JOURNAL OF BACTERIOLOGY, Dec. 1968, p. 1997-2003
Vol. 96, No. 6

Coproporphyrinogenase in a Respiration-deficient Mutant of Yeast Lacking All Cytochromes and Accumulating Coproporphyrin

SETSUKO MIYAKE AND TAKASHI SUGIMURA

Biochemistry Division, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan

Received for publication 30 September 1968

In an earlier report, a respiration-deficient mutant of yeast which lacks all cytochromes and hemoproteins and accumulates coproporphyrin was described. This respiration-deficient mutant was temperature-sensitive and resulted from the single chromosomal gene(*cyt*). In this study, the activity of coproporphyrinogenase, catalyzing the conversion of coproporphyrinogen to protoporphyrinogen, was assayed in the *cyt* mutant and wild strains. Coproporphyrinogenase activity was 10 times higher in the *cyt* mutant than in the wild strains. Cells of the *cyt* mutant grown at 20 C had less activity than those grown at 35 C. The Michaelis constants, *p*H optima, and temperature activations of the enzymes of the *cyt* mutant and the wild strains were similar. The significance of the higher activity of this enzyme in the *cyt* mutant, in which this enzymatic step is apparently blocked in vivo, is discussed.

In previous papers (12, 13), we described a new type of respiration-deficient mutant of yeast (5b-1), *Saccharomyces cerevisiae*, which lacks all cytochromes. The absorption spectrum of a whole-cell suspension of this mutant strain, grown aerobically, showed two dominant peaks at 538 and 575 nm in the cytochrome region. The substance showing this absorption was extracted with a mixture of acetic acid and ethylacetate, transferred to HCl solution and identified as coproporphyrin III.

In cells, coproporphyrin existed possibly as zinc chelates and the HCl treatment released free coproporphyrin (5). The cells of this strain also lack other hemoproteins, catalase, and L-lactic dehydrogenase. Genetic analysis showed that the phenotypes of this strain resulted from a single chromosomal gene mutation (CYT to *cyt*), and many segregants were isolated (2; T. Sugimura et al., 7th Intern. Congr. Biochem, Tokyo, p. 959, 1967). The phenotypic expression of this mutation was temperature-sensitive. Thus, when grown at 35 C, the mutant phenotype appeared; when grown at 20 C, the wild phenotype was seen (2). There was a relatively poor yield of this mutant strain when grown aerobically at 35 C. Cells grown at 35 C in culture media supplemented with either protoporphyrin IX or protohemin IX showed respiratory activities and normal cytochrome patterns. Thus, the enzymes or proteins involved in the heme-synthesizing pathway after the synthesis of coproporphyrin seem to be temperature-sensitive in this mutant.

Many types of respiratory-deficient mutants of yeast with various cytochrome patterns have been described; some of them lacked cytochromes *a* and *b* but had cytochrome *c*, whereas others had cytochromes *a* and *b* but lacked cytochrome *c* (8–10). Pretlow and Sherman described mutants which accumulated porphyrin and their zinc chelates but had all the cytochromes (5). Our mutant (*cyt*) had no cytochromes (12). Yčas and Starr (15) also isolated a respiration-deficient mutant of yeast, induced by ultraviolet irradiation, which lacked all of the cytochromes. Their strain was found to have a defect in the pathway of synthesis of glycine, a porphyrin precursor, and grew normally in synthetic medium supplemented with protoporphyrin IX or glycine, but it was definitely different from the *cyt* mutant.

We studied the nature of the enzymatic lesion in this mutant of yeast (*cyt*). Results show that *cyt* mutant cells had coproporphyrinogenase activity under all growth conditions tested and that the activity of this enzyme in *cyt* mutant cells was considerably higher than in wild cells. The characteristics of this enzyme in *cyt* mutant cells did not differ from those of the enzyme in the wild strain. The significance of these results with respect to the function of this enzyme in vivo is discussed. This is the first report of the presence of coproporphyrinogenase in yeast.

MATERIALS AND METHODS

Strains. The respiration-deficient mutant strain accumulating coproporphyrin III used was a diploid

strain Z-1 (*cyt/cyt*) produced by the cross $R_3E5C \times R_3H4C$-1. The latter were haploid strains of respiration-deficient mutants, *cyt*, accumulating coproporphyrin III (2). The normal strain used was a cross of the normal haploid yeast strains ($R_2O1B \times R_3A1B$-2). For reference, a cytoplasmic respiration-deficient mutant (ρ^-) induced from the wild strain R_2Q2B by acriflavine was used.

Growth conditions. The medium for aerobic culture consisted of: Proteose Peptone No. 3 (Difco), 20 g; yeast extract (Difco), 10 g; glucose, 20 g; and water, 1,000 ml. Aeration was carried out by shaking culture flasks. For anaerobic culture, the medium described by Hebb and Slebodnik (3) was used. Anaerobic conditions were attained by filling up the bottles to the neck with medium. Cells were cultured under various conditions, harvested by centrifugation at the end of the logarithmic phase, and washed twice with 0.9% NaCl solution.

Enzyme preparation and assay. Washed cells were rapidly ground in the cold in a mortar in the presence of quartz sand [4 g of quartz sand, 200 mesh, per g (wet weight) of yeast] and suspended in 0.1 M tris-(hydroxymethyl)aminomethane (Tris)-chloride buffer (*p*H 7.4). After centrifugation for 10 min at $1,000 \times g$, the supernatant fluid was obtained as an enzyme extract. The activity of coproporphyrinogenase (coproporphyrinogen decarboxylase, oxidative) was measured by a slight modification of the method of Sano and Granick (7). The enzyme assay system contained: enzyme extract, 10 to 50 mg of protein; about 100 nmoles of coproporphyrinogen III; and 0.4 mmole of Tris-chloride buffer (*p*H 7.4), in a total volume of 4.0 ml. Coproporphyrin III was extracted from the filtrate of a culture of *Corynebacterium diphtheriae*, kindly supplied by H. Saito, Kitasato University, Tokyo. Coproporphyrin III, in 0.01 N KOH, was reduced to coproporphyrinogen III with 3% sodium amalgam; the mixture was bubbled with nitrogen gas, and the *p*H of the solution was adjusted to about 9.0 with dilute phosphoric acid.

Incubation was performed in the dark with shaking. Under these assay conditions, protoporphyrin IX formation was proprotional to the amount of enzyme extract and to the reaction time for at least 1 hr. After incubation for 40 min, extraction and determination of porphyrins were performed as described by Sano and Granick (7). Potassium thioglycollate and sodium diethyl dithiocarbamate were omitted from the incubation mixture in the present experiments. As an oxidizing agent, quinhydrone solution (1 μmole/ml) was added to the incubation medium at the rate of 0.25 ml/ml.

Paper chromatography. Coproporphyrin III, protoporphyrin IX, and the reaction products were developed by ascending paper chromatography, by using a solvent system of 2,6-lutidine-water (5:3, v/v) under an atmosphere of ammonia vapor.

Other chemicals. Protoporphyrin IX and protohemin IX were purchased from Sigma Chemical Co., St. Louis, Mo.

Spectra of whole cell suspensions. The absorption spectra of cell suspensions were taken with a Cary recording spectrophotometer, model 15, with an opalescent glass plate (11).

RESULTS

Absorption spectra of whole cell suspensions. The absorption spectrum of a whole cell suspension of the wild type strain ($R_2O1B \times R_3A1B$-2) showed maxima at 603, 563, and 550 nm, corresponding to cytochromes *a*, *b* and *c*, respectively (Fig. 1). Cytoplasmic respiration-deficient mutant cells (R_2Q2B, *CYT*, ρ^-) showed only the absorption of cytochrome *c*. Neither the absorption profile of the wild strain nor the ρ^- RD mutant strain was altered by changing the culture temperature from 20 to 35 C. Cells of *cyt* mutant (Z-1) grown at 35 C were completely devoid of cytochromes *a*, *b*, and *c*, and accumulated a considerable amount of coproporphyrin as represented by the absorptions at 538 and 575 nm. Cells of *cyt* mutant (Z-1) grown at 20 C definitely had cytochromes *a*, *b*, and *c*, but did not accumulate porphyrin.

Oxygen uptake by cells grown at various temperatures. Oxygen uptake of wild cells grown at 20, 25, 30, and 35 C and *cyt* mutant cells (Z-1) grown at 20 C was similar, but that of *cyt* mutant cells grown at 35 C was very low (Table 1). These findings on oxygen uptake are in accordance with those on the cytochrome patterns described above.

Coproporphyrinogenase activities in the cyt mutant (Z-1) and wild strains. Enzyme extracts of *cyt* mutant (Z-1) cells grown at 20 or 35 C exhibited coproporphyrinogenase activity (Table 2). The activity of Z-1 cells grown at 35 C was much higher than that of the wild strain. The coproporphyrinogenase activities of other wild strains and of *cyt* mutant strains (Table 3) indicate the presence of this enzyme activity in many segregants of the *cyt* mutant.

Localization of coproporphyrinogenase. The localization of this enzyme in various cellular fractions was investigated after centrifugation of the enzyme extract. Most of the enzyme activity was found in the supernatant fraction after centrifugation at $105,000 \times g$ for 60 min (Table 4). Similar distribution patterns of enzyme activity were found in the *cyt* mutant (Z-1) and in wild strains.

Properties of coproporphyrinogenase of cyt mutant (Z-1) and wild strains. The results indicate that synthesis of coproporphyrinogenase is not impaired in the *cyt* strain (Z-1). *Cyt* mutant cells (Z-1) accumulated coproporphyrin III and apparently did not synthesize heme in vivo, although coproporphyrinogenase activity was found on assay in vitro. It is not clear whether the enzyme observed in vitro actually functions in vivo. Accordingly, the enzymatic properties of the coproporphyrinogenase produced by the *cyt*

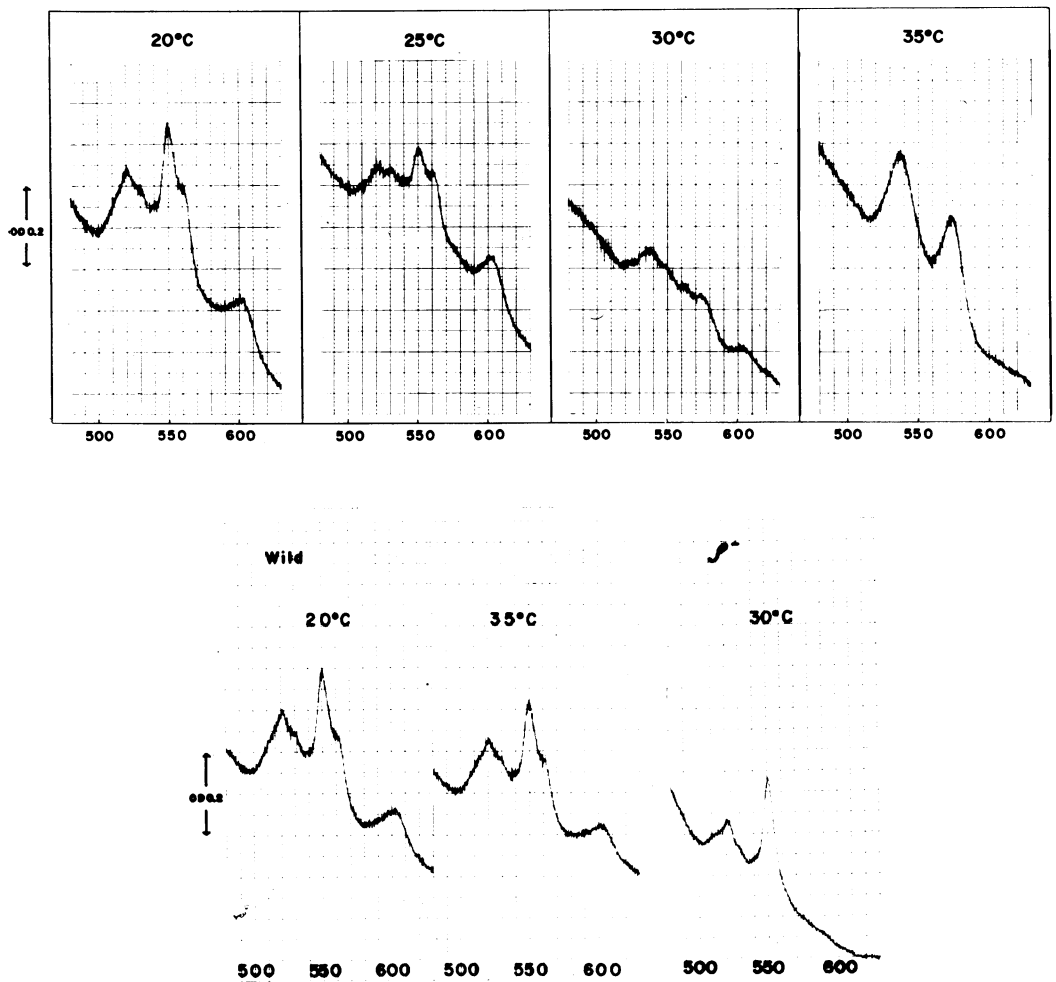

FIG. 1. *Absorption spectrum of cell suspensions of cyt mutant Z-1 and wild and ρ⁻RD mutants, grown aerobically at various temperatures.*

mutant (Z-1) were compared with those of wild strains.

The effects of *p*H on the enzyme activities of extracts from the *cyt* mutant (Z-1) and wild strains, under various culture conditions, are shown in Fig. 2. In all the preparations, the *p*H optimum was between 7.0 and 7.5, without any sharp increase in activity on either side of the optimum. These preparations had Michaelis constants (K_m values) of 1.2×10^{-5} M to 1.5×10^{-5} M for coproporphyrinogen III (Fig. 3), but these differences are not considered significant, since the enzyme preparations were crude.

The enzyme activities of preparations from both the wild type and the *cyt* mutant (Z-1) increased on raising the temperature from 25 to 38 C. This is illustrated by Arrhenius plots in Fig. 4, indicating the same value for the activation energy of the enzymes of the two strains. The enzyme from *cyt* mutant (Z-1) cells at elevated temperature did not seem to be more thermolabile.

The effects of various reagents on the enzyme activities of these extracts were investigated but, of five compounds tested, only *p*-chloromercuribenzene sulfonate was found to have an effect. It depressed enzyme activity by about 40% at a concentration of 10^{-2} M (Table 5). Its inhibitory effect was similar on extracts from the *cyt* mutant (Z-1) and wild strains. Mercaptoethanol, ethylenediaminetetraacetic acid, α,α'-dipyridyl, and *o*-phenanthroline had no effect, even at high concentrations of 10^{-2} M, and tended to stimulate enzyme activity slightly. α,α'-Dipyridyl and *o*-phenanthroline have been reported to be strong inhibitors of this enzyme in mammalian mitochondria (7).

Enzyme activities of various cyt mutants and wild strains, grown under aerobic and anaerobic

TABLE 1. *Oxygen uptake by cyt mutant and wild strains grown at various temperatures*

Strain	Temperature of growth	Oxygen uptake[a]
	C	
Z-1	20	16.4
	25	14.1
	30	10.1
	35	0.752
Wild	20	17.6
	25	23.1
	30	24.8
	35	22.7

[a] Microliters of O_2 per milligram (wet weight) per minute. Oxygen uptake was determined polarographically at 30 C in 0.02 M phosphate buffer (*p*H 5.7) containing 1% glucose.

TABLE 2. *Coproporphyrinogenase activities of cyt mutant and wild strains grown at 20 C and 35 C*

Strain	Activity[a] when grown at	
	20 C	35 C
Wild	8.1	6.8
Z-1	11.4	80.1

[a] Expressed in micromicromoles of protoporphyrin IX formed per minute per milligram of protein of enzyme extract.

TABLE 3. *Coproporphyrinogenase activities of several cyt and wild strains*

Strain	Activity[b]
Wild[a]	
R_3T1A	11.9
R_3T1B	7.2
R_2Q2B	8.3
R_2Q4A	8.9
R_2Q6B	7.7
R_2O1B	8.1
cyt Mutants	
Z-2($R_3E5D \times R_3H4C$-2)	54.8
Z-3($R_3E5D \times R_3H4D$-1)	32.7
Z-4($R_3E5D \times R_3H4D$-2)	74.1
R_4O29B	66.1
R_4C4A	40.4
R_4M4C	32.3

[a] The wild strains used in this experiment were haploids; the mutant strains were diploids and haploids. All were grown at 35 C.

[b] Expressed as in Table 2.

conditions. Since the reaction of coproporphyrinogenase requires oxygen, it is interesting to determine the amount of this enzyme in *cyt* mutant (Z-1) and wild cells when grown anaerobically. The enzyme activity of wild-type cells, grown anaerobically at 20 or 35 C, was as high as that of Z-1, grown aerobically at 35 C, and similar to that of Z-1 cells grown anaerobically (Table 6). The cytoplasmic respiration-deficient mutant (ρ^-) showed the same enzyme activity as the wild strain at various culture temperatures.

TABLE 4. *Intracellular distribution of coproporphyrinogenase in cyt and wild strains*

Fraction[a]	Z-1 (35 C)		Wild (35 C)	
	Total activity[b]	Total protein	Total activity[b]	Total protein
		mg		*mg*
Crude enzyme extract	825	319	40.2	329
Supernatant fluid	607	148	45.6	159
Particulate fraction	57.7	145	9.6	157

[a] Enzyme extract, obtained by centrifugation at 1,000 × *g* for 10 min, was centrifuged at 105,000 × *g* for 60 min. Particulate fractions were resuspended in an appropriate volume of 0.1 M Tris-chloride buffer (*p*H 7.4). Data obtained with 5 g (wet weight) of cells.

[b] Expressed as millimicromoles of protoporphyrin IX formed per 40 min.

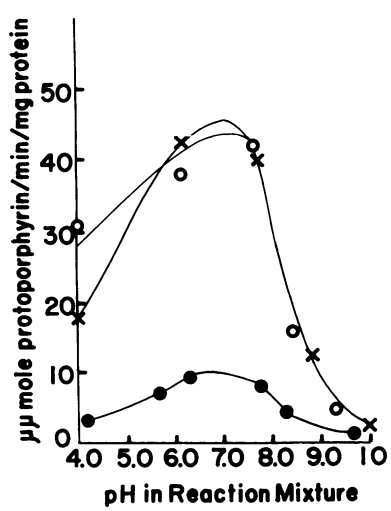

FIG. 2. *pH and activity-curves of coproporphyrinogenase from cyt mutant and wild strains. Symbols:* ○, *Z-1 cells grown at 35 C;* ×, *Z-1 cells grown at 25 C;* ●, *wild cells grown at 35 C.*

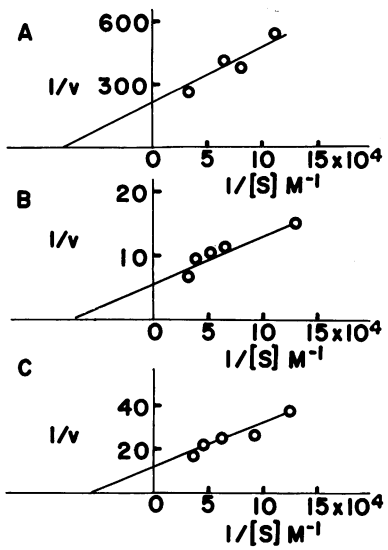

FIG. 3. *K_m values of coproporphyrinogenase from cyt mutant and wild strains. (A) Wild cells grown at 35 C (K_m = 1.18 × 10^{-5} M); (B) Z-1 cells grown at 35 C (K_m = 1.24 × 10^{-5} M); (C) Z-1 cells grown at 30 C (K_m = 1.52 × 10^{-5} M).*

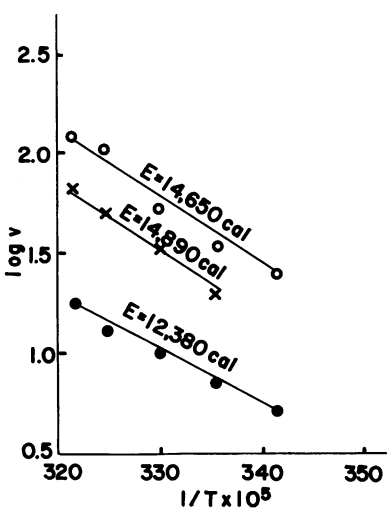

FIG. 4. *Effects of temperature on coproporphyrinogenase from cyt mutant and wild strains. Symbols: ○, Z-1 cells grown at 35 C; ×, Z-1 cells grown at 25 C; ●, wild cells grown at 35 C.*

Identification of the reaction products. The reaction mixture was extracted with 0.1 or 1.5 N HCl, and products were transferred to ethyl ether after adjusting the *p*H to 3.5. They were developed by paper chromatography with a mixture of 2,6-lutidine and water under ammonia vapor. The 0.1 N HCl extract contained coproporphyrin III as the main component; the 1.5 N HCl extract contained protoporphyrin IX as the main component (Table 7). Both extracts contained a minor spot travelling between coproporphyrin III and protoporphyrin IX. This was considered to be an intermediate of the reaction. These results show that no cross-contamination of the two extracts occurred.

DISCUSSION

The new type of respiration-deficient mutant yeast, cyt (Z-1), grows normally at 20 C but lacks all cytochromes and accumulates coproporphyrin III when grown at 35 C. The nature of its suspected enzymatic lesion was investigated. The enzyme coproporphyrinogenase, which converts coproporphyrinogen III to protoporphyrinogen IX, was definitely present in *cyt* (Z-1) when cultured either at 20 or 35 C. Moreover, when the

TABLE 5. *Effect of reagents on coproporphyrinogenase in extracts of cyt mutant and wild strains*

Reagent	Final concn	Activity (%)	
		Z-1	Wild type
	mM		
p-Chloromercuribenzenesulfonate..	1	92	
p-Chloromercuribenzenesulfonate..	10	66	61
Mercaptoethanol....	50	100	100
Ethylenediaminetetraacetate (disodium).....	20	95	114
α,α′-Dipyridyl	10	117	134
o-Phenanthroline....	5	107	111

TABLE 6. *Coproporphyrinogenase activities in cyt mutant and wild strains grown anaerobically*

Strain	Temperature at growth	Enzyme activity[a]
	C	
Z-1	20	214
	35	50.6
Wild	20	204.5
	35	125.9
ρ⁻RD strain	20	226
	35	13.7[b]

[a] Expressed as in Table 2.
[b] Aerobic growth. All others were grown anaerobically.

TABLE 7. *Paper chromatography of coproporphyrin, protoporphyrin, and reaction products*

Sample	R_F values of	
	Main spot	Minor spot
Coproporphyrin III	0.25	
Protoporphyrin IX	0.69	
Reaction product (0.1 N HCl extract)	0.25	0.43
Reaction product (1.5 N HCl extract)	0.70	0.43

cyt mutant was grown at 35 C, its enzyme activity was 10 times that of the wild-type strain. Enzyme preparations from *cyt* mutant (Z-1) and wild cells grown at 35 C showed similar enzymatic properties—they had a similar K_m value and optimal *p*H and similar sensitivities to various reagents. The enzyme activities of extracts from the *cyt* mutant (Z-1) and wild strains assayed at various temperatures showed the same features, that is, a more temperature-sensitive enzyme was not present in the *cyt* mutant. These data indicate that *cyt* mutant (Z-1) cells produce normal wild-type coproporphyrinogenase. The higher enzyme activity in *cyt* mutant cells grown at elevated temperature may result from the absence of some end product, such as heme, which represses synthesis of this enzyme. The same situation was noticed in wild-type cells when grown anaerobically. It is concluded that the phenotype of the *cyt* mutant cannot be an enzymatic lesion at the site of coproporphyrinogenase.

Other possible explanations of why the coproporphyrinogenase in *cyt* mutant (Z-1) cells apparently does not function in vivo are as follows. (i) *Cyt* mutant (Z-1) cells may contain an inhibitor of the enzyme and the enzyme-inhibitor-formed complex may dissociate when the cells are destroyed. (ii) The localization of the enzyme protein in the mutant cells may be distorted (14) so that the enzyme cannot react with the substrate. This would represent a so-called organizational mutant. (iii) The substrate coproporphyrinogen III may be rapidly oxidized to coproporphyrin III in Z-1 cells, so that it is not available as substrate, or (iv) the *cyt* mutant (Z-1) cells may have a disturbance of iron uptake. Ferrous ion is required for heme production.

With regard to explanation (i), no evidence was obtained for the presence of an enzyme inhibitor from the present experiments, and the natural occurrence of an inhibitor of this enzyme has not been described in the literature with regard to explanation (ii). There was a similar distribution of this enzyme in the cellular fractions obtained by centrifugation of *cyt* mutant (Z-1) and wild-strain cells. Explanations (iii) and (iv) are possible. This enzyme requires coproporphyrinogen III as a substrate. If coproporphyrinogen III is rapidly oxidized in *cyt* mutant (Z-1) cells by some unknown mechanism, it cannot serve as a substrate. An abnormal oxidation-reduction potential at the site where coproporphyrinogen III molecules exist may cause oxidation of the substrate. It is also probable that iron ion was not taken up efficiently by *cyt* mutant. The addition of protoporphyrin to the culture medium may help the uptake of iron ion.

Rietmuller and Tuppy (6) reported on the existence of the so-called cheletase or insertion enzyme in yeast. The studies on this enzyme of *cyt* mutant are now under investigation in our laboratory.

ACKNOWLEDGMENTS

This investigation was supported by a grant from the Ministry of Education, Japan. We thank Norio Gunge, Shichiro Sato, and Seiyo Sano for useful discussion of this work.

LITERATURE CITED

1. Erilksen, L. 1953. Paper chromatography of porphyrin pigments. Scand. J. Clin. Lab. Invest. **5**:155–157.
2. Gunge, N., T. Sugimura, and M. Iwasaki. 1957. Genetic analysis of a respiration-deficient-mutant of *Saccharomyces cerevisiae* lacking all cytochromes and accumulating coproporphyrin. Genetics **57**:213–226.
3. Hebb, C. R., and Slebodnik. 1958. The effect of prior growth conditions on the kinetics of adaptive enzyme formation in yeast. Exptl. Cell Res. **14**:286–294.
4. Nicholas, R. E., and C. Rimington. 1949. Qualitative analysis of porphyrins by partition chromatography. J. Clin. Lab. Invest. **1**:12–18.
5. Pretlow, T. P., and F. Sherman. 1967. Porphyrins and zinc porphyrins in normal and mutant strains of yeast. Biochim. Biophys. Acta **148**: 629–644.
6. Rietmüller, G., and H. Tuppy. 1964. Hämsynthetase (Ferrocheletase) in *Saccharomyces cerevisiae* nach aerobem und anaerobem Wachstum. Biochem. Z. **340**:413–420.
7. Sano, S., and S. Granick, 1963. Mitochondrial coproporphyrinogen oxidase and protoporphyrin formation. J. Biol. Chem. **236**:1173–1180.
8. Sherman, F. 1963. Respiration-deficient mutants of yeast. 1. Genetics. Genetics **48**:375–385.
9. Sherman, F. 1964. Mutants of yeast deficient in cytochrome *c*. Genetics **49**:39–48.
10. Sherman, F., and P. P. Slonimski. 1964. RD mutants of yeast. II. Biochemistry. Biochim. Biophys. Acta **90**:1–15.
11. Shibata, K., A. A. Benson, and M. Calvin. 1954.

The absorption spectra of suspensions of living microorganisms. Biochim. Biophys. Acta **15**: 461–470.

12. Sugimura, T., K. Okabe, M. Nagao, and N. Gunge. 1966. Respiration-deficient mutant of *Saccharomyces cerevisiae* which accumulates porphyrins and lacks cytochromes. Biochim. Biophys. Acta **115**:267–275.
13. Sugimura, T., and H. Tanooka. 1967. Photoreactivation of hemeprotein lacking yeast. Biochim. Biophys. Acta **136**:154–155.
14. Wagner, R. P., A. Berquist, T. Barbee, and K. Kiritani. 1964. Genetic blocks in the isoleucine-valine pathway of *Neurospora crassa*. Genetics **49**:865–882.
15. Yčas, M., and T. J. Starr. 1953. The effect of glycine and protoporphyrin on cytochrome deficient yeast. J. Bacteriol. **65**:83–88.