ORIGIN OF MITOCHONDRIAL ENZYMES. IV. ON THE CHARACTER OF THE PRODUCT OF CYTOCHROME c SYNTHESIS BY THE ENDOPLASMIC RETICULUM*

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Summary: Following an interval of incorporation of radioactive δ-aminolevulinic acid, the pattern of labeling of the multiple forms of cytochrome c of rat liver tissue was determined. The results obtained indicate that the product of the synthetic activity of the endoplasmic reticulum must be a minor, nonfunctional form (Form IV) and that it is this entity which is transferred to the mitochondrion from the endoplasmic reticulum.

Cytochrome c synthesis in liver tissue had been concluded in previous investigations (1-3) to be catalyzed in toto by the endoplasmic reticulum (ER). However, Kadenbach (4) now contends that the ER makes only apocytochrome c and that it is linked to protoheme in the mitochondrion. On the other hand, we believe that the ER catalyzes the linkage of the apoprotein and protoheme. This view was initially based upon measurements of the specific activities of the respective hematohemes isolated from the cytochrome c of liver microsomes and mitochondria of rats injected with ^{3}H-2,3-δ-aminolevulinic acid (^{3}H-ALA), and upon the changes in such specific activities with the injection of unlabeled ALA (5). Flatmark and Sletten (6) have reported that cytochrome c of various mammalian tissues exists in four forms (I,II,III and IV) which differ from each other by one in their content of asparaginyl and/or glutaminyl amide groups. Form I constitutes the major portion (α90%) of the cytochrome c of the tissues examined (6,7) and is the functional form (6). We present herein experiments which indicate that the product of ER synthesis is not Form I, but most likely is Form IV.

METHODS

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The methods used for : isolation of cell fractions; extraction of cytochrome c and its purification by aluminum sulfate and cation exchange resin chromatography; spectrophotometric estimations of cytochrome c; and administration of ^{3}H-ALA to the rats have been detailed previously (5). Polyacrylamide gel electrophoresis was performed on a portion of the cytochrome c which had been derived from the isolated cell fractions (6), using an alkali-soluble gel (8). The cytochrome c content of solubilized gel segments was determined by use of cysteine as a reductant using a Cary Model 14 spectrophotometer fitted with the sensitive slide wire. The tritium content of dissolved gel segments was determined in a toluene-Triton-X-100 scintillation mixture (5), using a Beckman Model LS-100 scintillation counter. Corrections for sample quenching were made by the channels ratio technique utilizing an external standard. Counting efficiency was approximately 30%, and samples were counted to 2% statistical error. Blank gel segments gave a counting rate of 20 cpm. In control experiments, when separate longitudinal segments of polyacrylamide gel containing a sufficiency of the forms of mitochondrial cytochrome c were stained for protein with Coomassie blue and for hemoproteins with benzidine, we found an absolute correspondence of staining of the four forms in the two segments with no detectable extra components. This indicated that the specific activities expressed for the various forms are valid and that any labeled contaminants, if present, either fail to migrate or move toward the anode. Other experiments established also that there is sufficient homology between the forms of cytochrome c of horse heart and rat liver that the former species can be used as markers for the migration of the latter during electrophoresis.

Results and Discussion

Table 1 presents the pattern of specific activities of the four forms of cytochrome c extracted from liver subcellular fractions after an interval of incorporation of ^{3}H-ALA. It is of interest that the specific activities of the forms of mitochondrial cytochrome c exhibit the following order: IV>III>II>I. Where analyses were possible, this pattern was mirrored in the specific ac-

Table 1

Labeling of the Multiple Forms of Cytochrome c Extracted From Rat Liver Subcellular Fractions

Conditions: 117 μCi of ^{3}H-ALA were injected IP into each of 12 rats, and at 15 minutes the animals were sacrificed. Cytochrome c was derived from the subcellular fractions and a portion was subjected to polyacrylamide gel electrophoresis. Results are expressed as the total radioactivity and total Soret absorbance found in each of the solubilized gel segments.

Cell Fraction	Form	Total ^{3}H dpm	Total $A_{415\ nm}$	Specific Activity
Mitochondria	I	430	0.220	1950
	II	321	0.092	3490
	III	410	0.070	5860
	IV	359	0.033	10880
Nuclei	I	500	0.270	1850
	II	358	0.121	2960
Cell Sap	I	363	0.108	3360
	II	421	0.124	3390
	III	345	0.078	4420
Microsomes	I	310	0.143	2170

tivities of the forms in extra-mitochondrial cytochrome c. This is to be expected inasmuch as the bulk of the extra-mitochondrial cytochrome c results from an unavoidable redistribution of part of the mitochondrial cytochrome c during cell fractionation (9). Because the absorbances were insufficient for accurate determination, specific activities for the minor forms of microsomal cytochrome c could not be calculated.

Table 2 presents the results of a second experiment (1a) wherein a pattern of specific activities for the four forms of cytochrome c very much like that of Table 1 is discernible. Experiment 1b gives the specific activity pattern observed for the forms of mitochondrial cytochrome c after an added interval of labeling. For reasons unknown the yields of cytochrome c extracted from the various cell fractions were low in experiment 1b but, nevertheless, the specific activities of the minor forms again exceed that of Form I.

Table 2

Specific Activities of the Multiple Forms of Cytochrome c of Rat Liver Tissue

Conditions: Ten rats in each of two groups were each injected IP with 75 μCi of ^{3}H-ALA. One group (Experiment 1a) was sacrificed at 20 minutes after injection, and the second (Experiment 1b) after 40 minutes. Manipulations of cytochrome c were performed as in Table 1.

Experiment	Cell Fraction	Specific Activity				
		Unfractionated	Fractionated			
			Form: I	II	III	IV
1a	Mitochondria	870	470	6290	8080	*
	Nuclei		660	5590	*	---
	Cell Sap		735	*	*	---
	Microsomes		625	*	*	---
1b	Mitochondria	2200	2460	4430	5920	*

* Significant tritium dpms were found in each of these solubilized gel segments, but absorbances at 415 nm were not measurable.

It is significant to note that the specific activities of total mitochondrial cytochrome c and of Form I after its separation by electrophoresis are both greater at 40 minutes than at 20 minutes, and that the specific activities of the minor mitochondrial forms have decreased during this interval. The significance of these findings is enhanced by comparing the percentage distribution of the mitochondrial forms in the two experiments with values obtained by Flatmark and Sletten (6):

	Form I	Form II	Form III	Form IV
Flatmark and Sletten (6)	89%	7.6%	2.9%	0.6%
Experiment 1a (Table 2)	89.6	9.6	0.8	---
Experiment 1b (Table 2)	91	6.2	2.3	---

It is apparent that the respective percentages of the total constituted by each form are approximately the same at both time intervals, and not unlike those observed by Flatmark and Sletten. Thus, if the differences in specific activities observed at 20 minutes (i.e., IV>III>II>I) were to be attributed

to a simultaneous incorporation of ^{3}H-ALA into each form, the pools of which differ in size, one would not see the pattern of changes in specific activities of the mitochondrial forms that occur between 20 and 40 minutes. The respective specific activities of Forms II and III of mitochondrial cytochrome c could be expected to increase in the interval from 20 to 40 minutes; however both specific activities declined, as would be expected were there a precursor product relationship between the minor forms and Form I.

Flatmark and Sletten found that the minor forms of cytochrome c arise from Form I as the products of successive deamidations, but the rate of this process is too slow (6) to have a bearing on the labeling patterns reported herein. However, from the work of Flatmark and Sletten we presume that Form IV undergoes conversion to Form I by a series of amidation reactions within the mitochondrion. In this regard it is noted that the existence in liver tissue of at least one enzyme with a capacity to amidate proteins, and to which no function has been ascribable, has been reported (10). From the results of these experiments, as well as those reported previously (5), we believe that Form IV is synthesized by the ER and that entity is transferred to the mitochondria. This belief is supported by the fact that we have consistently observed the same pattern of specific activities for the forms of mitochondrial cytochrome c in four separate experiments. Although the results are not presented herein, it should be noted also that in those four experiments the specific activities of total microsomal cytochrome c ranged from 2 to 8 times that found for total mitochondrial cytochrome c. Such results are consistent with our previous findings (5). Although we have been unable to obtain specific activities for the minor forms of microsomal cytochrome c, especially for Form IV, this does not negate the significance of the observed pattern of specific activities of mitochondrial cytochrome c.

Mitochondria have been reported to be impermeable to cytochrome c (11). Our experiments indicate that mitochondria must most likely be permeable to Form IV of cytochrome c and thus they would not seem to conflict with the

findings of Wojtczak and Zaluska (11). In their experiments, these workers were most likely using a cytochrome c preparation which could be expected to contain a maximum of 1% of the cytochrome c as Form IV* (6,7). Thus, any penetration of the mitochondria would have gone undetected for : a. There would have been only minute amounts of Form IV in the system; and b. Form IV is inactive as an electron carrier (6).

Jones and Jones have localized the bulk of the ferrochelatase of liver tissue to the mitochondria (12). This observation would seemingly impose a severe limitation on the significance of our results. However, these authors state that their experiments do not exclude the possibility that cytochrome c synthesis might be a special process catalyzed by a ferrochelatase which acts upon porphyrin cytochrome c (a porphyrin covalently bound to protein) or that, alternatively, the mitochondria might supply protoheme to the ER (12). Either of these mechanisms can be considered compatible with our findings.

The results of these experiments confirm those of Kadenbach (4) in indicating that the ER supplies a precursor rather than functional cytochrome c to the mitochondria. However, we believe that the data presented, considered with our previous findings (5), indicate that the ER most likely supplies Form IV of cytochrome c to the mitochondria. It is our hope that experiments presently in progress will enable us to characterize the mechanisms whereby mitochondria acquire Form IV of cytochrome c and how Form IV is converted to Form I.

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