lactose and additions as shown. Incubation was carried out for 3 hours with shaking. All values are expressed in nmoles per flask. [a]Chloramphenicol was added, 200 ug per ml.

	Experiment 1			
	Cysteine-^{35}S		DHP-^{3}H-cysteine	
Added	730	825[a]	4200	6250[a]
Found Extracellular	147	183	1460	1360
Intracellular	570	640	3040	4700
%	78	78	72	75

	Experiment 2			
	Cysteine-^{35}S		DHP-cysteine-^{35}S	
Added	182	128[a]	13900	12200[a]
Found Extracellular	57	55	11900	11300
Intracellular	124	63	2000	900
%	68	49	19	7.4

	Experiment 3			
	Leucine-^{14}C		DHP-cysteine-^{35}S	
Added	52.7	36.5[a]	18100	16800[a]
Found Extracellular	2.8	27.9	13500	13000
Intracellular	51.5	9.9	6550	3790
%	98	27	36	23

liquid scintillation counting. Ribosomal structural proteins were purified according to the method of Kaltschmidt et al (6).

RESULTS

In Table I a typical experiment shows labeled amino acid analogue penetrated the cellular membrane during a 3-hour incubation. When chloramphenicol was added to the incubation mixture, amino acid uptake by the cells was reduced in all experiments excepting one. Approximately one-third of the intracellular conjugate was found in the precipitate when protamine was used to remove nucleic acids. In several experiments, from 0.7 to 1.4% of the analogue, DHP-cysteine, was incorporated into the active β-galactosidase in 2 1/2 hours while cysteine-^{35}S in controls was incorporated to the extent of 2-10%. Table II shows approximately 80% of the analogue in the intracellular compartment was incorporated into active enzyme. After a 24-hour incubation, 1.6% of the analogue in the intracellular fraction was found in ribosomal structural proteins. The rest of the analogue was distributed among the various protein fractions, cellular debris, etc. Elution of a Sephadex G-25 column loaded with a tryptic digest of the purified, labeled enzyme produced a single peak of radioactive, 280 nm absorbing material emerging at a time corresponding to a molecular weight of approximately 30,000. This product was hydrolyzed with Pronase and the products were resolved by two-dimensional paper chromatography. This yielded principally one ninhydryn-positive, radioactive spot which was identified as the original dihydrohydroxyphenanthryl-cysteine on the basis of R_f values in two solvents. In control experiments in which cysteine-^{35}S was substituted for the amino acid analogue, the resulting chromatogram had no radioactivity at the spot at which the DHP-cysteine would be expected to appear. In a comparable experiment, the Pronase digest was evaporated to dryness and extracted with dry methanol. The methanol extract

TABLE II

INCORPORATION OF LABELLED AMINO ACID IN β-GALACTOSIDASE

Incubation and isolation of pure β-galactosidase are described in the text. (a) 20 µg/ml of chloramphenicol added (b) DHP-cysteine was added after the sonication. (c) No radioactivity and no galactosidase activity detected. All values are expressed in nmoles per incubation mixture.

	Cysteine-^{35}S		DHP-^{3}H-cysteine		Control
Added	270	240[a]	2500	2600	----[b]
Intracellular	93	31	72	45	(1400)
After protamine	89	29	75	38	130
After dialysis	37	4	59	18	4
Purified Galactosidase	30	-[c]	58	-[c]	0.1
Amino acid residues per mole enzyme	16		17		

was chromatographed in n-butanol, acetic acid, water (2:1:1). DHP-^{3}H-cysteine was found at the appropriate area on the chromatogram. Of 250 pmoles of analogue present in the β-galactosidase, 4.1 pmoles of free analogue were recovered from the Pronase digest.

DISCUSSION

Previous studies on the participation of polycyclic hydrocarbon derivatives of cysteine in protein biosynthesis have only demonstrated the incorporation of the analogue into ribosomal protein by the usual criteria of precipitation of protein containing labeled analogue (7). In the present work, a pure protein, β-galactosidase, has been isolated with phenanthrene residues bound to it by sulfide links. The incorporation of the analogue into the protein fabric was demonstrated unequivocally by isolation of a fragment after tryptic digestion and release of the

dihydrohydroxyphenanthrylcysteine after Pronase digestion. Chloramphenicol prevented incorporation into the protein. The same results were obtained whether the label on the analogue was tritium in the hydrocarbon moiety or sulfur-35 in the cysteine residue. This, as well as isolation of the analogue by paper chromatography demonstrated that the dihydrohydroxyphenanthryl-cysteine was transferred intact.

Previous work on inhibition studies from this laboratory (3) has suggested that the DHP-cysteine is activated and transferred to tRNA by the synthetase systems operating on histidine, phenylalanine and glutamic acid. It is probable that penetration of the cell wall is a limiting factor in incorporation of analogues with groups as bulky as phenanthrene. The number of residues incorporated was variable, i.e. from 1 to 17 in a protein containing 45 phenylalanine and 36 histidine residues. It is well known that analogues are used much less efficiently than natural amino acids (8). Incorporation of the analogue did not produce a noticeable alteration in the behaviour of active β-galactosidase in the isolation procedures. The protein with hydrocarbon residues attached appeared to be fully active as an enzyme.

REFERENCES

1. Heidelberger, C., J. Cell Comp. Physiol., 64, (Sup. 1) 129 (1964).
2. Boyland, E., and Sims, P., Biochem. J., 84, 564 (1962).
3. Bucovaz, E. T., Morrison, J. C., James, H. L., Dais, C. F., and Wood, J. L., Cancer Res., 30, 155 (1970).
4. Lennox, E. S., Virology, 1, 190 (1955).
5. Craven, G. R., Steers, Jr., E., and Anfinsen, C. B., J. Biol. Chem., 240, 2468 (1965).
6. Kaltschmidt, E., Dzionara, M., Donner, D., and Wittman, H. G., Mol. Gen. Genet., 100, 364 (1967).
7. Bucovaz, E. T., and Wood, J. L., J. Biol. Chem., 239, 1131 (1964).
8. Conway, T. W., Landsford, Jr., E. M., and Shive, W., J. Bact., 85, 141 (1963).