INCORPORATION OF AMINO ACID CARBON INTO PROTEINS BY SHEEP THYROID GLAND MITOCHONDRIA*

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Received April 10, 1964

In this laboratory, Raghupathy et al. (1963, 1964) demonstrated the incorporation of ^{14}C-amino acids into protein by (a) slices prepared from the thyroid glands of rats, guinea pigs and sheep and (b) monolayer cultures of isolated thyroid gland cells. In vitro protein synthesis by subcellular fractions of the thyroid gland, however, has not hitherto been studied.

Since the early report of McLean et al. (1958), considerable evidence has accumulated demonstrating the incorporation of amino acids into protein by mitochondria isolated from a variety of animal and plant tissues as well as from microorganisms (Simpson, 1962). The present communication deals with the incorporation of ^{14}C-amino acids into protein by mitochondria isolated from sheep thyroid glands. An attempt has been made to elucidate the steps involved in this process by studying the cofactor requirements for and the

* This investigation was supported by grants from the U. S. Public Health Service.

effects of various inhibitors, such as DNase,* RNase, actinomycin D and puromycin, on the amino acid incorporating activity of thyroid mitochondria.

MATERIALS AND METHODS

Sheep thyroid glands were homogenized in a 0.25 M sucrose solution, and the homogenate was centrifuged at 500 x g for 10 minutes to remove nuclei and cell debris. The supernatant fraction was spun at 10,000 x g for 15 minutes, and the mitochondrial pellet so obtained was resuspended in the sucrose solution. The mitochondrial suspension was then spun at 8,000 x g for 15 minutes. This process of resuspending and spinning was repeated until the fluffy layer, consisting of lighter particles, was completely removed. This was considered achieved when the washings became clear; generally 4 to 5 washings were sufficient. Low-speed centrifugation was employed in order to provide mitochondria as free as possible of microsomes. The supernatant fraction obtained after removal of the mitochondria was spun at 100,000 x g for 1 hour to obtain the cell sap. The pH 5-enzyme fraction was prepared from the cell sap according to the method of Hoagland et al. (1956). Protein was estimated by the biuret method of Gornall et al. (1949), and RNA by the orcinol method described by Volkin and Cohn (1954).

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* Abbreviations: DNase for deoxyribonuclease; RNase for ribonuclease; ATP for adenosine triphosphate; GTP for guanosine triphosphate; PEP for phosphoenol pyruvic acid; PK for pyruvate kinase; RNA for ribonucleic acid; DNA for deoxyribonucleic acid; FDNB for 1-fluoro-2,4-dinitrobenzene; EDTA for ethylenediamine tetraacetic acid; GSH for glutathione; Tris for tris(hydroxymethyl)aminomethane; U for uniformly labeled; DNP for dinitrophenyl.

The isolated mitochondria were suspended in the sucrose solution, and aliquots of the suspension were incubated under the conditions described in Table I. At termination of the incubation, the reaction was stopped by addition of an equal volume of 10 % trichloroacetic acid to the incubation mixture. The precipitated protein was washed free of acid-soluble compounds, nucleic acids and lipids (McLean et al., 1958). The protein was dissolved in formic acid, and plated for counting (Raghupathy et al., 1964).

RESULTS AND DISCUSSION

Significant amounts of all six amino acids tested (leucine, valine, serine, glutamic acid, tyrosine and phenylalanine) were incorporated into protein by the mitochondria isolated from sheep thyroid glands (Table I).

TABLE I

INCORPORATION OF AMINO ACIDS INTO PROTEIN BY SHEEP THYROID MITOCHONDRIA

The reaction mixture consisted of 1 µmole ATP, 5 µmoles PEP, 50 µg PK, 10 µmoles magnesium acetate, 50 µmoles KCl, 0.5 µmole EDTA, 2.1 mµmoles ^{14}C-labeled amino acid (0.5 µc), 35 µmoles Tris-HCl buffer (pH 7.6) and mitochondria (3-6 mg protein), all in a total volume of 1 ml. The reaction was carried out at 37.5° in air for 60 minutes.

Amino acid	CPM per mg of isolated protein
L-Leucine-U-^{14}C	157
L-Valine-U-^{14}C	228
L-Serine-U-^{14}C	220
L-Glutamate-U-^{14}C	90
L-Tyrosine-U-^{14}C	417
L-Phenylalanine-U-^{14}C	316

To determine whether the observed incorporation of amino acids represents true peptide bond formation, the ^{14}C-labeled mitochondrial protein was treated with FDNB (Sanger, 1952), which reacts with the free amino groups of the protein. The protein was then hydrolyzed, and the DNP-amino acids were extracted with ether. The finding that less than 2 % of the ^{14}C of the protein (before FDNB treatment) was recovered in the ether-soluble DNP-amino acid fraction (Table II) suggests that the observed amino acid incorporation by the thyroid mitochondria represented, for the most part, true peptide formation and did not result from artifactious labeling of proteins by nonspecific acylation reactions.

TABLE II

TREATMENT OF ^{14}C-LABELED MITOCHONDRIAL PROTEIN WITH FDNB

Sheep thyroid mitochondria were incubated with L-leucine-U-^{14}C under the conditions described in Table I. The ^{14}C-protein was isolated and dinitrophenylated as described in text.

Fraction	CPM in fraction
Total protein (before treatment)	4800
DNP-amino acids (ether extract)	60

The findings presented in Table III demonstrate that, under the experimental conditions employed in the present study, GSH, GTP and cell sap (or the pH 5-enzyme fraction) are not obligatory requirements for incorporation of the ^{14}C of ^{14}C-leucine into sheep thyroid mitochondrial protein.

TABLE III

COFACTOR REQUIREMENTS FOR AND EFFECT OF INHIBITORS ON INCORPORATION OF L-LEUCINE-U-^{14}C INTO PROTEINS BY SHEEP THYROID MITOCHONDRIA

The reaction mixture was essentially the same as that described in Table I, unless otherwise mentioned.

Expt. no.	Omission or addition	CPM per mg protein
1	Complete system	463
	- Energy system (ATP, PEP & PK)	89
	- PEP, - PK	48
	- Mg^{++}	310
2	Complete system	450
	+ GTP (0.5 µmole)	375
	+ GSH (1 µmole)	340
	+ Cell sap (0.5 mg protein)	337
	+ pH 5-enzyme (0.5 mg protein)	315
3	Complete system	309
	+ RNase (50 µg)	210
	+ DNase (50 ug)	272
	+ Actinomycin (100 µg)	115
	+ Puromycin (100 µg)	86

Indeed, they tended to inhibit the reaction. Omission of ATP, PEP and PK from the reaction mixture lowered considerably the extent of the amino acid incorporation. It is clear, therefore, that amino acid incorporation into thyroid mitochondrial protein is energy-dependent. However, in the absence of PEP and PK, ATP did not meet the energy requirements of the system, a finding which suggests that the continuous generation of ATP is essential for incorporation of amino acids into mitochondrial protein.

The effects of various inhibitors on the ability of thyroid mitochondria to incorporate leucine ^{14}C into proteins are shown in Table III. DNase had no significant effect.

RNase tended to inhibit, but the inhibition was not pronounced. On the other hand, actinomycin D and puromycin brought about a substantial inhibition of amino acid incorporation, the effect of the latter being much more pronounced than that of actinomycin D.

Actinomycin D is known to inhibit cellular RNA synthesis by combining with DNA to block the action of DNA-dependent RNA-polymerase (Hurwitz et al., 1962; Goldberg et al., 1962). Reports of Staehelin et al. (1963) and Korner and Munro (1963) attribute the actinomycin effect on protein synthesis to an inhibition of messenger RNA formation. Thus, the inhibition of amino acid-incorporating activity of thyroid mitochondria by actinomycin observed here indicates that a continuous generation of RNA-template is an obligatory requirement for protein synthesis by thyroid mitochondria.

Puromycin has been shown to inhibit protein synthesis in preparations of rat liver and of bacteria by preventing the transfer of amino acids from amino acyl-sRNA to ribosomal protein (Yarmolinsky and de la Haba, 1959; Nathans et al., 1962). The inhibitory action of puromycin reported here, therefore, suggests that amino acid transfer from amino acyl-sRNA to ribonucleoprotein particles is involved in the synthesis of protein by sheep thyroid mitochondria.

The RNA contents of the mitochondrial preparations were measured to assess their contamination with microsomes. The very low RNA contents (7 to 10 μg per mg of mitochondrial protein) show that the preparations were not heavily contaminated. Furthermore, under the incubation conditions used here, the incorporation of amino acids into protein by mitochondria exceeded that observed with microsomes.

CONCLUSIONS

The findings of the present study demonstrate that thyroid mitochondria are capable of incorporating amino acids into protein by a process apparently involving formation of true peptide linkage. That the amino acid incorporation required an energy-generating system, and that it was inhibited by actinomycin D and puromycin, indicate that protein synthesis in sheep thyroid mitochondria is directed essentially via the same steps as those involved in microsomal or ribosomal protein synthesis of other tissues and microorganisms.

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