Evidence for Formation of Two Thioether Bonds to Link Heme to Apocytochrome c by Partially Purified Cytochrome c Synthetase*

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(Received for publication, January 10, 1983)

Cytochrome c synthetase has been solubilized from yeast mitochondria using Triton X-100 and fractionated with ammonium sulfate. Use of this partially purified enzyme has permitted us to isolate a quantity of iso-1-cytochrome c from 125I-labeled apocytochrome c and hemin in the presence of a NADPH-generating system. Visible absorption spectra (pH 8.0 or 5.0) including α, β, and Soret bands and their molar absorption coefficients of this enzymatically synthesized cytochrome c in the oxidized and reduced states are the same, within experimental error, as those of native cytochrome c. Pyridine ferrohemochrome (pH 13) of the synthesized species also exhibits the same α and β bands as those of iso-1-cytochrome c and similar to those reported for heme peptides of cytochrome c. If only one or no thioether bond was formed between the two vinyl side groups of heme and the cysteine residues of apocytochrome c, all these α and β bands would have shifted to red (Pettigrew, G. W., Leaver, J. L., Meyer, T. E., and Ryle, T. E. (1975) Biochem J. 147, 291-302). Thus, two thioether bonds appear to be formed to link heme to apocytochrome c by cytochrome c synthetase, completing information of the three-dimensional structure of cytochrome c.

Cytochrome c synthetase present in mitochondria catalyzes covalent bonding between hemin (or heme) and apocytochrome c in the presence of NADPH (or NADH) to form cytochrome c or cytochrome c-like species (1-3).

A heme moiety of native cytochrome c is linked through two thioether bonds to the polypeptide chain (cf. Ref. 4). It is yet to be established, however, whether enzymatically synthesized cytochrome c contains two thioether bonds or only one of them to link heme. This information is necessary to assess the biological significance of cytochrome c synthetase and also precisely define the enzymatic reaction.

It is well known that cytochrome c, particularly the ferrous form, exhibits characteristic absorption at 550 and 521 nm (α and β bands, respectively) (cf. Ref. 5). On the other hand, α and β bands of proteins containing noncovalently bound heme (6) and those of some protozoan cytochrome c are all shifted to red (7, 8). This is also true with pyridine ferrohemochromes of these proteins (9). The origin of such red shift has been attributed to effects of unsaturated vinyl side groups at positions 2 and 4 of the porphyrin ring on the wavelength of absorption (10, 11). In the case of protozoan cytochrome c, one of the two vinyl side groups remains unsaturated, i.e.

these cytochromes contain only one thioether bond to link a heme moiety to apocytochrome c (7-9). Thus, measurement of visible absorption spectra is a sensitive method in evaluating the involvement of the vinyl side groups in linkage to apocytochrome c.

Preparation of enzymatically synthesized cytochrome c in a quantity required for examination of visible absorption spectra has been hampered since the activity of cytochrome c synthetase bound with intact mitochondria is very low (1-3). In order to overcome this problem, we have for the first time solubilized and concentrated the enzyme. Using this partially purified enzyme we have been able to isolate the synthesized cytochrome c in a quantity and purity sufficient for spectrophotometric studies. A preliminary account of this work has appeared (12).

MATERIALS AND METHODS

RESULTS AND DISCUSSION

Triton X-100 (0.5 to 0.7%) is found to be effective in solubilizing cytochrome c synthetase (up to 64%) from mitochondria of Saccharomyces cerevisiae (ATCC 24883). The solubilized enzyme was fractionated by 40% saturation with ammonium sulfate. The activity of such partially purified enzyme is almost absolutely dependent on hemin (apparent saturation with 0.1 mM hemin). A NADPH-generating system (NADPH, isocitrate, and isocitric dehydrogenase) has also increased the activity (see Miniprint). The partially purified enzyme is heat-labile and also apparently saturated with 8 µM apoiso-1-cytochrome c.

Using optimal conditions thus found, preparation of enzymatically synthesized iso-1-cytochrome c from 125I-labeled apoprotein and hemin was carried out (see Miniprint). The synthesized iso-1-cytochrome c (9.5 nmol) thus isolated exhibited absorption spectra from 390 to 580 nm (pH 8.0) indistinguishable from the native protein in either the oxidized (Soret band and a maximum at 528 nm) or the reduced (Soret, α, and β bands) form (see Miniprint, Figs. 4 and 5) (5) (an estimated maximum contamination from endogenous cytochrome c.

1 Portions of this paper (including "Materials and Methods," part of "Results," Tables I and II, Figs. 1 to 6, and additional references) 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, 360 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M57, cite the authors, and include a check or money order for $6.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 Information about the effect of hemin and the concentration of apoiso-1-cytochrome c has been obtained using the partially purified enzyme from the cell debris, which is assumed to be the enzyme of mitochondria contaminating the cell debris (see Miniprint).

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chrome c, 4%). The ratio, 1.2) of absorbance of the Soret band of the reduced to the oxidized state also agrees with that reported (5). The specific radioactivity of the synthesized cytochrome c was found to be 8.6 ± 0.9 × 10^6 cpm/nmol on the basis of absorbance at 409 nm (Soret band of the oxidized form). This value is close to 7.7 ± 1.5 × 10^6 cpm/nmol (corrected for decay) of the original apocytochrome c, indicating stoichiometric incorporation of hemin.

After treatment of the sample with trypsin to remove contaminating labeled apocytochrome c, if any, the values for ε of Soret (416 nm), α (550 nm), and β (520.5 nm) bands (the reduced state, pH 5.0) were equal to 1.56 ± 0.3 × 10^5, 2.8 ± 0.5 × 10^4, and 1.5 ± 0.3 × 10^4 M⁻¹cm⁻¹, respectively (see Miniprint, Fig. 5), which are, within experimental error, consistent with those reported for horse cytochrome c (5). The value (1.46 ± 0.3 × 10^5 M⁻¹cm⁻¹) for ε (416 nm) and ε (460 nm) also agrees, within experimental error, with that (1.23 × 10^5 M⁻¹cm⁻¹) reported for yeast cytochrome c (13).

Pyridine ferrohemochrome of the synthesized cytochrome c (a denatured state, pH 13) also exhibited α and β bands at 550 and 520.5 nm, respectively, in agreement with standard iso-1-cytochrome c (see Miniprint, Fig. 6), or heme fragments of cytochrome c (14) (ε (550 nm), 3.8 ± 0.9 × 10^4 M⁻¹cm⁻¹ of the synthesized species agreeing, within experimental error, with that (2.9 × 10^4 M⁻¹cm⁻¹) reported (5)). On the other hand, α and β bands of pyridine ferrohemochrome of free heme or myoglobin are shifted to 557 and 524 nm, respectively (see Miniprint, Fig. 6). These results indicate that the synthesized cytochrome c contains, in a significant extent, neither noncovalently bound heme nor a heme moiety in which only one of the two vinyl side groups is used for covalent bonding and the other remains unsaturated (9).

The previous studies including acetone HCl and silver sulfate treatments have supported the idea that the action of cytochrome c synthetase links heme through thioether bond(s) to the apoprotein (1, 2). The present studies provide evidence for the concept that actually two thioether bonds are formed by the action of this enzyme to complete information of the three-dimensional structure of cytochrome c (cf. Ref. 1).

Availability of solubilized cytochrome c synthetase opens a new way for attachment of heme in chemical synthesis of heme fragments (15) or cytochrome c. Studies of stereochimistry of the two thioether bonds thus formed would shed light on the origin and significance of the stereochemical specificity involved in these bonds of native cytochrome c (1). Solubilization of cytochrome c synthetase may also be useful for studies of transportation of cytochrome c across mitochondrial membranes (3, 16) or investigation of gene of cytochrome c synthetase (17).

Acknowledgments—We acknowledge the excellent technical assistance of Linda Jackson who has grown yeast cells and prepared mitochondria and the ammonium sulfate fraction from the cell debris. We are indebted to Barbara S. Berlett for her thoughtful help in the fermentation. We also thank Dorothy Stewart and Laura Barry for their help in preparation of the manuscript.

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Additional references are found on p. 10965.
Solubilization of Cytochrome c Synthetase

RESULTS

Partial Purification and Characterization of Cytochrome c Synthetase—Preliminary experiments (II) have indicated that the 0.5 to 0.78 Tris buffer (pH 7.5) are effective in solubilizing cytochrome c synthetase from tobacco leaves. However, these experiments did not indicate the presence of any material that might be considered to be cytochrome c synthetase. Therefore, the experiments were repeated using the 0.5 to 0.78 Tris buffer (pH 7.5) and the material that was solubilized was fractionated into 500,000 g supernatant, membrane, and mitochondrial fractions. The membrane fractions were subjected to a series of solubilization experiments (see Table 1), and the results are summarized in Table 1. The fractions that were obtained by the solubilization experiments are indicated in Table 1.

The activity of cytochrome c synthetase of intact mitochondria was previously found to decrease upon addition of detergent to the reaction mixture. The activity of the synthetase was lost almost entirely when the membrane fraction was treated with 0.5 M NaCl. The activity of cytochrome c synthetase in the membrane fraction was not significantly increased upon addition of NaCl alone or a combination of NaCl and Triton X-100. However, the addition of Triton X-100 alone to the reaction mixture did not significantly increase the activity of the synthetase. The results of these experiments are summarized in Table 1. The results of these experiments are summarized in Table 1.



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Solubilization of Cytochrome c Synthetase

TABLE I

| Experiment | Solubilization c | Ammonium Sulfate Fraction | Incubation Time | Solubilization c |
|------------|------------------|---------------------------|-----------------|------------------|
| 1. Mitochondria | 0-60 min | 10 min | 1.7 (85) | 0.107 |
| 2. The cell debris | 0-60 min | 10 min | 0.62 (26) | 0.12 |

TABLE II

| Solubilization and Ammonium Sulfate Fraction of Cytochrome c Synthetase |
|--------------------------------------------------------------------------|
| In experiment 1 the ammonium sulfate fractions were prepared from mitochondria as the basis of the procedure described under MATERIALS AND METHODS. The ammonium sulfate fraction was prepared by the cell debris (see MATERIALS AND METHODS). The specific enzymatic activity was measured according to the method described under MATERIALS AND METHODS with the exception as indicated. The specific enzymatic activities were expressed, in each experiment, as a mole of substrate formed per mg protein at 37°C during the specified incubation time. |

Solubilization of Cytochrome c Synthetase

The enzymatic activity was measured according to the method described under MATERIALS AND METHODS. The ammonium sulfate fraction was prepared from mitochondria (see MATERIALS AND METHODS). The specific enzymatic activity was measured according to the method described under MATERIALS AND METHODS. The specific enzymatic activity was expressed, in each experiment, as a mole of substrate formed per mg protein at 37°C during the specified incubation time. The specific enzymatic activity was expressed, in each experiment, as a mole of substrate formed per mg protein at 37°C during the specified incubation time. The specific enzymatic activity was expressed, in each experiment, as a mole of substrate formed per mg protein at 37°C during the specified incubation time.
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J. Biol. Chem. 1983, 258:10963-10966.

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