Formation of an Iso-1-cytochrome c-like Species Containing a Covalently Bonded Heme Group from the Apoprotein by a Yeast Cell-free System in the Presence of Hemin*

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Incubation of the $^{125}$I-labeled apoprotein, prepared from $^{125}$I-labeled iso-1-cytochrome c, with a yeast mitochondrial fraction in the presence of hemin, NADPH, and an extract of the postmitochondrial fraction at 32 ± 2°C for 180 min has resulted in formation of cytochrome c-like species in yields of up to 85%. This radioactive synthesized species contains a functional group which responds to reduction with ascorbate and oxidation with K$_2$Fe(CN)$_6$ in that it is resistant in the reduced form and susceptible in the oxidized form to trypsin action in a manner characteristic of native cytochrome c. The functional group cannot be removed from the protein by cold HCl-acetone or 8 M urea treatment. The reduced form of the synthesized species exhibits resistance against autoxidation and the oxidized form can be reduced also by cytochrome b. The synthesized species exhibits the same compact hydrodynamic volume of native cytochrome c. Treatment with silver sulfate followed by incubation with diithiothreitol converts the synthesized species to the original apoprotein as judged by an increase in the hydrodynamic volume. Thus, the synthesized species is indistinguishable from the original iso-1-cytochrome c by these measurements; i.e. the synthesized species consists of the apoprotein to which heme is covalently attached through the thioether bond(s).

The active factor of the mitochondrial fraction is heat-labile. The synthetic activity is strongly dependent on pH with a maximum approximately at pH 7.0. Hemin (or heme) appears to be required for this synthesis. The postmitochondrial fraction is inactive by itself. However, its addition markedly increases the synthetic activity. This factor is heat-stable, soluble in 80% methanol (or 75% ethanol), and insoluble in ethyl ether or ethyl acetate. Addition of NADP(H) (or NAD(H)) also increases the synthetic activity, the reduced form being more effective than the oxidized form. The postmitochondrial factor and the pyridine nucleotides appear to enhance the effect of each other. Thus, it seems that cytochrome c or a cytochrome c-like species is formed from the apoprotein and heme (or hemin) by an enzyme, cytochrome c synthetase, present in mitochondria.

The covalent attachment of the heme moiety to cytochrome c apoprotein in cells is stereochemically specific. For example,

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cytochrome c. The covalent attachment of the heme moiety to cytochrome c apoprotein in cells is stereochemically specific. For example,
purified by ion exchange chromatography and gel filtration. Iso-I-
medium described by Jayaraman were found (29) to be 2.3 mol (a control sample of unlabeled
The spheroplasts were lysed with 0.25 M sucrose, 0.05 M
labeled apoprotein is prepared from this purified 125I-labeled iso-I-
chondy c (18, 19) was obtained by purification (20, 21) of yeast
cytochrome c from Sigma (type VIII) and characterized (22, 23) by
from the apoprotein (see above). The principle of the assay of cytochrome c synthesis is as follows. The validity of this assay method is confirmed by determination of
A quantity of the stock solution of 125I-labeled apoprotein (see miniprint supplement) was pretreated by incubation with 0.5 M β-
mercaptotetanol, 20 mM potassium phosphate, pH 8.0, at 37°C for 2
to 3 h prior to use and the radioactivity was remeasured. A stock solution of 0.5 mM hemin (Sigma) was made in 0.075 M NaOH and
stored under N2 in the dark at 4°C until use. A typical assay mixture without the postmitochondrial factor (see below) was made as follows. Approximately 500 mg, wet weight, of washed mitochondria (see miniprint supplement) were suspended in 1 ml of Krebs-Ringer bicarbonate solution (40) containing 2 mM succinate (Krebz-Ringer bicarbonate/succinate) and then NADH (Sigma) (a final concentration, 0.4 mM), 1 mM potassium phosphate, pH 7.7 (a final concentration, 125 mM), and 36 μl of the 125I-labeled apoprotein solution were added. The mixture was 7.3 ± 0.1° and the total volume was approximately 2.2
1.4 M NaCl, and centrifuged. The second extract thus obtained was combined with the <br>Experimental Procedures
The details of the following procedures are described in the miniprint supplement immediately following this paper. Yeast cytochrome c (18, 19) was isolated from a 1% glucose growth
Saccharomyces cerevisiae (ATCC 24653) was grown in the growth
medium described by Jayaraman et al. (30) (1% glucose) under aerobic conditions and spheroplasts were prepared from the cells
The principle of the assay of cytochrome c synthesis is as follows. Since the structure of apocytochrome c is highly disordered (17, 32), the covalent bonding of the heme group to the apoprotein should result in folding of the polypeptide chain which, in turn, results in development of cytochrome c function. Native cytochrome c is strongly resistant in the ferrous form but susceptible in the ferric form to trypsin action (33). Biologically active, ordered complexes formed by noncovalent interactions of two or three overlapping fragments of horse cytochrome c also exhibit similar properties in that incubation of these complexes in the reduced form with trypsin has resulted in digestion of only the redundant sequences (34-36). These phenomena are explained by cooperativity of interatomic interactions such that reduction of the heme iron strengthens interatomic interactions throughout the three-dimensional structure (37). This stabilization occurs without a detectable change in the time average atomic coordinates at 2 Å resolution (38). It is assumed that the redundant segments of such complexes are superfluous to and therefore not incorporated into this cooperative interaction system, which has been proposed as specifically operating only in the ordered structure of native protein to stabilize the structure (36, 39). Then,
assay method is confirmed by determination of the radioactivity associated with intact cytochrome c from an incubation mixture in which the mitochondrial fraction is omitted, resulting in a negligible value (0.02%) (Table I, Series F). Similarly a zero time incubation resulted in a very low value (0.04% yield, see Table

to 8%. The mixture was subjected to gel filtration on a Sephadex G-50 column (1 x 50 cm) using 0.05 M ammonium acetate, pH 5.0, at 25°C. Measurements of absorbance at 410 nm and the radioactivity of the fractions established the radioactivity associated with intact cytochrome c (see, for example, Fig. 2, A and B, below). From this the yield of the radioactive synthesized species (or percentage of incorporation of radioactivity) was determined after correction for the loss occurring during the treatments on the basis of recovery of carrier iso-1-cytochrome c (416 ng = 190,000 cpm) (see miniprint supplement). The radioactive sample thus obtained contained the synthesized species and carrier (unlabeled) iso-1-cytochrome c and is referred to below as the radioactive synthesized species.
The validity of this assay method is confirmed by determination of the radioactivity associated with intact cytochrome c from an incubation mixture in which the mitochondrial fraction is omitted, resulting in a negligible value (0.02%) (Table I, Series F). Similarly, a zero time incubation resulted in a very low value (0.04% yield, see Table

7 Where indicated, NADPH was used in place of NADH.
8 The optimal pH is approximately 7.0 (see Fig. 1). However, the early experiments were performed at pH 7.2.
In each series of experiments, approximately 500 to 700 mg, wet weight, of washed mitochondrial pellets obtained from a 13-liter fermentation (see miniprint supplement) was divided in four to eight portions (the concentrations of mitochondrial protein are shown). An assay mixture contained, in a total volume of approximately 1.9 ml, 0.4 ml of Krebs-Ringer bicarbonate/succinate, washed mitochondrial pellets (0.2 to 0.3 ml), 1 ml of the lysis solution or 1 ml of the postmitochondrial fraction, 0.65 mM NADH, a given amount of hemin, and approximately 0.1 M potassium phosphate, pH 7.3. The following changes were made with the components of the assay mixture in Series C, D, and E. Series C: Krebs-Ringer bicarbonate/succinate, 0.6 ml; NADPH (instead of NADH), 1.3 mM; the 80% methanol extract (see miniprint supplement) (in place of the postmitochondrial fraction), 0.8 ml; potassium phosphate, approximately 0.15 M, pH 7.0; Series D: a mixture of a mitochondrial suspension in 0.5 ml of Krebs-Ringer bicarbonate/succinate plus 0.15 ml of 1 M potassium phosphate, pH 7.5, was heated in a boiling water bath for 10 min and used as such, a seven times concentrated 75% ethanol extract (see miniprint supplement) (in place of the postmitochondrial fraction), 1 ml; NADH, 0.59 mM; potassium phosphate, pH 7.3, 0.08 M. Series E: 1 ml of the postmitochondrial fraction was heated in a boiling water bath for 10 min and used as such; Krebs-Ringer bicarbonate/succinate, 0.5 ml; potassium phosphate, pH 7.3, 0.08 M; NADH, 0.59 M. A given amount of the labeled apoprotein (20 to 40 µl) was added to each assay mixture. The incubation was performed at 32 ± 1°C for 30 min with the exception that in Experiment 1 (zero time incubation) of Series A addition of the labeled apoprotein to the assay mixture was immediately followed by addition of 144 nmol of unlabeled apoprotein and the mixture was placed in an ice bath for 30 min. The yield of the radioactive synthesized species and the specific activity were determined by the procedure described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Series</th>
<th>Experiment</th>
<th>Components</th>
<th>Yield</th>
<th>Specific activity</th>
</tr>
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<td></td>
<td></td>
<td>Mitochondrial fraction</td>
<td>Hemin</td>
<td>Postmitochondrial fraction</td>
</tr>
<tr>
<td>A. Apoprotein:</td>
<td>1&quot;</td>
<td>3.2</td>
<td>2</td>
<td>3.0</td>
</tr>
<tr>
<td>2 nmol</td>
<td>2</td>
<td>2.3</td>
<td>2</td>
<td>3.0</td>
</tr>
<tr>
<td>2.4 × 10³ cpm</td>
<td>1</td>
<td>4.0</td>
<td>2</td>
<td>3.5</td>
</tr>
<tr>
<td>B. Apoprotein:</td>
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<td>2</td>
<td>None</td>
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<tr>
<td>1.59 × 10³ cpm</td>
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<td>None</td>
<td>2</td>
<td>4.0</td>
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<td>C. Apoprotein:</td>
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<td>10.3</td>
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<td>8.33&quot;</td>
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<tr>
<td>2.40 × 10³ cpm</td>
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<td>8.85</td>
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<td>1.2&quot;</td>
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<td>2.365 × 10³ cpm</td>
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<td>None</td>
<td>8</td>
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<tr>
<td>F. Apoprotein:</td>
<td>10 nmol, 8.4 × 10³ cpm</td>
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<td></td>
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</tbody>
</table>

* Zero incubation time.
*" Referred to the protein of the postmitochondrial fraction.
" The 80% methanol extract of the postmitochondrial fraction.
 Boiled (see above).
 The 75% ethanol extract of the postmitochondrial fraction.

I, Series A, Experiment 1). Thus, 1% incorporation of the radioactivity (20 pmol of the synthesized species/incubation mixture) or less can be detected by this method.

When the postmitochondrial fraction was present in the assay mixture (see Table I), the protein concentration of the assay mixture containing the mitochondrial fraction was determined before and after addition of the postmitochondrial fraction; then, the protein concentration of the postmitochondrial fraction was calculated as a difference. The specific activity is in unit (picomoles of the radioactive synthesized species formed in 30 min) per mg of mitochondrial protein.

RESULTS

Properties of the Yeast Cell-free System for Formation of the Radioactive Synthesized Species from the Labeled Apoprotein in the Presence of Hemin—As presented in Table I (Series A and B), the yeast mitochondrial fraction was found to be active in the formation of the radioactive synthesized species from the labeled apoprotein in the presence of hemin.

Specific activity referred to the total protein of the mitochondrial fraction varied depending on the preparation. The reason for this is unknown at the present time. Therefore, in the present study the effects of various factors on the synthetic activity are compared for the assay mixtures containing the same preparation of the mitochondrial fraction in each series of experiments.

This active mitochondrial factor is heat-labile, as it is completely inactivated by heating in a boiling water bath for 10 min (Table I, Series D). The postmitochondrial fraction is inactive by itself (Table I, Series B). However, addition of the postmitochondrial fraction markedly increases the synthetic activity (Table I, Series B). This factor is heat-stable (active after heating in a boiling water bath for 10 min) (Table I, Series E). Soluble in 80% methanol or 75% ethanol, and insoluble in ethyl ether or ethyl acetate (see the miniprint supplement). The synthetic activity is highly dependent on pH with a maximum approximately at pH 7.0 (Fig. 1). Incubation of the labeled apoprotein with an increased amount of the mitochondrial fraction (relative to the apoprotein) and hemin in the presence of the postmitochondrial extract (described below) and NADPH at pH 7.0 (at 32 ± 1°C, 30 min) has resulted in an increase in the yield of the radioactive synthesized species up to 35% (Table I, Series C).

Effect of Hemin—In the absence of added hemin in the assay mixture the radioactive synthesized species was formed. However, addition of hemin increased the yield up to three times whether or not the postmitochondrial fraction was present (Table II, Series A experiments and Series C, Experiments 1 and 2). If endogenous heme present in mitochondria


(10, 43) is utilized in the absence of added hemin, removal of the endogenous heme results in lack of synthesis. It is known that human serum albumin binds to heme (43). Indeed, addition of human serum albumin resulted in strong inhibition (approximately 90%) of the synthetic activity (Table II, Series B). Prior incubation of the mitochondrial fraction with human serum albumin, followed by washing the mitochondrial pellets to remove the albumin, also resulted in approximately 40% decrease in the synthetic activity in the absence of added hemin (Table II, Series C, Experiments 1 and 3). Addition of hemin to this treated mitochondrial fraction restored the synthetic activity closely to that of the untreated mitochondrial fraction in the presence of added hemin (Table II, Series C, Experiments 2 and 4). Thus, heme or hemin appears to be required for formation of the radioactive synthesized species from the labeled apoprotein.

Characterization of the Radioactive Synthesized Species—

On the basis of the treatments used for isolation (see above) the radioactive synthesized species should exhibit resistance against trypsin action under reducing conditions. This point has been confirmed as follows.

The radioactive synthesized species was treated with cold HCl-acetone, conditions which cause dissociation of heme from hemoglobin or myoglobin (44), as described in the miniprint supplement. The precipitated carrier iso-1-cytochrome c plus the radioactive synthesized species were subjected to digestion with trypsin after reduction with ascorbate (see miniprint supplement). Gel filtration analysis of the products indicated that the radioactivity distributed 71 and 29% between intact cytochrome c and the digest, respectively. The specific radioactivity associated with cytochrome c decreased by approximately 25% after this experiment. A similar decrease (24%) of the specific radioactivity was also observed when labeled iso-1-cytochrome c was treated by the same procedure in a control experiment. In this case the radioactivity distributions between cytochrome c and the digest was 90 and 10%, respectively.

The radioactive synthesized species recovered after this treatment was subjected to a second and a third cycle of the same treatments for digestion with trypsin (under reducing conditions) with the exception that HCl-acetone treatment was omitted. In the second cycle, the radioactivity distributed between intact cytochrome c and the digest was 94 and 6%, respectively, and in the third again 94 and 6%. In the case of labeled iso-1-cytochrome c these distributions were 89 and 11%, respectively, in the second cycle and 92 and 8%, respectively, in the third. (A decrease in the specific radioactivity by 42 and 16% observed with the synthesized species after the second and the third cycle of treatments, respectively. In the

![Graph](https://via.placeholder.com/150)

**Fig. 1. Effect of pH on the formation of the radioactive synthesized species from the labeled apoprotein in the presence of hemin by a yeast cell-free system.** The assay mixture contained, in a total volume of approximately 2.0 ml, washed mitochondrial pellet (approximately 0.2 ml), 0.8 ml of ethyl acetate-treated methanol extract of the postmitochondrial fraction (see miniprint supplement), 0.6 ml of Krebs-Ringer bicarbonate/succinate, 2.5 μM hemin, 0.89 mM NADPH, 0.6 mM NADH, 0.6 mM NADP, 0.6 mM NAD, 0.7 mM ATP, and approximately 0.15 M potassium phosphate, at pH 7.0. The pH of the mixtures were readjusted with 1 N NaOH before addition of the labeled apoprotein (30 μl, 5.75 × 10⁶ cpm, 2.17 mmol). After incubation at 32 ± 1°C for 30 min, the pH of the mixtures remained unchanged. The radioactive synthesized species thus formed and the specific activity were determined as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Series</th>
<th>Experiment</th>
<th>Mitochondrial fraction</th>
<th>Albumin</th>
<th>Hemin</th>
<th>Postmitochondrial fraction</th>
<th>Yield of the radioactive synthesized species</th>
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<td></td>
<td></td>
<td>mg protein/ml</td>
<td>nmol/ml</td>
<td>mg protein/ml</td>
<td>%</td>
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<tr>
<td></td>
<td>4.8 nmol, 1.49 × 10⁶ cpm</td>
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<td>None</td>
<td>None</td>
<td>0.8</td>
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<tr>
<td>B. Apoprotein:</td>
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<td>2.5</td>
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<td>12.7</td>
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<td>1.6 nmol, 3.65 × 10⁶ cpm</td>
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<td>11.8</td>
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<td>3.8</td>
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<td>Present*</td>
<td>9.8</td>
<td></td>
</tr>
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</table>

* The 80% methanol extract (see miniprint supplement).

* Heme-depleted mitochondria (see above).
case of labeled iso-1-cytochrome c these decreases were 17 and 15%, respectively.)

The radioactive synthesized species recovered after the third cycle of treatments was oxidized with K$_3$Fe(CN)$_6$, and, after removal of K$_3$Fe(CN)$_6$, divided in two equal portions. One of these was incubated immediately with trypsin and the other after reduction with ascorbate. As shown in Fig. 2, A and B, while the reduced species was completely protected from digestion the oxidized was essentially completely digested. (The same results were obtained with the reduced and the oxidized form of labeled iso-1-cytochrome c obtained after the third cycle of treatments.) These results indicate that the radioactive synthesized species contains a functional group which responds to reduction with ascorbate and to oxidation with potassium ferricyanide in a manner characteristic of native cytochrome c and that this functional group cannot be removed by cold acetone-HCl treatment. It follows that this functional group is heme covalently bound, through thioether bond(s), to the apoprotein. It is assumed that only the native three-dimensional structure of cytochrome c changes stability against trypsin as a function of the valence of the heme iron (see "Experimental Procedures"). This hypothesis is supported by the following result.

Ion Exchange Chromatography of the Radioactive Synthesized Species—On ion exchange chromatography on SP-Sephadex C-25 the behavior of the radioactive synthesized species, including those presumably deamidated and polymerized, was the same as that of carrier iso-1-cytochrome c (see Fig. 1 of the miniprint supplement). To show that the radioactive synthesized species consists of a single polypeptide chain, the radioactive synthesized species was subjected to ion exchange chromatography in the presence of 8 M urea as described in the miniprint supplement. These conditions have previously permitted separation of the apo fragment and the heme-containing fragment from noncovalently bound complexes (34, 36). As shown in Fig. 2 (right) of the miniprint supplement, a single radioactive peak (representing the radioactive synthesized species, approximately 40% recovery) and a single absorbance peak measured at 408 nm (representing carrier iso-1-cytochrome c, the recovery being similar to the radioactivity (see miniprint supplement)) were obtained, the absorbance peak eluting slightly earlier than the radioactive peak. In a control experiment in which labeled iso-1-cytochrome c was similarly subjected to ion exchange chromatography, the absorbance peak also eluted slightly earlier than the radioactivity peak, although in this case a second, minor radioactivity peak eluting earlier than the absorbance peak was also observed (see Fig. 2 (left) of the miniprint supplement).

To show that the functional group is bound to the polypeptide chain after urea treatment, the sample obtained from the fractions comprising the radioactivity peak (see Fig. 2 (right) of the miniprint supplement) was subjected to removal of urea followed by digestion with trypsin under reducing conditions as described in the miniprint supplement. The results indicated that the extent of renaturation of the radioactive synthesized species after removal of urea was approximately 47%. The specific radioactivity of this renatured sample was 91% that before trypsin treatment, indicating that the ability of the radioactive synthesized species to renature after urea denaturation is essentially the same as that of carrier iso-1-cytochrome c (see miniprint supplement). A similar extent of renaturation was also obtained for labeled iso-1-cytochrome c subjected to the same treatments with 8 M urea in the control experiment (see miniprint supplement). Thus, the functional group remained bound to the apoprotein after this treatment.

Autoxidizability—Ferrous native cytochrome c is known to be resistant against autoxidation (45-47). Perturbation of the "native state" by changing solution conditions (pH, salt concentration, etc.) or alteration of the protein molecule results in an increase in autoxidizability (21, 46-50). The autoxidizability of the "reduced," radioactive synthesized species was measured as a function of pH by the method described in the miniprint supplement. The qualitative results are presented in Fig. 3 and also in the miniprint supplement (Table I). The radioactive synthesized species changed its autoxidizability in the same manner as labeled iso-1-cytochrome c. That is, the autoxidation was slow at pH 8.0 and very rapid at pH 3.6 (see miniprint supplement, Table I): At pH 5.7 the autoxidation was accelerated with respect to that at pH 8.0, but it was much slower than at pH 3.6 (Fig. 3) (see miniprint supplement, Table I). However, both labeled species (synthesized species and iso-1-cytochrome c) exhibited approximately through the column. After washing with 2 ml of 50 mM potassium phosphate, pH 8.0, cytochrome c was eluted with 1 ml of 0.5 M potassium phosphate, pH 8.0. The eluate was divided in two equal portions. One of these, B, was immediately incubated with trypsin for 1 h at 36°C and then under reducing conditions, and gel filtration (see miniprint supplement). The mixture was subjected to gel filtration on a Sephadex G-50 column (0.7 x 50 cm) at 25°C, using 0.05 M ammonium acetate, pH 5.0. Absorbance at 416 nm (A) and the radioactivity (○) of the fractions (1.0 ml each) were measured.

![Fig. 2. Resistance in the reduced state (A) and susceptibility in the oxidized state (B) to trypsin action of the radioactive synthesized species.](image-url)
The autoxidizability of the radioactive synthesized species as a function of pH. A solution of the reduced radioactive synthesized species at pH 5.7 (left) or 3.6 (right) was allowed to stand at 25°C for air oxidation. At indicated times, an aliquot was subjected to trypsin digestion at pH 7.7 under air oxidation. At indicated times, an aliquot was subjected to trypsin digestion at pH 7.7 under N₂ as described in the miniprint supplement. The products were analyzed by gel filtration on a Sephadex G-50 (fine) column (1 x 20 cm) using 0.05 M ammonium acetate, pH 5.0, at 25°C. The radioactivity peaks emerging earlier (14 to 24 ml) and later (26 to 38 ml) represent the undigested and the digested species (fragments), respectively. The former species corresponds to the species remaining reduced and the latter to the species oxidized when the aliquot was withdrawn. The experimental details, quantitative results, and interpretation are described in the miniprint supplement. Units of elution volume, ml.

Biological Activity—The radioactive synthesized species was essentially completely reduced upon incubation with lactate dehydrogenase (cytochrome b₅, Refs. 51 to 53) in the presence of lactate in a manner similar to labeled iso-1-cytochrome c or carrier iso-1-cytochrome c (Table III).

The results indicate that the standard reduction-oxidation potential of the radioactive synthesized species is the same as or similar to that of iso-1-cytochrome c and that lactate dehydrogenase binds with and transfers electron to the radioactive synthesized species in the same manner as that with native iso-1-cytochrome c.

Conversion of the Radioactive Synthesized Species to the Apoprotein by Silver Sulfate Treatment followed by Incubation with Dithiothreitol—The radioactive synthesized species exhibited the same compact hydrodynamic volume of native iso-1-cytochrome c by gel filtration on a column (1 x 210 cm) of Sephadex G-50 (fine) using 0.05 M ammonium acetate, pH 5.0, at 7°C (see also Fig. 2). In contrast, the labeled apoprotein eluted earlier than native cytochrome c under the same conditions (17). Upon treatment with silver sulfate (which cleaves the thioether bonds of cytochrome c) (28), and then with dithiothreitol (which generates free sulfhydryl groups) (17), the entire radioactivity of the radioactive synthesized species eluted at the elution position of the apoprotein (Fig. 4). These observations are consistent with the idea that the radioactive synthesized species contains a covalently bound heme group attached through thioether bond(s) and that this covalent bonding of the heme group to the apoprotein results in folding of the polypeptide chain.

Effects of Pyridine Nucleotides and Ca²⁺—Beside the postmitochondrial factor, the pyridine nucleotides are also found to markedly increase the synthetic activity (Fig. 4). NADH is less effective than NADPH (Fig. 5). The effect of NADP and NAD is also significant, although less than the reduced forms (Fig. 5). The postmitochondrial factor and the pyridine nucleotides appear to enhance the effect of each other (see miniprint supplement, Table II), indicating that the postmitochondrial factor is not pyridine nucleotide itself. Note that while in the absence of the postmitochondrial factor, the effect of both NADP and NAD was less than that of NADH, in the presence of the factor, the effect of both NADP and NAD was similar to that of iso-1-cytochrome c by gel filtration on a column (1 x 50 cm) using 0.05 M ammonium acetate, pH 5.0, at 25°C. The radioactive synthesized species was allowed to stand at 25°C for air oxidation. At indicated times, an aliquot was subjected to trypsin digestion at pH 7.7 under air oxidation. At indicated times, an aliquot was subjected to trypsin digestion at pH 7.7 under N₂ as described in the miniprint supplement. The products were analyzed by gel filtration on a Sephadex G-50 (fine) column (1 x 50 cm) using 0.05 M ammonium acetate, pH 5.0, at 7°C (see also Fig. 3). In contrast, the labeled apoprotein eluted earlier than native cytochrome c under the same conditions (17). Upon treatment with silver sulfate (which cleaves the thioether bonds of cytochrome c) (28), and then with dithiothreitol (which generates free sulfhydryl groups) (17), the entire radioactivity of the radioactive synthesized species eluted at the elution position of the apoprotein (Fig. 4). These observations are consistent with the idea that the radioactive synthesized species contains a covalently bound heme group attached through thioether bond(s) and that this covalent bonding of the heme group to the apoprotein results in folding of the polypeptide chain.

Reduction of the radioactive synthesized species and labeled iso-1-cytochrome c by a yeast lactate dehydrogenase (cytochrome b₅)-lactate system

The experimental details are described in the miniprint supplement. The complete incubation system contained the oxidized form of the radioactive synthesized species or labeled iso-1-cytochrome c, yeast lactate dehydrogenase (50-52), and L(+) lactic acid in approximately 0.05 M potassium phosphate, pH 8.0. After incubation at 25°C for 20 min, the quantity and the specific activity of the reduced species were determined. The percentage of the specific radioactivity refers to the sample before the incubation.

<table>
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<tr>
<th>Reduction</th>
<th>Radioactive synthesized species</th>
<th>Labeled iso-1-cytochrome c</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>90</td>
<td>85</td>
</tr>
<tr>
<td>Minus lactic acid</td>
<td>12</td>
<td>30</td>
</tr>
<tr>
<td>Minus lactate dehydrogenase</td>
<td>1</td>
<td>13</td>
</tr>
</tbody>
</table>

* N.D., not determined.

Conversion of the radioactive synthesized species to the apoprotein after treatment with silver sulfate followed by dithiothreitol treatment. The treated sample, mixed with unlabeled iso-1-cytochrome c, was applied to a Sephadex G-50 column (0.7 x 170 cm) equilibrated with 0.05 M ammonium acetate, pH 5.0, at 25°C. The column was eluted with the same buffer. Radioactivity (●) and absorbances at 276 (○) and 410 (▲) nm of the fractions were measured. The experimental details are described in the miniprint supplement.
FIG. 5. Effect of pyridine nucleotides on the formation of the radioactive synthesized species from the labeled apoprotein in the presence of hemin. In the case of the presence of the postmitochondrial factor (upper) the assay mixture contained, in a total volume of approximately 2.0 ml of Krebs-Ringer bicarbonate/succinate, 2.5 μM hemin, 0.8 ml of the methanol extract of the postmitochondrial fraction (see the miniprint supplement), 0.15 M potassium phosphate, pH 7.0, washed mitochondrial pellets (approximately 0.2 ml), and one of the following nucleotides, 0.96 mM NADPH, 0.55 mM NADH, 0.56 mM NADP, 0.57 mM NAD, or 0.72 mM ATP as indicated. None and all indicate the presence of none and all of these cofactors at the concentrations specified above. The labeled apoprotein (22 μl, 3.7×10⁵ cpm, 1.6 nmol) was added to each assay mixture. In the case of the absence of the postmitochondrial factor (lower), the assay mixture was the same as that described above with the following exception. The methanol extract was replaced by 0.8 ml of the lysis solution, and Krebs-Ringer bicarbonate/succinate by 0.6 ml of 150 mM potassium phosphate, pH 7.0, in three of the assay mixtures as indicated. NADPH, NADP, NADH, NAD, MgCl₂, and CaCl₂ were present at concentrations of 1.3, 1.3, 1.4, 1.45, 2.5, and 2.5 mM, respectively, in the indicated mixtures. The labeled apoprotein (22 μl, 3.7×10⁵ cpm, 1.6 nmol) was added to each assay mixture. In both cases, the incubation was carried out at 32 ± 1°C for 30 min. The radioactive synthesized species formed and the specific activity were determined as described under "Experimental Procedures."

...presence of the factor the relative effects for increasing the synthetic activity of NADP and NAD become similar to that of NADH (Fig. 5). Addition of ATP alone did not increase the synthetic activity (Fig. 5).

In the absence of Krebs-Ringer bicarbonate/succinate in the assay mixture the synthetic activity decreased (Fig. 5). While addition of Mg²⁺ to this assay mixture did not affect the synthetic activity, addition of Ca²⁺ increased the activity by approximately 20% (Fig. 5).

**DISCUSSION**

Since Sano and Tanaka's success in chemical synthesis of cytochrome c or cytochrome c-like species (9), the suggestion that nonenzymatic synthesis of porphyrin cytochrome c from the apoprotein and protoporphyrinogen (present in mitochondria) may be a step in biosynthesis of cytochrome c (10) has received wide attention (e.g. Ref. 54), especially since combination of protoporphyrinogen with the apoprotein could proceed at neutral pH (55). Since insertion of iron into porphyrin cytochrome c requires rather drastic conditions (e.g. 12.5% acetic acid, 75°C (56)), a hypothetical special ferrochelatase catalyzing insertion of iron has also been considered (54). Thus, although direct attachment of heme to the apoprotein in cells has been suggested (8), formation of porphyrin cytochrome c in mitochondria (9, 10) must also be taken into account in the investigation of the hypothetical cytochrome c synthetase. In this context, a sensitive assay method which differentiates cytochrome c or cytochrome c-like species containing a covalently bound heme group from porphyrin cytochrome c is essential. For such a sensitive assay one may consider an immunological reaction (57), as in the study of the biosynthesis of cytochrome c in a cell-free system of N. crassa (15, 16). However, since the conformation of porphyrin cytochrome c resembles cytochrome c (17), it would not be easy to obtain a specific anticytochrome c-antibody completely devoid of cross-reactivity with porphyrin cytochrome c. It is unknown whether the antihemoglobin antibody used in the Neurospora cell-free system (15, 16) does not cross-react with porphyrin cytochrome c and also with a noncovalent complex of the apoprotein and a heme fragment (34-37).

In the present study, we have developed a functional assay method which makes use of a specific oxidation-reduction property of cytochrome c, that is, change in an all or none fashion, in the susceptibility to trypsin action with change from the ferric to the ferrous state (33) (see description of "Experimental Procedures").

The radioactive synthesized species consists of a single labeled polypeptide chain and the extent of renaturation after urea treatment is comparable to native iso-1-cytochrome c. This synthesized species exhibits the specific properties associated with the covalently bound heme group of native cytochrome c in that 1) it responds to reduction with ascorbate and oxidation with K₃Fe(CN)₆ in a manner characteristic of native cytochrome c as measured by susceptibility to trypsin action; (2) this oxidation-reduction property (or change of susceptibility to trypsin action as a function of valence of the iron atom) is not affected by HCl-acetone treatment; 3) the change of autoxidizability as a function of pH is the same for the radioactive synthesized species and the original labeled iso-1-cytochrome c; 4) the radioactive synthesized species can be essentially fully reduced by a yeast lactate dehydrogenase (cytochrome b₅)-lactate system; 5) while the labeled apoprotein exhibits a higher hydrodynamic volume than native cytochrome c by gel filtration, the radioactive synthesized species exhibits the same compact hydrodynamic volume of native cytochrome c; 6) the functional group contained in the radioactive synthesized species is removed by silver sulfate treatment as measured by an increase in the hydrodynamic volume of the protein. Although direct observation (e.g. absorption spectrum) of the heme group contained in the radioactive synthesized species was not made due to the small quantity of the synthesized sample obtainable at this stage, observations 1, 3, and 4 clearly indicate the presence of a heme group, observations 2 and 6 and the treatment with 8 M urea indicate covalent bonding of the heme group, through thioether bonds, to the apoprotein, and observations 5 and 6 indicate a compactly folded structure of the radioactive synthesized species in contrast to the disordered state of the apoprotein (Fig. 4) (17, 32). Furthermore, observations 1 and 3 through 5 indicate that the ordered structure of radioactive synthesized species is functional in the same manner as that of the labeled iso-1-cytochrome c. In summary, by these criteria the radioactive synthesized species is not distinguishable from the original labeled iso-1-cytochrome c. Thus, we conclude that cytochrome c or a cytochrome c-like species containing a covalently bound heme group, through thioether bonds, is formed from the labeled apoprotein. Two thioether bonds (at residues 19 and 22 (22)) are more likely formed than
Cytochrome c Synthetase

one (at one of these residues), since the functional properties (observations 1, 3, and 4) of the radioactive synthesized species are the same as those of the labeled iso-1-cytochrome c. That is, iso-1-cytochrome c itself may be formed. Then, the mode of formation of the thioether bonds (one by one or simultaneously, the order of formation in the former case) remains to be understood.

In agreement with studies of in vivo labeling of cytochrome c with 1-[35S]lysine (13) the mitochondrial fraction is active for formation of the radioactive synthesized species from the labeled apoprotein in the presence of hemin. In this respect the present results also agree with the Neurospora cell-free system (15, 16), provided that the species precipitated by the antiholoprotein antibody in the Neurospora cell-free system is proved to form cytochrome c instead of porphyrin cytochrome c. Being consistent with the hypothesis of the enzyme catalyzing the formation of the configurationally "correct" thioether bonds, the active factor of the present mitochondrial fraction exhibits enzymatic properties in that it is heat-labile and its activity is highly dependent on pH with a maximum at pH 7.0. Furthermore, nonenzymatic synthesis of cytochrome c (9) does not require heme or hemin (9, 10, 56). In contrast, even though the radioactive synthesized species is formed without exogenous hemin, addition of hemin increases the synthetic activity up to three times. It is also unlikely that the hypothetical ferrochelatase requires hemin or heme for its activity since the activity of known ferrochelatase is inhibited by heme (11). Although it is reported that serum albumin inhibits conversion of protoporphyrinogen to protoporphyrin in mitochondria (10), our results indicate rather that binding of heme (or hemin) to the albumin (43) is the cause for inhibition of the synthetic activity. In fact, treatment of the mitochondrial fraction with albumin (followed by removal of albumin) has considerably decreased the synthetic activity. The addition of heme to this, presumably partially hemedepated, system restored the activity nearly to that of the untreated mitochondria in the presence of added hemin. These observations indicate that heme or hemin is required for formation of the radioactive synthesized species from the apoprotein. Thus, on the basis of these considerations, we conclude that cytochrome c synthetase catalyzing direct attachment of heme (or hemin) to the apoprotein is present in the mitochondrial fraction. This conclusion is in agreement with the suggestion of Colleran and Jones that heme is directly attached to the apoprotein in cells (8).

It is interesting to observe that the cytochrome c synthetase activity increases in the presence of NADPH or NADH, the former pyridine nucleotide being more effective than the latter. Moreover, NADP and NAD also increase the synthetase activity at a lesser extent than their respective reduced forms. A heat-stable factor(s) of the postmitochondrial fraction also increases the synthetase activity. The pyridine nucleotides and the postmitochondrial factor apparently enhance the effect of each other. Furthermore, in the presence of the postmitochondrial factor, the effect of NADP or NAD becomes similar to that of NADH. This may suggest that the postmitochondrial factor could be a hydrogen donor to NADP or NAD. Thus, it seems that an electron transfer system present in the mitochondrial fraction is coupled or related to the cytochrome c synthetase activity. Use of these cofactors and a large amount of the mitochondrial fraction relative to the amount of the labeled apoprotein has increased the yield of the radioactive synthesized species to approximately 35% after 30 min of incubation at 32 ± 1°C.

In the present studies, 14C-labeled residues are not identified. Considering the low degree of the labeling, only a few residues of a molecule could be iodinated (27). The 35S labeling appears to influence the charge of cytochrome c (27) since both labeled iso-1-cytochrome c and the radioactive synthesized species eluted slightly later than unlabeled iso-1-cytochrome c by ion exchange chromatography in the presence of 8 M urea. However, in the absence of denaturant, the behavior on ion exchange chromatography of the radioactive synthesized species was the same as that of carrier iso-1-cytochrome c. An increase in the autoxidizability of both the radioactive synthesized species and labeled iso-1-cytochrome c is also indicated. Generation of free sulfhydryl groups are less (2.3 mol) than that of theory (3.0 mol (22)) with both labeled and unlabeled apoproteins. The reason for this apparent incomplete generation is not known, although the same treatment resulted in essentially complete generation of SH groups (theory, 2 mol (4)) in horse apoprotein. A decrease in the specific radioactivity was observed when the radioactive synthesized species or labeled iso-1-cytochrome c was subjected to a series of treatments (see above). The reason for this decrease is also not clear. Photoactivated dissociation of 125I label is known (27). Although these points are yet to be clarified, the present functional assay method is specific and sensitive and can be applied to other species as far as the susceptibility to proteolysis of cytochrome c specifically changes by reduction and oxidation of the heme iron atom (33). For example, using this assay method a preliminary experiment in this laboratory has indicated that yeast cytochrome c synthetase is capable of catalyzing covalent bonding of heme or hemin to horse apocytochrome c to form cytochrome c or cytochrome c-like species.

Acknowledgments—We thank Dr. Dulce Veloso for her help in preparation of the synthesized radioactive species used in one of the experiments reported in this communication. We also thank Mrs. Dorothy Stewart for her help in preparation of the manuscript.

REFERENCES

1. Theorell, H. (1939) Enzymologia 6, 88-89

A preliminary experiment using sonication has indicated that the enzyme is bound to particulate material (presumably broken mitochondria).

D. Veloso, G. Basile, and H. Taniuchi, unpublished results.
Recherche Scientifique, Paris
Fusion of one-tissue-cytochrome oxidase activity was studied in a series of experiments designed to determine the effects of different factors on the activity of the enzyme. The results of these experiments are presented in Table 1, which shows the activity of the enzyme in different tissues under various conditions.

Table 1: Activity of Cytochrome c Oxidase in Different Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>5.8</td>
</tr>
<tr>
<td>Liver</td>
<td>4.2</td>
</tr>
<tr>
<td>Heart</td>
<td>6.5</td>
</tr>
<tr>
<td>Muscle</td>
<td>3.4</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.8</td>
</tr>
</tbody>
</table>

The activity of the enzyme was determined using a spectrophotometric method. The results indicate that the activity of the enzyme is highest in the brain and lowest in the kidney. Further studies are needed to understand the factors that influence the activity of the enzyme in different tissues.
Table I

The experimental details are described in the miniprint text. The specific radiactivity per centage refers to the specific radioactivity of the reduced species before the incubation. The percentage of carrier cytochrome c oxidized in the absence of the radiolabeled species.

| Species | Reduced | Specific Radioactivity | % Oxidation of Carrier Cyt. | Specific Radioactivity
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>a) labeled</td>
<td>85</td>
<td>7.7</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>b) non-radiolabeled</td>
<td>91</td>
<td>7.7</td>
<td>9.3</td>
<td></td>
</tr>
</tbody>
</table>

\( \text{N.D.} \), not determined.

Table II

The effect of the pyridine-2-carboxylic acid on the cytochrome c synthetase activity.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Yield of the radioactivity</th>
<th>Specific Activity on Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>nmol</td>
<td>( \mu )mol/mg protein</td>
</tr>
<tr>
<td>2</td>
<td>ethanolic extract</td>
<td>( \mu )mol/mg protein</td>
</tr>
<tr>
<td>3</td>
<td>ether extract</td>
<td>( \mu )mol/mg protein</td>
</tr>
<tr>
<td>4</td>
<td>ether extract residue</td>
<td>( \mu )mol/mg protein</td>
</tr>
<tr>
<td>5</td>
<td>ethylacetate extract</td>
<td>( \mu )mol/mg protein</td>
</tr>
<tr>
<td>6</td>
<td>ethylacetate extract residue</td>
<td>( \mu )mol/mg protein</td>
</tr>
</tbody>
</table>

Fig. 1. Exchange chromatography of labeled cytochrome c (left) and non-radiolabeled cytochrome c (right) on the pyridinyl-2-carboxylic acid column. The experimental details are described in the miniprint text. The elution volumes of the radioactive and non-radioactive controls were determined as described in the miniprint text. The elution pattern on left is compared on opposite with that of right, due to accidental loss of a portion of the buffer in the mixing chamber at the beginning of the gradient elution, resulting in an increase in the concentration gradient.

Fig. 2. Exchange chromatography of labeled cytochrome c and non-radiolabeled cytochrome c on the pyridine-2-carboxylic acid column. The experimental details are described in the miniprint text. The elution pattern on left is compared on opposite with that of right, due to accidental loss of a portion of the buffer in the mixing chamber at the beginning of the gradient elution, resulting in an increase in the concentration gradient.