PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 174, 415–420 (1983)

Regulation of Protein Synthesis in Isolated Mitochondria from Regenerating Rat Liver (41756)

JOSEPH F. TARSIO[1] AND DIPAK HALDAR[2]

Department of Biological Sciences, St. John's University, New York, New York 11439

Abstract. Possible sites in the mitochondrial protein synthetic/degradative scheme that might be responsible for the increased incorporation of [^{3}H]leucine into protein by mitochondria isolated from regenerating liver over the levels found for mitochondria isolated from sham-operated controls were examined.

The rate of degradation of newly synthesized protein in mitochondrial preparations from regenerating liver was not decreased but proceeded approximately 30% greater than that found for the sham-operated controls. The increased incorporation of [^{3}H]leucine into protein could also not be accounted for by a stimulation in the extent of formation of leucyl-tRNA^{Leu}. In another experiment, isolated mitochondria were incubated with [^{3}H]leucine and the input of mitochondrial ribosomes from regenerating liver and sham-operated controls equalized for analysis on sucrose density gradients. The 55-S ribosomes from regenerating liver mitochondria contained 2.7-fold greater radioactivity in their nascent polypeptide chains than those from control mitochondria. These results indicate that the stimulation in amino acid incorporation into protein due to liver regeneration is the direct result of enhanced polypeptide bond formation on mitochondrial ribosome-messenger RNA complexes.

Regeneration of the liver after partial hepatectomy is an excellent model for the study of the molecular regulation involved in the biogenesis of mammalian mitochondria. After the removal of approximately two-thirds of the liver (1) there is a tremendous hypertrophy and increase in cell number in the remaining lobes. At 2–3 weeks after partial hepatectomy, the preoperative weight of the liver is restored concomitantly with a large synthesis of mitochondria (2). It has been shown (3) that the enzymes of the mitochondrial inner membrane and matrix, with the exception of cytochrome C oxidase, appear synchronously during this massive period of mitochondrial biogenesis. We report in the present study that mitochondria isolated from rat livers, 2–3 days after partial hepatectomy, are 2.5–3 times more active in amino acid incorporation into protein than mitochondria isolated from corresponding sham-operated animals, in confirmation with Malkin (4). However, to extend these studies we have also focused upon the 2-day time point and the use of paired groups of sham-operated and regenerating animals in order to determine the step(s) in the mitochondrial protein synthetic/degradative scheme responsible for the observed stimulation in amino acid incorporation due to liver regeneration.

Materials and Methods. *Partial hepatectomy.* Male rats (Taconic Farms, Sprague–Dawley), 100–125 g, were partially hepatectomized (1) under ether anesthesia. Operations were performed at 9:00 AM or 9:00 PM depending on whether the animals were to be killed on full-day or half-day time points, respectively, after partial hepatectomy. The rats were fed *ad libitum* before and after the operation and all rats were sacrificed at 9:00 AM. Sham operations were performed by the same procedure but excision of the liver was omitted.

Amino acid incorporation into protein. Rats were killed by decapitation and the right lateral and caudate lobes from at least three rats were pooled for the isolation of mitochondrial fraction (5, 6) from either the regeneration or sham-operated animals. Amino acid incorporation into protein by isolated mitochondria was determined as previously described (5, 6) using approximately 3 mg of mitochondrial protein/ml of incubation medium and L-[4,5-

[1] Present address: University of Minnesota, 17-226 Health Sciences Unit A, Minneapolis, Minn. 55455.

[2] Author to whom all correspondence should be addressed.

0037-9727/83 $1.50

^{3}H(N)] leucine (specific activity 1.0 Ci/mmole, unless otherwise stated) added to the incubation medium to a final concentration of 0.67 μCi/ml. Prevention and checking for bacterial contamination was done as previously described (5). Protein was estimated by the method of Lowry *et al.* (7) using bovine serum albumin as standard.

Measurement of the degradation of newly synthesized proteins in isolated mitochondria. The rate of decay of newly labeled proteins was followed after adding a 500-fold excess of nonradioactive leucine ("chaser") to the incubation medium at 10 and 20 min after the initiation of the incorporation period (8). The leucine concentration before and during the chase was 1.8×10^{-5} *M* and 5.8×10^{-3} *M*, respectively. The samples were incubated for different chase times. Incubation for each sample and time was performed in triplicate at 37°C. Two additional samples were incubated for determining any change in pH of the incubation medium that might occur upon addition of chase and subsequent incubation. After each chase, the reaction was stopped and the radioactive counts in the samples determined as previously described (6).

Measurement of the formation of aminoacyl-transfer RNA. The method used for this purpose was that previously described by Moldave (9) as modified by Haldar and Freeman (5). Since leucyl-tRNA is stable to ice-cold 5% (w/v) trichloroacetic acid (cold precipitate) but labile to dilute alkali and heat (alkali–heat-resistant precipitate remains), the difference between the incorporation of [^{3}H]leucine into alkali–heat-resistant material and cold precipitate is taken as a measure of [^{3}H]leucyl-tRNA formed in isolated mitochondria.

Measurement of the extent of polypeptide bond formation on mitochondrial ribosomes in isolated mitochondria. Mitochondrial fraction from 2-day regenerating liver and the corresponding sham-operated controls was prepared as above and the extent of labeling of mitochondrial ribosomes with nascent ^{3}H-labeled polypeptide chains was determined essentially according to Ashwell and Work (10) by pulse-labeling these ribosomes with [^{3}H]leucine for 7 min at 30°C. The reaction was stopped by the addition of incubation medium to ice-cold KTM-NH_4 buffer (50 m*M* KCl, 5 m*M* Tris–HCl, 10 m*M* $MgCl_2$, 50 m*M* NH_4Cl, pH 7.5) and all subsequent steps carried out at 4°C. The mitochondrial fractions were resedimented at 10,000*g* for 10 min, washed two times with buffer, resuspended at 5 mg of protein/ml, and lysed with Triton X-100 (1% v/v). The lysate was clarified by low-speed centrifugation and further purified of membranous residue by centrifugation through a 1.0 *M* sucrose cushion (11) for 14 hr at 105,000*g* in the Spinco 65 rotor. "Crude mitoribosome" pellets obtained were resuspended by gentle homogenization in KTM-NH_4 buffer. Any large aggregates were sedimented at 15,000*g* for 15 min and 1 ml of the clear supernatant was layered on a 10–30% (w/v) sucrose gradient for each preparation from regenerating liver or sham-operated controls and run concomitantly in the Spinco SW 27.1 rotor at 88,600*g* for 4.5 hr along with cytoplasmic ribosomes and dissociated subunits prepared according to Greco *et al.* (11) in a third gradient tube serving as gradient markers. The input of crude mitoribosomes from regenerating liver mitochondria or control mitochondria was equal per gradient at 0.1 $A_{254\ nm}$ units and 1-ml fractions were collected after the run and total radioactivity per fraction determined as described (10).

Materials. All chemicals were of the analytical reagent grade. Amino acids, ADP (equine muscle), Tris, chloramphenicol, and cycloheximide were obtained from Sigma; ultrapure sucrose (ribonuclease-free) was obtained from Schwarz/Mann. Blood agar plates were from Difco. Triton X-100, L-[4,5-^{3}H(N)]leucine (specific activity 5 Ci/mmole), and Biofluor were obtained from New England Nuclear.

Results. *Amino acid incorporation.* Figure 1 shows the amino acid incorporation into protein (cpm/mg) by mitochondria isolated from normal, sham-operated control, and regenerating liver. Mitochondria isolated from sham-operated animals over 1 to 4 days postoperatively showed very little difference in incorporation of [^{3}H]leucine into protein (mean ± SEM: 842 ± 125 cpm/mg, Fig. 1a). The amino acid incorporation with these mitochondria was approximately two times that of normal unoperated control mitochondria

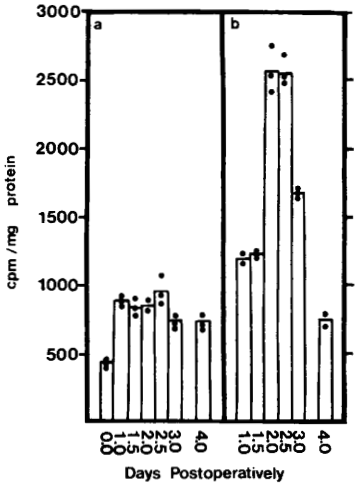

FIG. 1. Amino acid incorporation into protein by mitochondria isolated from normal (a, 0.0 days postoperative), sham-operated (a, 1.0–4.0 days postoperative), and regenerating (b, 1.0–4.0 days) rat liver. The samples were incubated for 20 min at 37°C in the presence of 0.67 μCi/ml of [^{3}H]leucine (1.0 Ci/mmole) and the reactions stopped and samples processed as described under Materials and Methods.

(mean ± SEM: 441 ± 25 cpm/mg). The mitochondria isolated from liver regenerating for 2–3 days postoperatively were even more active (Fig. 1b) than those isolated from sham-operated animals. The net stimulation in incorporation due to liver regeneration as compared to sham-operated controls was 33 ± 4% on Day 1, 46 ± 11% on Day 1.5, 198 ± 11% on Day 2, 165 ± 13% on Day 2.5, 128 ± 7% on Day 3, and 2 ± 6% on Day 4, after partial hepatectomy (values expressed as mean ± SEM, two or three incubation samples). The following experiments were performed with mitochondria isolated from sham-operated and regenerating livers 2 days after surgery.

Measurement of the rate of degradation of newly synthesized protein in isolated mitochondria. Before the addition of chase, the specific activity of protein of mitochondria isolated from regenerating liver (2 days postoperatively) was approximately 2-fold that of mitochondria isolated from corresponding sham-operated animals. After adding the chase, the pH of the incubation medium (pH 7.2) did not change significantly and Figs. 2a and b show that the degradation of newly synthesized protein in regenerating rat liver mitochondrial preparations was approximately 33% faster than that found for protein in mitochondria isolated from sham-operated control liver. Thus it appears that the higher specific radioactivity of protein synthesized in mitochondria isolated from regenerating liver is not due to any decrease in the rate of degradation of newly synthesized protein over the time span that we usually measure amino acid incorporation.

Measurement of the formation of aminoacylated-transfer RNA. To determine if the stimulation in the amino acid incorporation into protein by mitochondria isolated from regenerating liver is due to a difference in the aminoacylation of tRNA, the formation of [^{3}H]leucyl-tRNA^{Leu} was measured in mitochondria isolated from sham-operated control and regenerating (2 days postoperative) liver. Table I shows that after a 10-min incubation at 37°C there is a 5.7-fold increase in the specific radioactivity of newly synthesized protein (akali–heat-resistant precipitate) but only a 1.8-fold increase in the charging of leucyl-tRNA with radioactive leucine in mitochondria isolated from regenerating liver over that of the same preparation from sham-operated animals.

Measurement of the extent of polypeptide formation on mitochondrial ribosomes. Mitochondrial fractions were isolated from the remaining right lateral and caudate liver lobes of five partially hepatectomized rats (2 days

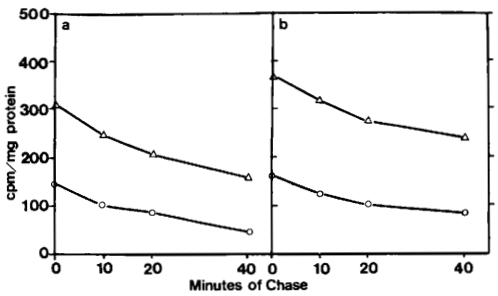

FIG. 2. Degradation of newly synthesized proteins in mitochondrial preparations isolated from regenerating (△) and sham-operated (○) rat livers was measured after (a) 10- and (b) 20-min incorporation periods (initial point in the graph). At these time points a "chaser" of unlabeled leucine (5.80×10^{-3} *M*) was added and the loss of radioactivity in the mitochondrial protein was followed in the ensuing 40 min.

TABLE I. INCORPORATION OF RADIOACTIVE LEUCINE INTO LEUCYL-TRANSFER RNA^{Leu}

	Incorporation (cpm/mg protein)[a]		
	Cold precipitate	Alkali–heat-resistant precipitate	Amount due to leucyl-tRNA^{Leu}
Regenerating	1065 ± 27	742 ± 12	323
Sham operated	323 ± 25	131 ± 9	192

Note. Mitochondrial preparations from regenerating and sham operated rat liver were incubated for 10 min at 37°C to incorporate [^{3}H]leucine into leucyl-tRNA^{Leu} plus protein (trichloroacetic acid cold precipitate) or into protein alone (trichloroacetic acid alkali–heat-resistant precipitate). The difference in radioactivity between the cold and alkali–heat-treated samples is a measure of the leucyl-tRNA^{Leu} formed.

[a] All values are expressed as the mean of four samples ± SEM.

postoperative) and from the corresponding liver lobes of eight sham-operated animals (equivalent wet weight). The pulse-labeling of mitochondrial ribosomes (mitoribosomes) in each of these preparations and hence the extent of polypeptide bond formation on mitoribosomes (10) was determined as described under the Experimental section. In each profile (Figs. 3a and b), one peak of radioactivity (fraction numbers 8–12), sedimenting at 50–60 S, is evident. In agreement with Ashwell and Work (10) this peak of radioactivity was also characterized functionally as the 55-S mitoribosomes because its labeling with [^{3}H]leucine under the same conditions employed above was inhibited by the addition of 100 μg of chloramphenicol/ml of incubation mixture but not by 100 μg/ml of cycloheximide (results not shown). The area under this peak and hence the extent of labeling of mitoribosomes with nascent polypeptide chains (10) was 2.7-fold greater for the mitoribosomes from regenerating liver than those obtained from the corresponding sham-operated animals. A concomitant experiment showed that for these specific preparations, the amino acid incorporation into protein by the isolated mitochondria from regenerating liver (1011 cpm/mg protein) was 3.3-fold higher than that by miochondria isolated from sham-operated controls (309 cpm/mg). All mitochondrial incubation mixtures and lysates showed less than 1500 bacteria/ml upon plating 0.1 ml samples on blood agar and subsequent incubating the plates for 48 hr at 37°C.

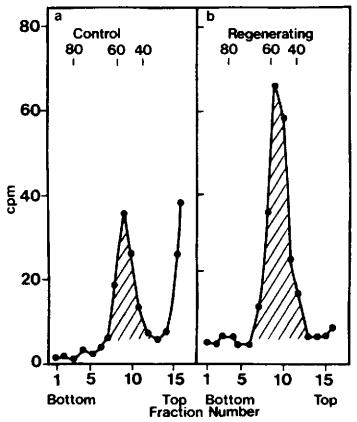

FIG. 3. Mitochondrial fraction was isolated from 2-day regenerating rat liver (b), and the corresponding sham-operated liver (a); and the extent of polypeptide bond formation on 55-S mitochondrial ribosomes determined using a 7-min pulse at 30°C of [^{3}H]leucine as described under Materials and Methods. The input of crude mitoribosomes was 0.1 $A_{254\,nm}$ units on each 10–30% sucrose gradient and they were run in the Spinco SW 27.1 rotor at 88,600*g* for 4.5 hr. The position of cytoplasmic ribosomes and dissociated subunits was determined concomitantly in a third gradient tube and is given above.

Discussion. It is now generally accepted (12) that isolated mitochondria from yeast and mammalian cells synthesize complete proteins identical to those synthesized in their cellular environments. Furthermore, the importance of finding mitochondria intrinsically more active in amino acid incorporation as a first step in understanding this process in mammalian systems has been previously emphasized (4, 13). In the present study we have shown that mitochondria isolated from regenerating rat liver 2 days postoperatively show approximately a 3-fold stimulation in amino acid incorporation into protein over that of sham-operated controls. We have also reported un-

der identical conditions to the present study (14) that the mitotic index of liver cells is 0.040, 0.284, 1.940, and 0.994% for Days 0, 1, 2, and 3, respectively, after partial hepatectomy. Taking together, these results indicate that some level of synchronization of mitochondrial protein synthesis with cell division may be taking place since the stimulation in amino acid incorporation into protein by isolated mitochondria and the mitotic index of regenerating rat liver were found by us to be at their highest levels 2 days after partial hepatectomy.

Another aim of the present study was the investigation of the factors that might be responsible for the stimulation in protein synthesis in mitochondria isolated from regenerating liver and therefore operating in the control of protein synthesis in mammalian mitochondria. The degradation of newly synthesized protein in isolated mitochondrial preparations from regenerating liver was seen to proceed at a rate approximately 30% greater than that found for the corresponding sham-operated animals (Fig. 2). The degradation rate found for the sham-operated controls in the present study was similar to that found by Wheeldon and Lehninger (8) for mitochondria isolated from normal rat liver. The increased incorporation of [^{3}H]leucine in mitochondria isolated from regenerating liver could also not be accounted for by an increase in the formation of leucyl-tRNA^{Leu}.

Numerous studies have shown that the 55-S monomeric mitoribosome of the mammalian mitochondrial ribosome–messenger RNA complex is actively involved in protein synthesis in mitochondria since it is isolated as the major ribosomal component from rat liver mitochondria (10, 11, 15–18), attains a specific activity 50 times that of mitochondrial protein pulse-labeled (19) as in the present study, can be stripped of nascent polypeptide chains by puromycin treatment (10, 16, 18, 20), and is highly active in catalyzing a poly(U)-dependent synthesis of polyphenylalanine (11). The 2.7-fold increase in the labeling of nascent polypeptides on these 55-S mitoribosomes of regenerating liver (Fig. 3) strongly indicates that the major stimulation of amino acid incorporation into protein by isolated mitochondria due to liver regeneration is a direct result of an increase in the extent of polypeptide bond formation on mitochondrial ribosome–messenger RNA complexes.

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Received April 8, 1983. P.S.E.B.M. 1983, Vol. 174.