J. Biochem. 121, 25-28 (1997)

Evaluation of Peroxisomal Heme in Yeast

Osamu Soga, Hiroshi Kinoshita, Mitsuyoshi Ueda, and Atsuo Tanaka[1]

Laboratory of Applied Biological Chemistry, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606-01

Received for publication, July 15, 1996

Heme is supposed to be transported into peroxisomes to form peroxisomal catalase [EC 1.11.1.6] in harmony with proliferation of the organelle because of the absence of the heme synthetic pathway in peroxisomes. We tried to understand the transport mechanism of peroxisomal catalase through the peroxisomal membrane from the aspects of a cofactor, heme, by measuring cellular and subcellular heme contents with the pyridine hemochrome method, independent of measuring the catalase activity. *n*-Alkane-grown *Candida tropicalis* cells, in which peroxisomes develop profusely, contained a larger amount of heme than the glucose-grown cells, and the increase well matched that of the catalase activity. The results of subcellular fractionation of the *n*-alkane-grown cells showed that, in peroxisomes, the catalase subunit and heme existed in a molar ratio of 1:1, indicating that excessively transported catalase subunit proteins or heme could not be present in peroxisomes and they formed a tetramer having four molecules of heme. From this quantification of peroxisomal heme, it was strongly suggested that the amounts of the catalase subunit protein and heme transported into peroxisomes should be stoichiometrically regulated at the step of their synthesis or/and transport.

Key words: *Candida tropicalis*, catalase, heme, peroxisomes, pyridine hemochrome method.

The yeast *Candida tropicalis* can grow in a medium containing *n*-alkane as the sole carbon source. Under these conditions, catalase, as well as other enzymes related to alkane or alkane-derived fatty acid assimilation, is induced concomitantly with peroxisome proliferation and transported posttranslationally into peroxisomes (*1, 2*). Catalase [EC 1.11.1.6] is a hydrogen peroxide-degrading enzyme composed of the apo-protein, which consists of four identical subunit proteins, and four molecules of heme as a cofactor (*3, 4*). Catalase is mainly localized in peroxisomes, in which this enzyme degrades hydrogen peroxide produced by various kinds of oxidases (*5*).

The mechanism of protein import into peroxisomes has not been completely and in detail comprehended even though peroxisome-targeting signals (PTSs I and II) have been found as candidate transport signals. In the cases of enzymes with cofactors, the mechanisms of import or synthesis of cofactors have been little studied. As for catalase, it has been supposed to be transported into peroxisomes as a subunit protein without heme after its synthesis, and heme attachment and tetramerization of four molecules of the subunit protein having heme to occur inside the peroxisomes, affording active catalase (*6, 7*). However, this simple pathway can not explain the following findings: peroxisomal catalase was present in cytosol as an active tetrameric form in peroxisome-deficient cells (*8, 9*) and the existence of cytosolic catalase (for example, catalase T in *Saccharomyces cerevisiae*) has been found in various organisms (*10, 11*). These facts suggest that the assembly of catalase subunit protein to the tetrameric form does not necessarily occur in peroxisomes, but may take place in cytosol.

In this paper, to clarify the actual function of peroxisomes in the transportation of catalase into peroxisomes, we made the first trial to measure the content of the cofactor, heme, in subcellular fractions. The quantitative analysis of heme inside and outside peroxisomes was carried out by the pyridine hemochrome method, independently of measuring the catalase activity. The results suggested the possibility of the stoichiometrical transport mechanism of the catalase subunit protein and heme through the peroxisomal membrane.

MATERIALS AND METHODS

Cultivation of Yeast—*C. tropicalis* pK233 (ATCC 20336) was cultivated aerobically at 30°C for 8 h on glucose or for 17 h on *n*-alkane mixture (C_{10}-C_{13}) as the sole source of carbon and energy (*12*).

Preparation of Cell-Free Extracts and Subcellular Fractionation—Cell-free extracts of exponentially grown cells were prepared by disintegrating the cells by sonication (2.4 A, 20 kHz, 2.5 min) in 50 mM potassium phosphate buffer (pH 7.2) (*12*). Subcellular fractionation of *n*-alkane-grown *C. tropicalis* was performed by the procedure described previously (*13, 14*). The cell wall was lysed with Zymolyase 20T and the protoplasts obtained were homogenized with a Teflon homogenizer. The homogenate was fractionated by differential centrifugation. The fractions obtained were as follows: S_1 fraction (5,000 × *g* supernatant), containing all the following fractions; P_2 fraction

[1] To whom correspondence should be addressed. Tel: +81-75-753-5524, Fax: +81-75-753-5534

(20,000×g pellet), peroxisomes and mitochondria; S_3 fraction (139,000×g supernatant), cytosol; P_3 fraction (139,000×g pellet), microsomes. The P_2 fraction was further subjected to discontinuous sucrose density gradient centrifugation [sucrose concentrations from top to bottom, 20, 30, 40, 41.3, 42.5, and 50 (w/v)%, each 2.5 ml; 49,600×g; 2 h] to separate peroxisomes and mitochondria (*13*).

Measurement of Cellular and Subcellular Heme Contents—Contents of cellular and subcellular heme, including both protein-associated heme and free heme, were determined by the pyridine hemochrome method (*15, 16*) as follows: 1 M NaOH (0.5 ml) and pyridine (0.5 ml) were added to 3 ml of a protein solution or cell suspension. The difference spectrum of the reduced minus oxidized pyridine hemochrome was recorded and the heme content was quantified by using the molar extinction coefficient of $\varepsilon_{mM}^{557-541}=20.7$ (*15*).

Enzyme and Protein Assays—Cytochrome *c* oxidase and protein were assayed by the methods described previously (*17, 18*). Catalase activity was measured spectrophotometrically by following the decrease of H_2O_2 at 240 nm (*17*).

Native-PAGE—Electrophoresis was done in 4-24% polyacrylamide gel (3-15% glycerol) in TBE buffer (89 mM boric acid, 20 mM EDTA, pH 8.0) for 24 h at 4°C (20 V/cm) (*19, 20*).

Western Blot Analysis—Western blot analysis using the anti-*C. tropicalis* peroxisomal catalase polyclonal antibody to detect the tetrameric form, other oligomeric forms, monomeric form, and degradation products, was carried out as reported (*21, 22*).

Chemicals—The electrophoresis calibration kits for native-PAGE (669 kDa, thyroglobulin; 440 kDa, ferritin; 232 kDa, catalase; 140 kDa, lactate dehydrogenase; 67 kDa, bovine serum albumin) were purchased from Pharmacia (Uppsala, Sweden).

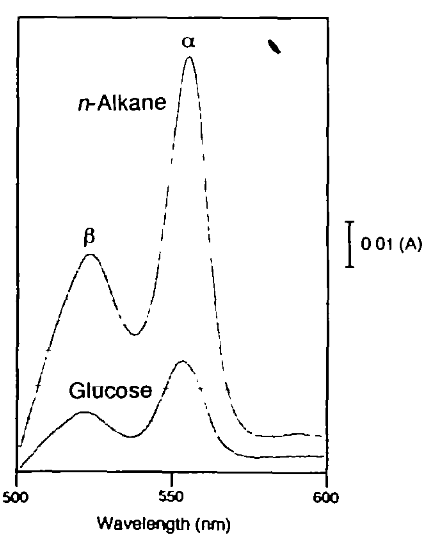

Fig. 1. **Pyridine hemochrome spectra of cell-free extracts obtained from exponentially grown cells of *C. tropicalis*.** Heme contents were calculated from these spectra. Protein concentration in each reaction mixture was 5 mg/ml. *n*-Alkane: cell-free extract from *n*-alkane-grown cells. Glucose: cell-free extract from glucose-grown cells.

RESULTS

Cellular and Subcellular Heme Contents and Heme Occupancy by Active Catalase—The biosynthesis of active catalase was strongly induced in *n*-alkane-grown *C. tropicalis*, and these cells had about 30-fold higher catalase activity than the glucose-grown cells (*1*). As it is of interest to know whether or not a change of the intracellular heme level is observed during the induction of catalase, the cellular heme content was measured by the pyridine hemochrome method. Figure 1 shows typical pyridine hemochrome spectra of cell-free extracts of glucose- and *n*-alkane-grown *C. tropicalis*. As shown in Table I, the increase of the heme content was 0.42 (nmol/mg protein) in comparison of the heme contents in the cell-free extracts of the *n*-alkane-grown cells and the glucose-grown cells. Similar results were obtained with cell suspensions (data not shown). Then, the contribution of catalase biosynthesis to the increase of the cellular heme content was examined by using the data obtained with purified *C. tropicalis* peroxisomal catalase; that is, catalase showing 1 U of

TABLE I. **Cellular heme content and catalase activity in *C. tropicalis*.** Cell-free extracts were prepared from the cells grown to the exponential phase. [a]One unit (U) of catalase activity was defined as the amount which catalyzed the degradation of 1 μmol of H_2O_2 per min. [b]Amount of catalase subunit protein was calculated by using the value for the purified protein (*4*). [c]Heme content was measured as described (*15, 16*).

Carbon source	Glucose	*n*-Alkane
Catalase specific activity[a] (kU/mg protein)	0.12	4.9
Amount of subunit protein forming active catalase[b] (nmol/mg protein)	0.011	0.44
Amount of heme binding to active catalase (subunit)[b] (nmol/mg protein)	0.011	0.44
Cellular heme content[c] (nmol/mg protein)	0.16	0.58
Heme occupancy by active catalase (%)	6.9	76

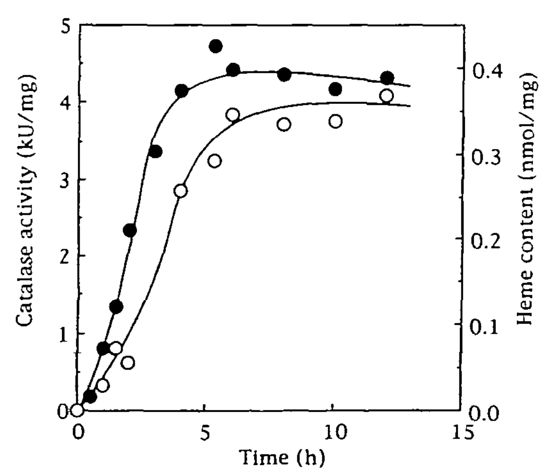

Fig. 2. **Time-course of changes of catalase activity and cellular heme content in *C. tropicalis*.** Cells exponentially grown on glucose were suspended in *n*-alkane medium at a high cell density [*A*(absorbance) 570=5] and cultivated at indicated intervals. Catalase activity and heme content were expressed as the increment from those of the glucose-grown cells (0 h), respectively. ○, Heme content; ●, catalase activity.

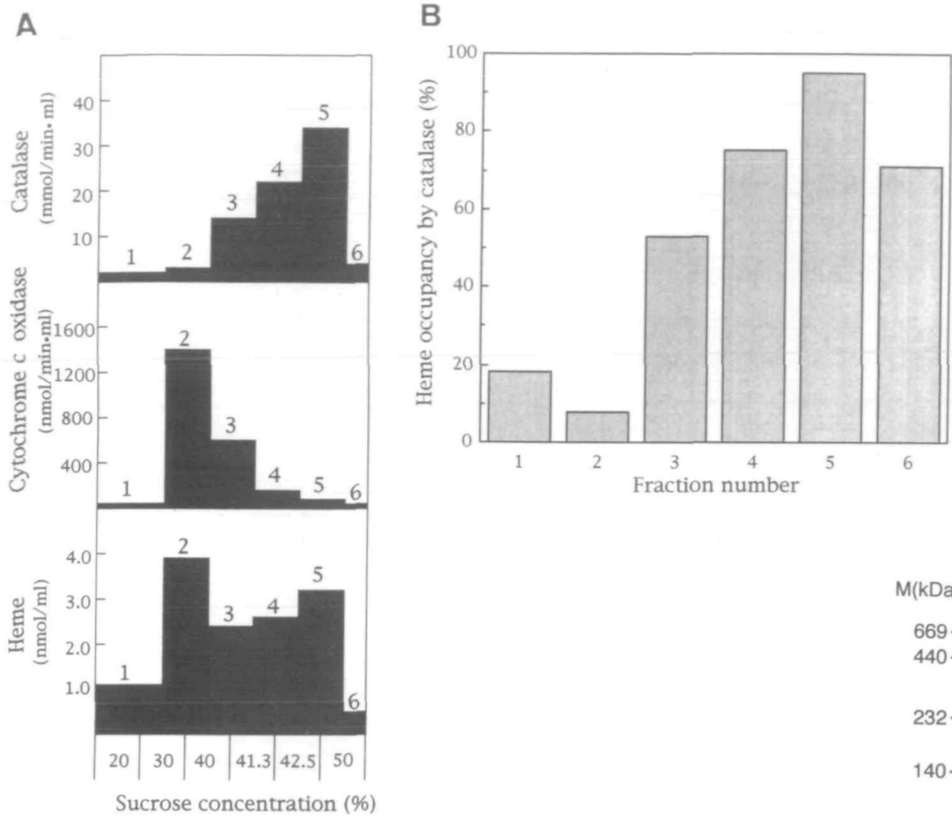

Fig. 3. **Particulate distribution of catalase and heme (A) and heme occupancy by active catalase (B) in *n*-alkane-grown *C. tropicalis*.** A: P_2 fraction was subjected to discontinuous sucrose density gradient centrifugation to separate peroxisomes and mitochondria. The volumes of the fractions were as follows: 1, 3.75 ml; 2-5, 2.5 ml each; 6, 1.25 ml. B: Heme occupancy by catalase in the fractions mentioned above.

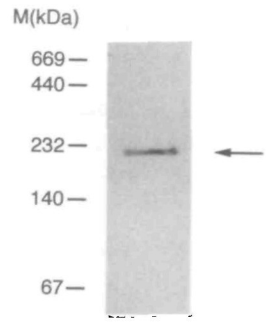

Fig. 4. **Molecular form of catalase in peroxisomes.** Native-PAGE and Western blot analysis using anti-*C. tropicalis* peroxisomal catalase antibody were carried out with an aliquot (10 μg protein) of peroxisomal fraction (fraction 5 in Fig. 3A). An arrow represents native catalase protein.

TABLE II. **Subcellular distribution of heme in *n*-alkane-grown *C. tropicalis*.** Cells were grown to the exponential phase in *n*-alkane medium and fractionated as described in "MATERIALS AND METHODS." S_3, P_2, and P_3 represent the fractions containing cytosol, peroxisomes and mitochondria, and microsomes, respectively. The methods of measurements were the same as in Table I.

Fraction	S_3	P_2	P_3
Catalase specific activity (kU/mg protein)	5.4	5.4	1.0
Amount of subunit protein forming active catalase (nmol/mg protein)	0.49	0.49	0.090
Amount of heme binding to active catalase (subunit) (nmol/mg protein)	0.49	0.49	0.090
Cellular heme content (nmol/mg protein)	0.58	0.75	0.99
Heme occupancy by active catalase (%)	84	65	9.1

activity corresponded to 9.0×10^{-5} nmol of the catalase protein (as subunit) (*4*). The increase of active catalase in the *n*-alkane-grown cells (0.43 nmol/mg protein) corresponded well to that of the cellular heme content. In the *n*-alkane-grown cells, 76% of total heme content was found to be occupied by active catalase, in marked contrast to the case of the glucose-grown cells (Table I). From these results, it was concluded that the increase in the cellular heme content contributed to the increase in the formation of active catalase and the *n*-alkane-grown cells contained a larger amount of heme than the glucose-grown cells.

Time-Course of Changes in Heme Content and Catalase Activity—To examine the relationship between the increase of the cellular heme content and that of the catalase activity in detail, their time-courses were investigated by transferring the glucose-grown cells to the *n*-alkane medium (Fig. 2). The results strongly suggested that the biosynthesis of active peroxisomal catalase and the synthesis of cellular heme were mutually regulated in this yeast.

Subcellular Distribution of Heme and Its Stoichiometric Relationship to the Subunit Protein of Catalase in Peroxisomes—As mentioned above, biosynthesis of heme was induced strongly in *C. tropicalis* under peroxisome-proliferating conditions. Then, the distribution of heme was examined by subcellular fractionation of the cells. The results (Table II) indicated that a large portion of heme found in the P_2 fraction (65%) was occupied by active catalase. This fraction was composed of peroxisomes and mitochondria, and mitochondria contained a variety of hemoproteins, such as cytochrome *c*. The high occupancy by active catalase in spite of this fact meant that catalase was the extremely major particulate hemoprotein in peroxisome-proliferating *C. tropicalis*, contributing to the large increase in the heme content (Table I and Fig. 2). The reason why high occupancy of heme by active catalase was observed in the S_3 fraction is probably due to the contribution of active catalase leaked from peroxisomes or the presence of cytosolic catalase. The P_2 fraction was then subjected to sucrose density gradient centrifugation to separate peroxisomes and mitochondria. Fraction No. 2 was a mitochondrial fraction and fraction No. 5 was a peroxisomal fraction, as indicated by the localization of the marker enzymes (Fig. 3A). The peaks of heme were seen in these two fractions. Figure 3B shows the heme occupancy by active catalase in each fraction. The results demonstrated that almost all the heme in fraction No. 5 (about 95%) was occupied by active catalase. Furthermore, the form of catalase protein inside peroxisomes in the exponentially grown cells was examined by native-PAGE. As can be seen in Fig. 4, only tetrameric catalase was detectable in peroxisomes. Other multimers or oligomeric forms and degradation products were not observed. All of the tetramer was active enzyme containing heme. It could be said from these results that the peroxisomal heme existed as the form of the heme-containing tetrameric catalase and the excessively transported and pooled heme was almost not present in yeast peroxisomes.

DISCUSSION

A stoichiometric relationship between the catalase subunit protein and heme in peroxisomes, implied by the time-course of changes of these species in *C. tropicalis* cells under peroxisome-proliferating conditions, was proved by subcellular fractionation of the cells. Heme was present in a larger amount in the peroxisome-proliferating cells than in the non-proliferating cells, and most of the increased heme was occupied by active catalase (Table I). Furthermore, the increase in heme corresponded well to that in the catalase activity (Fig. 2). This good correspondence between catalase activity and heme also suggested that, once heme was supplied in the cells, the catalase subunit protein immediately formed the active tetramer. This supports a possibility that heme supplement in yeast peroxisomes and synthesis of catalase subunit protein may have some relationship in the regulation of gene expression.

The results obtained by the subcellular fractionation and the native-PAGE studies showed that the peroxisomal heme and the catalase subunit protein exist stoichiometrically (ratio, 1 : 1) in peroxisomes, and excessively transported catalase subunit protein and heme were not detected. These facts suggested that heme is not pooled in peroxisomes prior to the transport of the catalase subunit protein. It seems likely that the transportation of the catalase subunit protein and heme is stoichiometrically coordinated through the peroxisomal membrane, including the transportation of the heme-binding tetrameric form.

REFERENCES

1. Teranishi, Y., Tanaka, A., Osumi, M., and Fukui, S. (1974) Catalase activities of hydrocarbon-utilizing *Candida* yeasts. *Agric. Biol. Chem.* **38**, 1213-1220
2. Osumi, M., Fukuzumi, F., Teranishi, Y., Tanaka, A., and Fukui, S. (1975) Development of microbodies in *Candida tropicalis* during incubation in a *n*-alkane medium. *Arch. Microbiol.* **103**, 1-11
3. Ruis, H. (1979) The biosynthesis of catalase. *Can. J. Biochem.* **57**, 1122-1130
4. Yamada, T., Tanaka, A., and Fukui, S. (1982) Properties of catalase purified from whole cells and peroxisomes of *n*-alkane-grown *Candida tropicalis*. *Eur. J. Biochem.* **125**, 517-521
5. Tanaka, A., Osumi, M., and Fukui, S. (1982) Peroxisomes of alkane-grown yeast: fundamental and practical aspects. *Ann. N.Y. Acad. Sci.* **386**, 183-199
6. Lazarow, P. and de Duve, C. (1973) The synthesis and turnover of rat liver peroxisomes. IV. Biochemical pathway of catalase synthesis. *J. Cell Biol.* **59**, 491-506
7. Lazarow, P. and de Duve, C. (1973) The synthesis and turnover of rat liver peroxisomes. V. Intracellular pathway of catalase synthesis. *J. Cell Biol.* **59**, 507-524
8. Wanders, R.J.A., Strijiland, A., Roermund, C.W.T., Bosch, H., Schutgens, R.B.H., Tager, J. M., and Schram, A. (1987) Catalase in cultured skin fibroblasts from patients with the cerebro-hepato-renal (Zellweger) syndrome: normal maturation in peroxisome-deficient cells. *Biochim. Biophys. Acta* **923**, 478-482
9. Erdmann, R., Veenhuis, M., Mertens, D., and Kunau, W.-H. (1989) Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **86**, 5419-5423
10. Seah, T.C.M. and Kaplan, J.G. (1973) Purification and properties of the catalase of baker's yeast. *J. Biol. Chem.* **248**, 2880-2893
11. Yamamoto, K., Volkl, A., Hashimoto, T., and Fahimi, H.D. (1988) Catalase in guinea pig hepatocytes is localized in cytoplasm, nuclear matrix and peroxisomes. *Eur. J. Cell. Biol.* **46**, 129-135
12. Ueda, M., Okada, H., Tanaka, A., Osumi, M., and Fukui, S. (1983) Induction and subcellular localization of enzymes participating in propionate metabolism in *Candida tropicalis*. *Arch. Microbiol.* **136**, 169-176
13. Ueda, M., Yamanoi, K., Morikawa, T., Okada, H., and Tanaka, A. (1985) Peroxisomal localization of enzymes related to fatty acid β-oxidation in an *n*-alkane-grown yeast, *Candida tropicalis*. *Agric. Biol. Chem.* **49**, 1821-1828
14. Ueda, M., Tanaka, A., and Fukui, S. (1982) Peroxisomal and mitochondrial carnitine acetyltransferases in alkane-grown yeast *Candida tropicalis*. *Eur. J. Biochem.* **124**, 205-210
15. Porra, R.J. and Jones, O.T.G. (1963) Studies on ferrochelatase. 1. Assay and properties of ferrochelatase from a pig-liver mitochondrial extract. *Biochem. J.* **87**, 181-192
16. Urban-Grimal, D. and Labbe-Bois, R. (1981) Genetic and biochemical characterization of mutants of *Saccharomyces cerevisiae* blocked in six different steps of heme biosynthesis. *Mol. Gen. Genet.* **183**, 85-92
17. Kawamoto, S., Tanaka, A., Yamamura, M., Teranishi, Y., Fukui, S., and Osumi, M. (1977) Microbody of *n*-alkane-grown yeast. Enzyme localization in the isolated microbody. *Arch. Microbiol.* **112**, 1-8
18. Kurihara, T., Ueda, M., Okada, H., Kamasawa, N., Naito, N., Osumi, M., and Tanaka, A. (1992) β-Oxidation of butyrate, the short-chain-length fatty acid, occurs in peroxisomes in the yeast, *Candida tropicalis*. *J. Biochem.* **111**, 783-787
19. Anderson, L.O., Borg, H., and Mikaelsson, M. (1972) Molecular weight estimations of proteins by electrophoresis in polyacrylamide gels of graded porosity. *FEBS Lett.* **20**, 199-202
20. Clos, J., Westwood, J.T., Becker, P.B., Wilson, S., Lambert, K., and Wu, C. (1990) Molecular cloning and expression of a hexameric *Drosophila* heat shock factor subject to negative regulation. *Cell* **63**, 1085-1097
21. Ueda, M., Mozaffar, S., Atomi, H., Osumi, M., and Tanaka, A. (1989) Characterization of peroxisomes in *n*-alkane-utilizable yeast *Candida tropicalis*, grown on glucose and propionate. *J. Ferment. Bioeng.* **68**, 411-416
22. Okamoto, H., Ueda, M., Yamanoi, K., Ukita, R., Naito, N., Osumi, M., and Tanaka, A. (1991) Degradation of peroxisomal catalase in an *n*-alkane-utilizable yeast, *Candida tropicalis*. *J. Ferment. Bioeng.* **72**, 254-257