In vitro synthesis and posttranslational uptake of cytochrome c into isolated mitochondria: Role of a specific addressing signal in the apocytochrome

(membrane biogenesis/rat liver/3,3',5'-triiodothyronine)

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ABSTRACT Administration of the thyroid hormone 3,3',5'-triiodo-L-thyronine (T3) to rats leads to a marked increase in hepatic levels of mRNA for cytochrome c. Messenger RNA prepared from the free polysomes of T3-treated rats directed the in vitro synthesis of a polypeptide which only differed in amino acid sequence from mature cytochrome c in that it contained an NH2-terminal methionine. The in vitro product was incorporated specifically into purified rat liver mitochondria and became inaccessible to added trypsin when the mitochondria were added after translation was completed. Horse heart apocytochrome c, but not the holocytochrome, could compete with the in vitro synthesized polypeptide for its uptake into mitochondria. This suggests that the primary structural features of apocytochrome c, which serve as an addressing signal for mitochondria, are masked after cleavage of horse apocytochrome c, extending from residue 66 to the carboxy end of the molecule, could compete with the in vitro product for its transfer into mitochondria.

Within eukaryotic cells, mitochondria grow and reproduce by incorporation of newly synthesized proteins and lipids into preexisting organelles (cf. 1). However, most mitochondrial proteins are synthesized in the cytoplasm (cf. 2), and it is not known how these proteins are transferred to their intramitochondrial destination. We have investigated the mechanism by which cytoplasmic polypeptides are selected for incorporation into the organelle, using rat liver cytochrome c as a model system.

Cytochrome c is a peripheral membrane protein found in the intermembrane compartment of mitochondria, where it transfers electrons between cytochrome c reductase and cytochrome c oxidase, both integral protein complexes of the inner mitochondrial membrane (3). It is known that cytochrome c is coded for by a nuclear gene (4; cf. 5) and that the polypeptide is synthesized outside mitochondria on cytoplasmic ribosomes (6-9).

We found that treatment of rats with the thyroid hormone 3,3',5'-triiodo-L-thyronine (T3) increases the level of translatable cytochrome c mRNA in liver cells. This enabled the demonstration that in rat liver, as is the case in neurospora (9), cytochrome c is synthesized on free ribosomes. Conditions were then developed for uptake of the in vitro synthesized cytochrome c by mitochondria, which allowed a characterization of structural elements of the molecule that participate in the mechanism mediating its translocation across the outer mitochondrial membrane.

MATERIALS AND METHODS

Albino male Sprague-Dawley rats (≈150 g) received daily intraperitoneal injections of T3 (300 μg per 150-g rat) for 5 days. Before sacrifice, 1 day after the last injection, animals were starved for 14–16 hr.

Free and membrane-bound polysomes (10) and total polysomes (11) were prepared by published procedures, and poly(A)+ mRNA was isolated as described (12).

In Vitro Protein Synthesis. Translation of mRNA (0.05 A260 unit/50 μl) was carried out in the reticulocyte lysate (13) for 1 hr at 25°C with 200 units of Trasylol (Mobay Chemical, New York) per ml as a protease inhibitor, and 50 μCi (1 Ci = 3.7 × 1010 becquerels) of [35S]methionine (800 Ci/mmole; American) per 50 μl.

Preparation of Anti-Cytochrome c Antibodies. Mouse cytochrome c, which has the same amino acid sequence as rat cytochrome c (14), was used to raise antisera in rabbits and separate total specific IgG by immunoabsorption, as described (15, 16).

Chromatographic Separation of Cyanogen Bromide Fragments from Horse Apocytochrome c. Horse heart apocytochrome c (80 mg) was prepared (17), subjected to CNBr fragmentation (18), lyophilized, and dissolved in 1 ml of 10% (vol/ vol) formic acid. The sample was applied to a Bio-Gel P6 column (1 × 100 cm) equilibrated and eluted with 10% formic acid.

Four fractions (I–IV) were recovered as distinct peaks containing polypeptide fragments, which were identified after dansylation of NH2-terminal amino acids. Fraction I contained a 9:1 mixture of fragments composed of residues 1–65/1–80; but fractions II, III, and IV contained single fragments composed of residues 66–104 (fragment II), 81–104 (fragment III), and 66–80 (fragment IV), respectively.

RESULTS

There is a well-established relation between thyroid hormone activity and the cellular concentration of cytochrome c. Hyperthyroid rat tissues contain higher than normal levels and hypothyroid tissues contain lower than normal levels of the protein (19, 20). Furthermore, administration of T3 to rats has been shown to lead to a rapid increase in the activity of respiratory enzymes in the liver (21). This suggested that high levels of spe-

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Abbreviation: T3, 3,3',5'-triiodo-L-thyronine.

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cific mRNAs for components of the mitochondrial electron transport chain may be induced by the thyroid hormone. Indeed, whereas with control mRNA the in vitro synthesis of cytochrome c was almost undetectable (Fig. 1a), we found that with mRNA from T3-treated rats an immunoprecipitable polypeptide with the mobility of holocytochrome c (Fig. 1, lane b) represented 0.1% of the total incorporation.

To determine the cytoplasmic site of synthesis of cytochrome c, the products made under the direction of mRNAs from free and membrane-bound polysomes obtained from T3-treated rats were characterized. Two clearly different sets of polypeptides, which spanned a wide size range, were encoded by these mRNA preparations (Fig. 2, lanes a and b), and analysis of immunoprecipitates demonstrated that the mRNA directing the synthesis of cytochrome c was largely confined to the free polysomes (Fig. 2, lanes c and e).

It appears most likely that the segregation of the cytochrome c mRNA to the free polysomes of T3-treated rats reflects a similar distribution in free polysomes of normal animals. Indeed, immunoprecipitates with anti-albumin antibodies showed that T3 treatment did not alter the normal distribution of the mRNA for this secretory protein, which is synthesized in bound polysomes (Fig. 2, lanes d and f).

Previous work with neurospora (9) also has indicated that cytochrome c is synthesized on free polysomes. Analysis of the

![Figure 1](image1.png)

**Figure 1.** Effect of T3 on the level of translatable cytochrome c mRNA in rat liver. Translation was carried out in the reticulocyte lysate with mRNA extracted from total polysomes prepared from control (lane a) or 13 treated rats (lanes b–d), with [3H]methionine as a label. After incubation, translation mixtures were made 2% (wt/vol) in NaDodSO4 and heated to 100°C for about 2 min. Samples were then diluted 1:5 with 190 mM NaCl/50 mM Tris HCl, pH 7.4/6 mM EDTA/2.5% (wt/vol) Triton X-100 and centrifuged at 12,800 × g for 2 min in an Eppendorf Microfuge. The supernatant was removed, mixed with anti-mouse cytochrome c antiserum (30 μg of IgG was added to 250 μl of the translation mixture) and kept overnight at 4°C. Protein A coupled to Sepharose CL-4B (Pharmacia, Sweden) was added (10 μg of protein per 30 μg of IgG), and the mixture was stirred at room temperature for 2–3 hr. The antigen–antibody–protein A-Sepharose complex was collected by centrifugation at 12,800 × g for 2 min in an Eppendorf Microfuge and washed at least four times with the same buffered solution. Washed immunoprecipitates were dissolved in sample buffer and analyzed by electrophoresis at room temperature in linear polyacrylamide gradient gels (6–13%) (22) with a current of 23 mA for 18 hr. The location of radioactive bands in stained and unstained gels was determined by fluorography. In lanes c and d, the acetyl-trapping system was added, and translation was carried out with (lane d) or without (lane c) a mixture of proteinase inhibitors. [3H]-Labeled horse holocytochrome c was used as a marker (lane e).

**Figure 2.** Site of biosynthesis of cytochrome c. Fluorographs show NaDodSO4/polyacrylamide gel electrophoretic patterns of total translation products obtained with mRNA from free (lane a) or bound (lane b) polysomes and the products immunoprecipitated from translation mixtures programmed with free (lanes c and d), or bound (lanes e and f) polysomal mRNA by anti-mouse cytochrome c antiserum (lanes c and e) or anti-rat albumin antiserum (lanes d and f). Translation mixtures used for immunoprecipitation contained 10–106 cpm (lanes c and e) and 3.5 × 105 cpm (lanes d and f) of incorporated radioactivity. The polypeptides eluted from corresponding positions in the gels after the bands were identified by fluorography contained 7100 cpm (lane c), 20 cpm (lane d), 40 cpm (lane e), and 6900 cpm (lane f). 125I-Labeled horse holocytochrome c was used as a marker (lane g).

In vivo synthesized cytochrome c from neurospora (9), and DNA sequence determination of the yeast cytochrome c gene (23) have shown that, in these lower eukaryotes, cytochrome c is not synthesized as a higher molecular weight precursor. The similar mobilities in NaDodSO4 gels of the polypeptide synthesized in vitro and native rat liver cytochrome c (Fig. 2, lanes c and g) suggested that this is also the case for vertebrates. This was confirmed by comparison of a partial NH2-terminal amino acid sequence of the polypeptide synthesized in vitro with the known sequence of the holoprotein (14). Cytochrome c was synthesized in vitro in a translation mixture containing [35S]methionine or [3H]lysine, or both, to which the acetyl trap, introduced by Palmiter (24) to prevent NH2-terminal acetylation, was added. It also became necessary to add a mixture of proteinase inhibitors [1 mM O-phenanthroline, L-leucyl-L-leucyl-L-lysine (0.5 mg/ml) and pepstatin (0.2 mg/ml)], because in their absence the in vitro synthesized, nonacetylated, labeled cytochrome c was rapidly and almost completely degraded to nonimmunoprecipitable products (Fig. 1, lanes c and d). Because in the same system newly synthesized albumin was not degraded (results not shown), this observation suggests that in the intact cell NH2-terminal acetylation may play a role in protecting cytochrome c from attack by cytoplasmic peptides.

Automated Edman degradation showed that [35S]methionine was NH2-terminal and that [3H]lysyl residues occurred at positions 6, 8, 9, and 14 (Fig. 3). Because in rat liver cytochrome c glycine is the NH2-terminal acetylated amino acid and lysine residues are present at positions 5, 7, 8, and 13, these results indicate that, as with neurospora cytochrome c (9), only methionine is normally removed from the NH2-terminal end of the primary translation product.

**Transfer After Translation of Newly Synthesized Cytochrome c into Mitochondria.** Because cytochrome c is synthesized exclusively on free polysomes, the completed polypeptide must be incorporated into the mitochondria after translation and
release into the cell sap. Therefore, the capacity of isolated mitochondria to accept the in vitro product was investigated. Labeled polypeptides were synthesized in vitro in translation mixtures programmed with free polysomal messenger RNA. A suspension of isolated rat liver mitochondria was then added together with cycloheximide to ensure that any uptake of labeled polypeptides into mitochondria in the ensuing 1-hr incubation did not involve a cotranslational mechanism. It was found that, during the incubation after translation, almost all (>90%), as estimated from densitometric tracings) of the newly synthesized cytochrome c became associated with the sedimentable mitochondria (Fig. 4, lane c) and resistant to digestion by exogenously added trypsin (Fig. 4, lane d). Thus, cytochrome c did not remain exposed on the mitochondrial outer membrane but probably was transferred to its normal intermembrane destination, where it was protected from the protease. Because under similar conditions labeled preproalbumin failed to bind to mitochondria (Fig. 4, lane e), it appears that the outer mitochondrial membrane bears sites for the specific recognition of cytochrome c.

Similar results were obtained when the labeled cytochrome c used for these experiments had been synthesized in the presence of the acetyl-trapping system with proteinase inhibitors (data not shown), as when acetylation of the in vitro product was not prevented. This indicates that removal of methionine and acetylation of the next residue is not necessary for uptake after translation.

Experiments were undertaken to determine if other subcellular membrane fractions can take up cytochrome c synthesized in vitro. It was found that more than 90% of the newly synthesized cytochrome c did not become associated with erythrocyte plasma membranes, when these were added to the translation mixtures instead of mitochondria (Fig. 5, lane c). The capacity of rat liver microsomes (Fig. 5, lane d) for uptake of the in vitro product, although not negligible, was much less than (∼10–20%) that of whole mitochondria (Fig. 5, lane b). It probably reflects the extent of contamination of the preparation with damaged mitochondria or mitochondrial fragments, which, on the basis of the cytochrome oxidase content, represent at least 6% of the total microsomal protein. Since the different added membrane fractions contained equal amounts of protein, these observations indicate that the uptake of cytochrome c after translation is a specific property of mitochondrial outer membranes.

To determine the configuration of the molecule that participates in the posttranslational uptake by mitochondria, the capacity of hol- and apo-cytochrome c to compete with the in vitro synthesized peptide for uptake by mitochondria was examined. It was found that whereas apocytochrome c almost completely inhibited uptake of the in vitro product (Fig. 5, lane f), an excess of holocytochrome did not prevent the transfer into mitochondria (Fig. 5, lane e). Moreover, as expected from the results of these competition experiments, 125I-labeled holocytochrome c failed to bind to mitochondria (Fig. 5, lane g). These observations suggest that the conformation of the newly synthesized product is functionally equivalent to that of apocytochrome c and, therefore, that conversion to the holocytocrome occurs after the newly synthesized polypeptide is transferred into the mitochondrial. A similar conclusion has been reached by Korb and Neupert (29), who studied the biosynthesis of cytochrome c in neurospora using antibodies specific for the apo and holo forms of the protein.

Inhibition of the Mitochondrial Uptake of Cytochrome c by a Fragment of the Protein. The above results suggest that a putative receptor in the outer mitochondrial membrane recognizes features of the cytochrome c polypeptide that are present in the apocytochrome but not in the fully folded heme-con-
DISCUSSION

The finding that rat liver cytochrome c is synthesized by free polysomes indicates that the completed polypeptide must be released into the cell sap before it is incorporated into the mitochondrion. This mechanism is consistent with our demonstration that isolated mitochondria have the capacity to take up the cytochrome c synthesized in vitro and protect it from attack by added trypsin, whereas other subcellular membranes do not take up the polypeptide. On the other hand, the mitochondria do not protect or bind preproalbumin, synthesized in the same system, or 125I-labeled holocytochrome c added after translation. Thus, the incorporation of cytochrome c is mediated by a mechanism that recognizes structural features characteristic of the newly synthesized molecule. The failure of mitochondria to take up or to bind the holocytochrome while incorporating the in vitro product suggests that heme binding and folding into the final spatial structure occur after or while the polypeptide is transferred across the outer mitochondrial membrane into the intermembrane space. A similar conclusion was reached by Neupert and his associates (29), who have studied the biosynthesis of cytochrome c in neurospora and demonstrated the existence of a cytoplasmic pool of newly synthesized apocytochrome c, from which molecules labeled in vitro (7) or in a cell-free homogenate (8) can be chased into mitochondria.

An outer-membrane localization for putative receptors involved in the recognition of cytochrome c and possibly other polypeptides destined to the mitochondria is also suggested by our observations (unpublished data) that mild trypsinization of mitochondria abolishes their capacity for uptake. Furthermore, mitoplasts, which lack the outer mitochondrial membrane, were found to be incapable of any significant uptake of the product synthesized in vitro, although they were able to bind substantial amounts of 125I-labeled holocytochrome c.

In contrast to other mitochondrial (30-40) or chloroplast (41-44) proteins that are synthesized in the cytoplasm, cytochrome c is not derived from a higher molecular weight precursor from which a peptide segment is removed proteolytically upon transfer of the polypeptide to its destination. Only one amino acid, the initiator methionine, is cleaved from the primary translation product, which is then acetylated at the adjacent glycine residue. However, these modifications, which take place in vitro in the absence of mitochondria and, therefore, are likely to occur in the cytoplasm, do not appear to be required for the uptake by mitochondria. Indeed, this was equally effective when methionine removal and acetylation had been prevented by carrying out synthesis of the polypeptide in the presence of a mixture of proteinase inhibitors and an acetyl-consuming trap.

Several observations suggest that the structural features of cytochrome c, which are recognized by the outer mitochondrial...
membrane and which may be considered its "addressing signal," are contained in a definite segment of its amino acid sequence and are masked in the holoprotein in its normal native conformation. Although addition of holocytochrome to the in vitro system did not affect the uptake of the in vitro product, removal of the heme allowed expression of the latent "addressing signal" because the apocytochrome effectively inhibited and, at sufficiently high concentration, completely prevented the uptake of the in vitro product. The signal appeared to be contained in a segment (fragment II) that encompasses the evolutionarily most highly conserved region of the cytochrome sequence, residues 70–80 (45, 46) and that in the native holoprotein is bound to the heme iron by the axial ligand, methionine-80 (47). Indeed, there is no obvious species specificity in the signal-like activity of fragment II. The fragment derived from horse heart cytochrome c was used to compete with the rat in vitro product, and we have recently demonstrated that equivalent fragments from rabbit heart or yeast cytochromes c are also effective competitors. The capacity of fragment II to compete was markedly diminished by cleavage at methionine-80 into two subfragments (fragments III and IV), indicating that direct primary continuity of the polypeptide through that residue is required for expression of the configuration recognized by the mitochondrial receptors.

It may be noted that in the competition experiments described in this paper, whether carried out with the apoprotein or the CNBr fragment, large molar excess of competing peptide over apoprotein synthesized in vitro (1000- to 10,000-fold, depending on the actual specific activity of the in vitro product) were used to show over 90% inhibition of uptake. The specificity of the competition, however, is apparent from the fact that at the same concentrations the holocytochrome and fragments of the apocytochrome other than fragment II showed no demonstrable competing activity.

Clearly, several intriguing possibilities must be considered concerning the uptake mechanism and the features of fragment II that may serve as addressing signals for cytochrome c. It would be of interest, for example, to determine if fragment II prevents the uptake of other mitochondrial proteins of cytoplasmic origin and if these contain, transiently or permanently, structural similarities to fragment II. This may be expected if only a limited number of receptors exist in the mitochondrial surface that are recognized by families of mitochondrial proteins with similar subcellular destinations. From a biogenetic standpoint, the processing reaction that binds heme to cytochrome c may be regarded as functionally equivalent to the proteolytic cleavage that affects the cytoplasmic precursors of other mitochondrial proteins. In both cases, a posttranslational modification results in a change in the conformation of the polypeptide, from that conformation which is released from the ribosome and expresses an addressing signal in the cytoplasm to that which functions within the organelle.

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