On the Specificity of Cytochrome c Synthetase in Recognition of the Amino Acid Sequence of Apocytochrome c*

(Received for publication, November 13, 1984)

Carlo Visco, Hiroshi Taniuchi, and Barbara S. Berlett

From the Laboratory of Chemical Biology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases and Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

Two forms of yeast cytochrome c synthetases with different specificities were resolved, one (synthetase I), solubilized from mitochondria or the cell debris with Triton X-100, recognizing not horse apocytochrome c but yeast apo-iso-1-cytochrome c as a substrate and the other (synthetase II) still bound with the particulate fraction from mitochondria after treatment with Triton, recognizing both horse and yeast apocytochromes c. The activity with labeled yeast apo-iso-1-cytochrome c as a substrate of cytochrome c synthetase I can be quantitatively inhibited by nonlabeled Candida krusei apocytochrome c and partially by nonlabeled tuna apocytochrome c but not by nonlabeled horse apocytochrome c indicating a specific amino acid sequence being recognized. However, an enzyme similarly solubilized from beef heart mitochondria recognized both horse apo-cytochrome c and yeast apo-iso-1-cytochrome c for attachment of heme. In view of the fact that the yeast synthetase II and the beef synthetase can both utilize either horse apocytochrome c or yeast apo-iso-1-cytochrome c as substrates, we suggest that these enzymes may also be involved in biosynthesis of cytochrome c1, that is, the ability to attach heme to apocytochrome c and apocytochrome c1 may have been conserved in eucaryotic cells, and that both synthetases may therefore be homologous.

Cytochrome c synthetase in yeast mitochondria catalyzes the NADPH (or NADH) dependent attachment of heme (or hemin) to yeast apo-iso-1-cytochrome c through two thioether bonds to form cytochrome c (1–3). A similar activity was also reported with Neurospora mitochondria (4). As reported previously (2), the yeast mitochondrial enzyme accepts horse apocytochrome c as well as yeast apo-iso-1-cytochrome c as a substrate. In fact, heme can be covalently attached to a chemically synthesized apo-fragment containing residues 1 to 25 of horse cytochrome c upon incubation with yeast mitochondria (5) (see Fig. 1). In contrast, we have found that after solubilization a cytochrome c synthetase from yeast mitochondria (3) can recognize yeast apo-iso-1-cytochrome c as a substrate but not horse apocytochrome c (Table I, experiment 2). Now we have resolved this paradox in finding that there are two enzymes with different specificities in yeast mitochondria, one solubilized by Na cholate or Triton X-100 (3) and recognizing yeast apo-iso-1-cytochrome c but not horse apocytochrome c and the other still bound with mitochondria after treatment with the surfactants and recognizing both yeast apo-iso-1-cytochrome c and horse apocytochrome c. We designate the former and the latter enzyme as yeast cytochrome c synthetases I and II, respectively. If this interpretation is correct, the particulate fraction of mitochondria, obtained after extraction of the synthetase I by treatment with e.g. Triton X-100 followed by centrifugation, would contain only the synthetase II and therefore its enzymatic activity with labeled yeast apo-iso-1-cytochrome c as a substrate would be completely inhibited in the presence of excess of nonlabeled horse apocytochrome c. Indeed, as shown in Fig. 4 in Miniprint, the formation of labeled cytochrome c from yeast 125I-apo-iso-1-cytochrome c and hemin by the particulate fraction decreased with increases in the concentration of nonlabeled horse apocytochrome c, approaching a value less than 20% of the activity measured in the absence of nonlabeled apocytochrome c. In a control experiment, yeast

* Portions of this paper (including “Materials and Methods,” References 23–26, Figs. 2–8, and Table II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84 M-3473, cite the authors, and include a check or money order for $5.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
mitochondria was tressed with Triton X-100 but used without centrifugation to separate the particulate fraction and the supernatant solution (see Miniprint). In this case, a maximum inhibition by nonlabeled Candida krusei apocytochrome c was approximately 60% (Fig. 4) as in the case of untreated mitochondria (Fig. 2).

Competitive Inhibition Tests with Solubilized Cytochrome c Synthetase from Yeast Cell Debris—It has been shown previously (3) that the cytochrome c synthetase activity can also be solubilized from yeast cell debris and fractionated with ammonium sulfate by the same procedures as those used for solubilization and fractionation of cytochrome c synthetase from yeast mitochondria (3). The enzymatic properties of these two enzymes are also found to be similar (3). Thus, we assume that the enzyme from yeast cell debris is associated with the enzyme of mitochondria contaminating the cell debris (3). Consistent with this assumption, the cell debris exhibits cytochrome c synthetase activity with either yeast apo-iso-1-cytochrome c or horse apocytochrome c as a substrate, whereas the solubilized enzyme from the cell debris is found to accept yeast apo-iso-1-cytochrome c as a substrate but not horse apocytochrome c (Table I, experiments 3 and 4). Thus, to save mitochondria, the solubilized enzyme (the ammonium sulfate fraction, see Miniprint) from the cell debris has been used in the following experiments.

Whereas formation of cytochrome c from yeast apo-iso-1-cytochrome c and hemin by the solubilized enzyme was not inhibited by more than a 10-fold excess of horse apocytochrome c over yeast apo-iso-1-cytochrome c (Fig. 5, Miniprint), incorporation of labeled yeast apo-iso-1-cytochrome c into cytochrome c by the solubilized enzyme was completely inhibited by nonlabeled yeast apo-iso-1-cytochrome c (Fig. 5). In a similar experiment quantitative inhibition was also observed when nonlabeled Candida krusei apocytochrome c was used (Fig. 6, Miniprint). In contrast, the inhibition by nonlabeled tuna apocytochrome c was much weaker than by nonlabeled Candida krusei apocytochrome c (Fig. 6, Miniprint).

Characterization of Yeast Cytochrome c Synthetase I—Purification of solubilized cytochrome c synthetase (synthetase I) has been hampered because of low activity (possibly due to a small quantity present in yeast mitochondria) (1–3) and also instability. Unless otherwise indicated, the characterization described below was carried out, using the partially purified synthetase I (an ammonium sulfate fraction) from the cell debris (see Miniprint). The synthetase I lost 60% of its activity after dialysis for 16 h at pH 7.0 at 4°C either in the presence or absence of 0.5% Triton X-100. The synthetase I was more stable at pH 7.0 than at pH 6.0 or below and the presence of 0.11 mM hemin appeared to stabilize the enzyme (Fig. 7, Miniprint). A preliminary gel filtration study of the synthetase I from the cell debris, prepared as described previously (3) using Sephadex G-100 and G-200 at pH 7.0 (0.05 M potassium phosphate) at 6°C in the presence of 0.5% Triton X-100, 1 mM EDTA, and 1 mM dithiothreitol, suggested a molecular weight of the enzyme in the order of 106. These gel filtrations the recovery of the enzymatic activity was 4%. The cause for this loss of the activity is unknown. Ion-exchange chromatography indicated the acidic nature of the synthetase I (Table II, Miniprint).

On the basis of these observations, the partially purified synthetase I (an ammonium sulfate fraction, 0–40% saturation) from yeast mitochondria was tested for its behavior in ion-exchange chromatography on DEAE-Sepharose at pH 7.0 in the presence of hemin at 4°C (Table II). Only part of the activity was adsorbed. A slight increase in the specific enzymatic activity was observed after elution with 0.1 M NaCl of the adsorbed protein (Table II). The reason for the partial adsorption is unknown.

Solubilization of Cytochrome c Synthetase from Beef Heart Mitochondria—The cytochrome c synthetase of beef heart mitochondria exhibits similar activity with either horse 125I-apocytochrome c or yeast 125I-apo-iso-1-cytochrome c as a substrate (Table I, experiment 5). This beef mitochondrial enzyme was solubilized using Triton X-100 and fractionated with ammonium sulfate (0-60% saturation) similarly to yeast cytochrome c synthetase I (see Miniprint). This partially purified beef cytochrome c synthetase also showed a similar level of activity with either horse 125I-apocytochrome c or yeast 125I-apo-iso-1-cytochrome c as a substrate (Table I, experiment 6).

DISCUSSION

The present studies have shown that cytochrome c synthetase I (solubilized from yeast mitochondria) differentiates between yeast apo-iso-1-cytochrome c and horse apocytochrome c while cytochrome c synthetase II (yeast mitochondria, not solubilized) and beef heart mitochondrial cytochrome c synthetase (solubilized) do not.

There are eight possible isomers in formation of two thioether bonds between two vinyl side chains of heme and two cysteine residues of apocytochrome c (cf. Ref. 1). Since noncovalent interactions between apocytochrome c and heme do not generate a native-like ordered structure (13, 14), it is unlikely that a “right” geometry of heme to apocytochrome c, which would determine the correct thioether bonds among the eight isomers, would be spontaneously attained without cytochrome c synthetase. Only the action of cytochrome c synthetase would permit the α-carbon atom of the vinyl side chain, for example, at position 4 of heme to bond to cysteine not at position 14 but at position 17 of apocytochrome c (in numbering of horse cytochrome c) (15). However, to differentiate between these two cysteine residues it would be suf-

---

2The apparent partial inhibition (40%) was observed at high concentrations of horse apocytochrome c (Fig. 4, Miniprint). Since precipitation occurred in the assay mixture containing a high concentration of the apocytochrome c, the significance of the partial inhibition is unclear.
Two Cytochrome c Synthetases in Yeast

The enzymatic activity was measured by the procedures described in the Miniunprint. One unit of the activity is pmol of radioactive cytochrome c formed from 151-apo-iso-1-cytochrome c or horse 151-apo-cytochrome c and hemin after incubation for 30 min at 25 °C unless otherwise indicated. The specific activity is unit/mg of protein. The protein was determined according to Lowry et al. (11). Unless otherwise indicated, the preparation of the enzyme samples are described in the Miniunprint.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme source</th>
<th>Enzyme preparations</th>
<th>Species of substrate [3H]-Apocytochrome c</th>
<th>Incorporation</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yeast</td>
<td>Mitochondria</td>
<td>Yeast</td>
<td>13.5*</td>
<td>22.6*</td>
</tr>
<tr>
<td>2</td>
<td>Yeast</td>
<td>Solubilized from mitochondria</td>
<td>Yeast</td>
<td>3.0</td>
<td>2.9</td>
</tr>
<tr>
<td>3</td>
<td>Yeast</td>
<td>Cell debris</td>
<td>Horse</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>Yeast</td>
<td>Solubilized from cell debris</td>
<td>Horse</td>
<td>&lt;0.07*</td>
<td>&lt;0.2*</td>
</tr>
<tr>
<td>5</td>
<td>Yeast</td>
<td>Mitochondria</td>
<td>Horse</td>
<td>4.6</td>
<td>6.5</td>
</tr>
<tr>
<td>6</td>
<td>Beef heart</td>
<td>Mitochondria</td>
<td>Horse</td>
<td>7.4*</td>
<td>2.7*</td>
</tr>
</tbody>
</table>

* Yeast cells were grown using 20-liter carboys instead of a fermenter.

The data from the previous work (3). Incubation: 20 min, 25 °C; solubilization with 0.5% sodium cholate instead of Triton X-100.

The data from the previous work (3). Incubation: 60 min, 30 °C.

To differentiate between yeast apo-iso-1-cytochrome c and horse apocytochrome c, cytochrome c synthetase I (solubilized from yeast mitochondria) would have to recognize an "extra" amino acid or amino acids. Assuming that the results obtained with the solubilized enzyme from yeast cell debris would be relevant, such an extra amino acid would be the one which is present in yeast, C. krueri, and tuna cytochromes c but not in horse cytochrome c. Looking at the amino acid sequence in proximity to the heme attachment site, one might consider threonine at position 18 (in numbering of horse cytochrome c) of yeast and such homology (e.g. lack of recognition of an extra amino acid or amino acids) would have been conserved for eucaryotic cells to synthesize not only cytochrome c but also cytochrome c. Then, if this hypothesis is correct, one would predict that cytochrome c might also axially ligate to heme at the active site of cytochrome c synthetase, serving as the second amino acid recognized by the enzyme.

Another exception is that cytochrome c55 of Desulfovibrio species contains four putative heme attachment sites, of which two are the sequence "Cys-X-Y-Cys-His" and the other two contain four intervening residues (instead of two, -X-Y) between cysteine residues (19). It is unknown whether both thioether bonds are enzymatically formed or the enzymatic formation of one of them is followed by nonenzymatic spontaneous formation of the other. Since protozoan cytochrome c containing only one thioether bond apparently folds to the "cytochrome c fold" (21), the enzymatic formation of a thioether bond between the vinyl side chain at position 4 of heme and cysteine at position 17 (in numbering of horse cytochrome c) could be sufficient to complete the information for such a folding. Then, this folding itself could direct the formation of a second thioether bond between the α-carbon atom of the vinyl side chain at position 2 of heme and cysteine at position 14 with the same sense of chirality (22) (with respect to the α-carbon atom) as that of the first thioether bond.

Acknowledgments—We thank Dr. Earl Stadtman for his critical reading of the manuscript. We also thank Laura Barry for her help in preparation of the manuscript.

REFERENCES

Two Cytochrome c Synthetases in Yeast


Additional references are found on p. 6137.
Two Cytochrome c Synthetases in Yeast

1637

Inhibitor Concentration Contraction

Preparation of partially purified cytochrome c synthetase and the particular fraction from yeast. The mitochondria of Saccharomyces cerevisiae were prepared according to previous procedures (1). The mitochondria were homogenized in 20 ml of 0.5 M sucrose solution containing 1 mM EDTA and 0.25 mM dithiothreitol. The homogenate was centrifuged at 10,000 g for 10 min and the supernatant was collected and diluted with 0.5 M sucrose solution. The cytochrome c synthetase was purified by isolation from the homogenate. The homogenate was centrifuged at 100,000 g for 60 min. The resulting supernatant was collected and dialyzed against 50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA. The purified enzyme was stored at 4°C.

Inhibitor Concentration Contraction

Preparation of partially purified cytochrome c synthetase and the particular fraction from yeast. The mitochondria of Saccharomyces cerevisiae were prepared according to previous procedures (1). The mitochondria were homogenized in 20 ml of 0.5 M sucrose solution containing 1 mM EDTA and 0.25 mM dithiothreitol. The homogenate was centrifuged at 10,000 g for 10 min and the supernatant was collected and diluted with 0.5 M sucrose solution. The cytochrome c synthetase was purified by isolation from the homogenate. The homogenate was centrifuged at 100,000 g for 60 min. The resulting supernatant was collected and dialyzed against 50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA. The purified enzyme was stored at 4°C.

Inhibitor Concentration Contraction

Preparation of partially purified cytochrome c synthetase and the particular fraction from yeast. The mitochondria of Saccharomyces cerevisiae were prepared according to previous procedures (1). The mitochondria were homogenized in 20 ml of 0.5 M sucrose solution containing 1 mM EDTA and 0.25 mM dithiothreitol. The homogenate was centrifuged at 10,000 g for 10 min and the supernatant was collected and diluted with 0.5 M sucrose solution. The cytochrome c synthetase was purified by isolation from the homogenate. The homogenate was centrifuged at 100,000 g for 60 min. The resulting supernatant was collected and dialyzed against 50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA. The purified enzyme was stored at 4°C.

Inhibitor Concentration Contraction

Preparation of partially purified cytochrome c synthetase and the particular fraction from yeast. The mitochondria of Saccharomyces cerevisiae were prepared according to previous procedures (1). The mitochondria were homogenized in 20 ml of 0.5 M sucrose solution containing 1 mM EDTA and 0.25 mM dithiothreitol. The homogenate was centrifuged at 10,000 g for 10 min and the supernatant was collected and diluted with 0.5 M sucrose solution. The cytochrome c synthetase was purified by isolation from the homogenate. The homogenate was centrifuged at 100,000 g for 60 min. The resulting supernatant was collected and dialyzed against 50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA. The purified enzyme was stored at 4°C.

Inhibitor Concentration Contraction

Preparation of partially purified cytochrome c synthetase and the particular fraction from yeast. The mitochondria of Saccharomyces cerevisiae were prepared according to previous procedures (1). The mitochondria were homogenized in 20 ml of 0.5 M sucrose solution containing 1 mM EDTA and 0.25 mM dithiothreitol. The homogenate was centrifuged at 10,000 g for 10 min and the supernatant was collected and diluted with 0.5 M sucrose solution. The cytochrome c synthetase was purified by isolation from the homogenate. The homogenate was centrifuged at 100,000 g for 60 min. The resulting supernatant was collected and dialyzed against 50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA. The purified enzyme was stored at 4°C.

Inhibitor Concentration Contraction

Preparation of partially purified cytochrome c synthetase and the particular fraction from yeast. The mitochondria of Saccharomyces cerevisiae were prepared according to previous procedures (1). The mitochondria were homogenized in 20 ml of 0.5 M sucrose solution containing 1 mM EDTA and 0.25 mM dithiothreitol. The homogenate was centrifuged at 10,000 g for 10 min and the supernatant was collected and diluted with 0.5 M sucrose solution. The cytochrome c synthetase was purified by isolation from the homogenate. The homogenate was centrifuged at 100,000 g for 60 min. The resulting supernatant was collected and dialyzed against 50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA. The purified enzyme was stored at 4°C.

Inhibitor Concentration Contraction

Preparation of partially purified cytochrome c synthetase and the particular fraction from yeast. The mitochondria of Saccharomyces cerevisiae were prepared according to previous procedures (1). The mitochondria were homogenized in 20 ml of 0.5 M sucrose solution containing 1 mM EDTA and 0.25 mM dithiothreitol. The homogenate was centrifuged at 10,000 g for 10 min and the supernatant was collected and diluted with 0.5 M sucrose solution. The cytochrome c synthetase was purified by isolation from the homogenate. The homogenate was centrifuged at 100,000 g for 60 min. The resulting supernatant was collected and dialyzed against 50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA. The purified enzyme was stored at 4°C.

Inhibitor Concentration Contraction

Preparation of partially purified cytochrome c synthetase and the particular fraction from yeast. The mitochondria of Saccharomyces cerevisiae were prepared according to previous procedures (1). The mitochondria were homogenized in 20 ml of 0.5 M sucrose solution containing 1 mM EDTA and 0.25 mM dithiothreitol. The homogenate was centrifuged at 10,000 g for 10 min and the supernatant was collected and diluted with 0.5 M sucrose solution. The cytochrome c synthetase was purified by isolation from the homogenate. The homogenate was centrifuged at 100,000 g for 60 min. The resulting supernatant was collected and dialyzed against 50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA. The purified enzyme was stored at 4°C.
Two Cytochrome c Synthetases in Yeast

Table 11
Fractionation by Ion-Exchange Chromatography of Partially Purified Cytochrome c Synthetase

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Partially Purified Cytochrome c Synthetase</th>
<th>Chromatographic Fractions</th>
<th>Total Protein Activity (units)</th>
<th>Specific Activity (activity/mg protein)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>From cell DEAE-Sephacel</td>
<td>Diaion 2</td>
<td>120</td>
<td>2.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>solution</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>From cell DEAE-Sephacel</td>
<td>Diaion 2</td>
<td>100</td>
<td>4.2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>solution</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>From microcarrier</td>
<td>Diaion 2</td>
<td>210</td>
<td>14.6</td>
<td>100</td>
</tr>
</tbody>
</table>

*The samples before ion-exchange chromatography were passed through the ion-exchange column without adsorption
*Deviations are within 2% of the mean activity

Figure 1: Effects of Concentrations of Non- labeled Horse Cytochrome c and yeast Cytochrome c Nonsynthetase on the Activity of Non-labeled Horse Cytochrome c Synthetase. The activity of horse cytochrome c synthetase was determined in the assay mixture containing non-labeled horse cytochrome c and yeast cytochrome c nonsynthetase (100 units) and a substrate of cytochrome c synthetase (100 units). The enzyme concentration refers to the concentration of non-labeled cytochrome c. The assay procedure is described in the manuscript.

Figure 2: Effect of Concentrations of Non-labeled Horse Cytochrome c and yeast Cytochrome c Nonsynthetase on the Activity of Non-labeled Horse Cytochrome c Synthetase. The activity of horse cytochrome c synthetase was determined in the assay mixture containing non-labeled horse cytochrome c and yeast cytochrome c nonsynthetase (100 units) and a substrate of cytochrome c synthetase (100 units). The enzyme concentration refers to the concentration of non-labeled cytochrome c. The assay procedure is described in the manuscript.

Figure 3: pH Stability of Cytochrome c Synthetase. Cytochrome c synthetase from yeast cell debris was incubated at the indicated pH in 0.05 M Tris-Cl buffer for 24 hours at 35°C. The pH stability was determined by measuring the activity as described in the manuscript.

Figure 4: Effect of Purification on the Formation of Radiolabeled Cytochrome c. Partially purified cytochrome c synthetase was incubated overnight in the presence of [14C]phenylalanine and [14C]tyrosine. The formation of radiolabeled cytochrome c was determined by measuring the radioactivity as described in the manuscript.

Figure 5: Effect of Purification on the Formation of Radiolabeled Cytochrome c. Partially purified cytochrome c synthetase was incubated overnight in the presence of [14C]phenylalanine and [14C]tyrosine. The formation of radiolabeled cytochrome c was determined by measuring the radioactivity as described in the manuscript.

Figure 6: Effect of Purification on the Formation of Radiolabeled Cytochrome c. Partially purified cytochrome c synthetase was incubated overnight in the presence of [14C]phenylalanine and [14C]tyrosine. The formation of radiolabeled cytochrome c was determined by measuring the radioactivity as described in the manuscript.