ORIGIN OF MITOCHONDRIAL ENZYMES I. CYTOCHROME *c* SYNTHESIS BY ENDOPLASMIC RETICULUM

R.PENNIALL and Nancy DAVIDIAN *
Department of Biochemistry, School of Medicine, University of North Carolina, Chapel Hill, N.C. 27514, USA

Received 17 May 1968

1. Introduction

Considerable evidence indicates that isolated mitochondria cannot synthesize their entire complement of proteins [1,2] and, *in vivo*, the incorporation of labeled amino acids has been found detectably slower for the easily solubilized proteins of mitochondria than for structural protein and lipoproteins [3]. These observations caused us to undertake a study of the synthesis of a specific mitochondrial protein, cytochrome *c*. Using ^{3}H-δ-aminolevulinic acid (^{3}H-ALA) as a label for hemoproteins, we have studied its incorporation into the cytochrome *c* of isolated subcellular fractions of liver tissue. The results of our experiments establish that the synthesis of cytochrome *c* occurs in the endoplasmic reticulum. Similar results, using radioactive lysine as a precursor, were recently reported by Cadavid and Campbell [4].

2. Experimental procedures

Mature male rats of the Sprague-Dawley strain were fasted 18 hours prior to use. ^{3}H-ALA was administered intraperitoneally, and at appropriate intervals the livers were removed under ether anaesthesia following their perfusion with 0.9% NaCl. The tissue was homogenized in six volumes of medium containing 0.25 M sucrose: 3 mM $MgCl_2$: 5 mM triethanolamine, pH 7.0 using a Teflon-glass homogenizer (Size C, A.H.Thomas Co.). The nuclei were isolated as a crude sediment by 20 minutes centrifugation at 1000 × *g* and then washed once in additional medium. The nuclei were subsequently isolated in dense sucrose as described previously [5]. Mitochondria were isolated from the initial supernatant by centrifugation for 15 minutes at 10000 × *g* and were then washed at least twice in the medium of Ito and Johnson [6]. The post-mitochondrial fractions were derived from the 1000 × *g* supernatant by successive centrifugations at 30000 × *g* for 20 minutes and at 105000 × *g* for 1 hour to obtain the premicrosomal, microsomal and cell sap fractions.

The cytochrome *c* of all the subcellular fractions but for the cell sap was obtained by two extractions with 0.15 M KCl according to Jacobs and Sanadi [7] and the combined extracts were subjected, successively, to: precipitation of extraneous protein with aluminum sulfate; removal of excess aluminum as the hydroxide at pH 8.0; and chromatography on Amberlite CG-50 resin [8]. Nuclei required a pretreatment with ultrasound and DNase for complete extraction of their cytochrome *c*. The cell sap was treated with aluminum sulfate directly. The cation-exchange resin columns with adsorbed cytochrome *c* were washed with quantities of a sodium phosphate buffer, pH 8.0 (containing 0.02 g ion of sodium per liter) found sufficient in preliminary experiments to remove completely the extraneous counts of ^{3}H-ALA. Cytochrome *c* was then eluted with 0.02 M sodium phosphate, pH 8.0: 0.5 M KCl. When indicated, cytochrome *c* was further purified by rechromatography as described above.

The column eluates were treated with acid-acetone as described by Paul [9]. The hematohemin of cyto-

* Recipient of a predoctoral fellowship (1F1 GM-25744) from the U.S.P.H.S.

chrome *c* was derived by the use of Ag_2SO_4 and converted into the pyridine hemichrome as described by Paul [10] and Paul et al. [11]. Extinction measurements were made in cuvets with a 1 cm light path in a Cary Model 14 Spectrophotometer utilizing a 0.0 - 0.2 slide wire. The principal and reference wavelengths and reductants which were used to determine cytochrome *c* or its derivatives are as follows: cation-exchange resin column eluates – 550 mμ and 540 mμ, cysteine as a reductant; redissolved acid-acetone precipitates – 415 mμ and 500 mμ, dithionite as a reductant; pyridine hemichrome – 395 mμ and 500 mμ, no reductant.

The tritium content of various preparations was determined by scintillation spectrometry in a toluene-Triton-X-100 system [12]. The channels ratio technique employing an external standard was used to determine quench corrections and all samples were counted to less than 4% statistical error. 2,3-^{3}H-δ-ALA (5.4 c/mmole) was obtained from Schwarz BioResearch, Inc.

It should be emphasized that in these experiments a sufficiency of animals was used for each time interval to eliminate any need to add carrier cytochrome *c* prior to chromatography of the cytochrome *c* from the various cell fractions. Thus, no correction factors were applied in specific activity calculations, and our results contain none of the ambiguities possibly inherent in such a process.

3. Results

It was found in preliminary experiments that intraperitoneal injection of ^{3}H-ALA fostered detectable incorporation of tritium into acid-insoluble proteins of all the liver subcellular fractions in as little as 5 minutes, and that the process would proceed for at least 120 minutes. These experiments established that the pattern of labeling of each of the cell fractions was not greatly different at 120 minutes from that at 30 minutes and that each of the other subcellular fractions examined attained specific activities approaching or greater than that of mitochondria. Therefore, experiments were then performed wherein cytochrome *c* was extracted from each subcellular fraction. This was done to determine if any tritium appeared in materials in these fractions which had the properties of cytochrome *c*. The results of a typical experiment wherein animals were sacrificed at 30 minutes after administration of ^{3}H-ALA are given in table 1. It can be seen that in these circumstances, each subcellular fraction is substantially labeled. However, when tritium incorporation is expressed as a function of the cytochrome *c* content, the mitochondria are found to have acquired the least amount of tritium per unit of cytochrome *c*.

Table 1
Analyses of the cytochrome *c* purified from liver subcellular fractions of rats injected with ^{3}H-δ-Aminolevulinic acid.

Fraction	^{3}H (dpm)	*E* (units)	^{3}H (dpm/*E* unit)
Cell Sap	3044	0.023	132400
Microsomes	3780	0.037	102200
Premicrosomes	1767	0.032	55200
Nuclei	764	0.020	38200
Mitochondria	3620	0.340	10700

Conditions: Subcellular fractions from each of two groups of 6 animals (34 μC ^{3}H-ALA/animal) were pooled and subjected to the extraction and purification of cytochrome *c*. Results are expressed as that found per ml of cation-exchange resin column eluate.

It was realized that the specific activities found in experiments such as that of table 1 could not be considered indicative of the actual specific activity of cytochrome *c per se* in each fraction. Therefore, we sought to determine, by additional methods of analysis, the incorporation of ^{3}H-ALA into the prosthetic group of the various cytochrome *c* fractions. The use of acid-acetone, followed by isolation of the pyridine hemichrome, has established that there is one subcellular fraction, the cytochrome *c* of which exhibits a consistently greater specific activity than that of mitochondrial cytochrome *c* through these steps of purification. That fraction is the microsomes (table 2). Such results are quite reproducible; five separate experiments to date have yielded similar results. In all such experiments the specific activity of microsomal cytochrome *c* at Step 3 has consistently been greater (from 100% to 330%) than that of mitochondrial cytochrome *c*. It can be seen in table 2 that there was a large decrease in specific activities in proceeding from Step 1 to Step 2 and there

Table 2
Changes in specific activity, with purification, of cytochrome *c*, or derivatives, from rat-liver subcellular fractions.

Subcellular fraction	Specific activity		
	Step 1	Step 2	Step 3
Mitochondria	22345	1855	4313
Premicrosomes	18640	2000	3508
Microsomes	57320	5286	8655
Nuclei	50400	2341	4022
Cell sap	– *	4760	4582

Conditions: 43.2 μC of ^{3}H-δ-ALA was injected into each of 5 animals and the animals were sacrificed 30 minutes after injection. Specific activities (^{3}H dpm/unit *E*) expressed are as follows: Step 1, cation-exchange resin column eluates; Step 2, redissolved acid-acetone precipitates; Step 3, pyridine hemichromes.

* Extinction too small for accurate measurement.

Table 3
The effects of carrier ALA on incorporation of ^{3}H-ALA into the pyridine hemichromes of microsomal and mitochondrial cytochrome *c*.

Fraction	^{3}H (dpm/unit *E*)		
	Interval of chase		
	15 min	5 min	50 min
Mitochondria	2278	4585	5028
Microsomes	7407	4118	4377

Conditions: Groups of 6 animals were each injected with 135 μC of ^{3}H-ALA per animal. At 15 minutes 1 group of animals was sacrificed and companion groups of animals were injected intraperitoneally with 2.5 μmoles of carrier ALA. These groups were subsequently sacrificed at intervals of 5 and 50 minutes. Mitochondria and microsomes were isolated and their cytochrome *c* was extracted and purified by cation-exchange chromatography (3 times), treatment with acid-acetone, and isolation of the pyridine hemichromes.

was an increase in specific activities in proceeding from Step 2 to Step 3. There are several factors which could contribute in varying degree to the observed changes. Although the column eluates are known to be free of other hemoproteins by both conventional and low temperature spectroscopy [13], it is quite possible that the column eluate from each subcellular fraction contains a complement of the several protein-bound porphyrin precursors which are known to be present in the soluble portion of the cell [14]. These are removed to a large extent by acid-acetone, and there is a large loss of tritium in proceeding from Step 1 to Step 2. Lastly, the increase in specific activity occurs at Step 3 because we have chosen to determine the pyridine hemichromes in the oxidized state (with its smaller extinction coefficient) to avoid the difficulties their rapid autoxidizability imposes upon their determination in the reduced state [15].

Because the specific activity of the pyridine hemichrome of microsomal cytochrome *c* was consistently greater than that of mitochondria, we have sought to determine any possible precursor-product relationship between these two respective cytochrome *c* fractions. A pulse-chase experiment was performed in which carrier ALA was injected after 15 minutes incorporation of ^{3}H-ALA. It can be seen in table 3 that, after 15 minutes incorporation, the specific activity of the pyridine hemichrome of microsomal cytochrome *c* is 3.3 fold greater than that of the mitochondria. On the injection of carrier ALA, the specific activity of mitochondrial cytochrome *c* continues to rise, increasing 100% in the next five minutes. In the same interval the specific activity of microsomal cytochrome *c* decreases markedly. The effectiveness of the chase is proven by the negligible further incorporation of tritium in the 5 to 50 minute interval. This pattern of changes in the respective specific activities of the pyridine hemichromes of microsomal and mitochondrial cytochrome *c* is that to be expected if the former is a precursor of the latter. Further proof of the identity of the tritium content and 395 mμ extinction at Step 3 in the experiment of table 3 was obtained by paper chromatography of the pyridine hemichromes according to Chu and Chu [16]. It may be noted that the specific activity of microsomal cytochrome *c* drops only to 90% of that of mitochondrial cytochrome *c* in the presence of carrier ALA. This decrease was less than was expected; one possible explanation, which is borne out by other experiments of this laboratory [17] as well as by those of Cadavid and Campbell [18], is that microsomes cannot be isolated without some contamination by cytochrome *c* released from mitochondria during the cell fractionation.

Our results establish that the final assembly of the cytochrome *c* molecule is a process catalyzed by the endoplasmic reticulum. The data of table 2 suggest this; but the consistently greater specific activity of microsomal cytochrome *c* could also result from an ability of both organelles to synthesize the protein at the same rate, the products of each process being diluted by pre-existing pools of widely disparate size. These findings parallel those of Cadavid and Campbell [4] in their studies of cytochrome *c* synthesis employing radioactive lysine. However, the data of table 3 clearly indicate the precursor-product relationship between microsomal and mitochondrial cytochrome *c*. These results imply the existence of a transfer process for mitochondrial acquisition of newly synthesized cytochrome *c*; preliminary evidence for the existence of such a microsomal to mitochondrial transfer process has been obtained by Kadenbach [19]. Investigations are currently in progress regarding the control of cytochrome *c* synthesis, and the mechanism of its transport into mitochondria.

Acknowledgement

Supported in part by grants from the National Institutes of Health (HE-06088 and 5TI-GM404) and the National Science Foundation (GB-3760 and GB-4577).

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