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Hydrogen Peroxide Metabolism in Yeasts

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A catalase-negative mutant of the yeast *Hansenula polymorpha* consumed methanol in the presence of glucose when the organism was grown in carbon-limited chemostat cultures. The organism was apparently able to decompose the H_2O_2 generated in the oxidation of methanol by alcohol oxidase. Not only H_2O_2 generated intracellularly but also H_2O_2 added extracellularly was effectively destroyed by the catalase-negative mutant. From the rate of H_2O_2 consumption during growth in chemostat cultures on mixtures of glucose and H_2O_2, it appeared that the mutant was capable of decomposing H_2O_2 at a rate as high as 8 mmol · g of cells^{-1} · h^{-1}. Glutathione peroxidase (EC 1.11.1.9) was absent under all growth conditions. However, cytochrome *c* peroxidase (CCP; EC 1.11.1.5) increased to very high levels in cells which decomposed H_2O_2. When wild-type *H. polymorpha* was grown on mixtures of glucose and methanol, the CCP level was independent of the rate of methanol utilization, whereas the level of catalase increased with increasing amounts of methanol in the substrate feed. Also, the wild type decomposed H_2O_2 at a high rate when cells were grown on mixtures of glucose and H_2O_2. In this case, an increase of both CCP and catalase was observed. When *Saccharomyces cerevisiae* was grown on mixtures of glucose and H_2O_2, the level of catalase remained low, but CCP increased with increasing rates of H_2O_2 utilization. From these observations and an analysis of cell yields under the various conditions, two conclusions can be drawn. (i) CCP is a key enzyme of H_2O_2 detoxification in yeasts. (ii) Catalase can effectively compete with mitochondrial CCP for hydrogen peroxide only if hydrogen peroxide is generated at the site where catalase is located, namely in the peroxisomes.

Catalase (EC 1.11.1.6) plays a key role in the destruction of H_2O_2 by microorganisms. In yeasts, the enzyme is specifically induced during growth on compounds that require the action of H_2O_2-producing oxidases. Utilization of, among others, methanol, *n*-alkanes, fatty acids, amines, D-amino acids, and uric acid leads to drastic elevation of the catalase level in yeasts (10, 25, 23). The enzyme is located in an organelle that is specialized in the production and consumption of H_2O_2, namely the peroxisome (21, 22, 24). Growth of yeasts on methanol, for example, is associated with the proliferation of peroxisomes which contain large amounts of alcohol oxidase and catalase (8, 22, 25). The key role of catalase in methanol metabolism in yeasts is indicated by the fact that catalase-negative mutants are unable to grow on methanol as a sole carbon and energy source (8). Surprisingly, however, it was found that these mutants were able to utilize methanol in the presence of glucose and that they remained viable under these conditions (10a, 10b). This observation indicates that in the catalase-negative mutant, an alternative H_2O_2-destroying system is present. In this paper, evidence is presented that this alternative system is cytochrome *c* peroxidase (CCP; EC 1.11.1.5).

MATERIALS AND METHODS

Organisms and cultivation. The catalase-negative mutant 55/11 of *Hansenula polymorpha* (8) was donated by L. Eggeling, Kernforschungsanlage, Jülich, Federal Republic of Germany. (This strain is identical to ATCC 46059.) *H. polymorpha* CBS 4732 and *Saccharomyces cerevisiae* CBS 8066 were obtained from the Centraal Bureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands. All strains were maintained on agar slants at 4°C.

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The organisms were grown in carbon-limited chemostat cultures at a dilution rate of 0.10 · h^{-1} on a mineral medium (4) containing 5 g of glucose · liter^{-1}. The dissolved oxygen tension and pH were controlled at 60 to 80% of air saturation and 5.0, respectively. *H. polymorpha* was grown at 37°C, and *S. cerevisiae* was grown at 30°C.

During experiments with addition of either methanol or H_2O_2 to the medium feed, the medium vessel was stored in a refrigerator. Under these conditions, the concentration of both compounds in the reservoir remained constant for at least 1 week.

Preparation of cell extracts. Cells (30 ml) from steady-state cultures were harvested by centrifugation at 5,000 × *g* for 10 min, washed once with 50 mM potassium phosphate buffer, pH 7.5, and stored as a concentrated suspension at −40°C until further use (within 1 month). Extracts were prepared by sonic disintegration of the cells for 4 min at 4°C with a sonicator (150 W; Measuring & Scientific Equipment, Ltd.) in 100 mM potassium phosphate buffer, pH 7.5, containing 2 mM $MgCl_2$ and 1 mM dithiothreitol. Whole cells and cell debris were then removed by centrifugation at 75,000 × *g* for 15 min in a rotor (SS-34; Ivan Sorvall, Inc.). No difference in enzyme activities was observed between extracts of frozen or fresh cell suspensions.

Enzyme assays. Spectrophotometric assays were carried out with freshly prepared extracts at 37°C for *H. polymorpha* and at 30°C for *S. cerevisiae*. Reaction rates were linearly proportional to the amount of extract added in the assays described below. When necessary, extracts were diluted in sonication buffer. A correction was made for endogenous reactions.

(i) Catalase. Catalase was assayed by the method of Verduyn et al. (26).

(ii) CCP. CCP was assayed at 550 nm by monitoring the oxidation of dithionite-reduced cytochrome *c*. The reaction

mixture contained 50 mM sodium acetate buffer, pH 6.0, and reduced cytochrome *c* (20 μM). The reaction was started with the addition of fresh H_2O_2 (0.18 mM). An extinction coefficient of 27.6 · mM^{-1} · cm^{-1} was used for reduced horse heart cytochrome *c* (30).

It is sometimes suggested in the literature that an assay of CCP is not possible in the presence of catalase because of competition for H_2O_2. Therefore, CCP is often assayed with cumene hydrogen peroxide (which is not a substrate for catalase) or in the presence of a catalase inhibitor such as aminotriazole. By adding commercial catalase to cell extracts of the catalase mutant of *H. polymorpha* (which has no detectable catalase activity), it could be shown that the initial velocity of CCP is not significantly affected (i.e., less than 10%) by the addition of catalase, even at catalase/CCP activity ratios as high as 50.

(iii) Glucose-6-phosphate dehydrogenase. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed by the method of Bruinenberg et al. (4).

(iv) Glutathione peroxidase. Glutathione peroxidase (EC 1.11.1.9) was assayed by the method of Lawrence and Burk (14) with H_2O_2 as the substrate. The assay was tested with commercial glutathione peroxidase (Boehringer GmbH).

(v) Glutathione reductase. Glutathione reductase (EC 1.6.4.2) was assayed by the method of Bergmeyer (3).

Enzyme units are defined as micromoles of nucleotide converted per minute for nucleotide-dependent reactions and as micromoles of cytochrome *c* oxidized per minute for CCP. Catalase units are expressed as ΔE_{240} · $minute^{-1}$. Specific activities are expressed as units · milligram of $protein^{-1}$.

Analytical methods. Glucose was determined by the GOD/PAP method of Boehringer GmbH. Methanol was assayed by the colorimetric method of Verduyn et al. (26). H_2O_2 was assayed by the same method, except that alcohol oxidase was omitted from the reaction mixture. Linear regression coefficients for this H_2O_2 assay were at least 0.9998 in routine measurements. The detection limit was 0.1 mM.

In samples which contained both glucose and H_2O_2, the latter compound had to be removed before the glucose could be determined. Commerical catalase (5 μl) was added to 1.0 ml of sample, and the mixture was incubated for 30 min. Catalase was then inactivated by addition of 10 μl of 75% trichloroacetic acid, and denatured protein was removed by centrifugation. The sample was then diluted, and glucose was determined with the standard assay.

Dry weight of cultures was determined by drying washed 20-ml samples of culture to a constant weight at 70°C.

Protein was determined by the method of Lowry with bovine serum albumin (corrected for moisture content) as the standard.

RESULTS

Metabolism of endogenous H_2O_2 by *H. polymorpha*. As reported previously, the catalase-negative mutant of *H. polymorpha* was able to consume methanol during growth in the presence of glucose. Utilization of methanol by the mutant and wild type was tested up to a molar methanol/glucose ratio of 2 in carbon-limited chemostat cultures. Both methanol and glucose were completely consumed under these conditions. Since methanol utilization proceeds via an alcohol oxidase that produces H_2O_2, and since H_2O_2 could not be detected in the culture fluid, it can be concluded that the mutant is capable of destroying H_2O_2 at a rate of at least 2 mmol · g of $cells^{-1}$ · h^{-1} (Fig. 1A). The cell yield of both

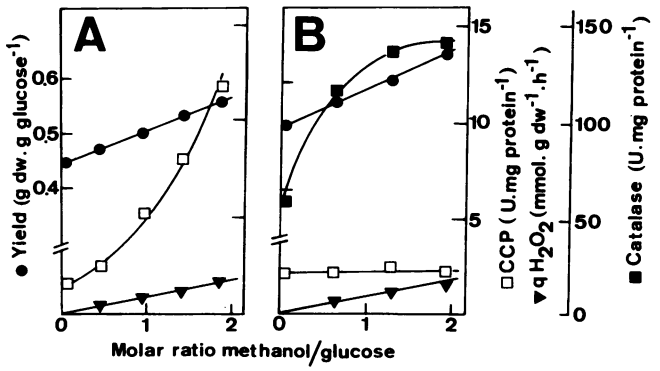

FIG. 1. (A) Cell yield, specific activity of CCP, and in situ rate of H_2O_2 destruction (q H_2O_2) in chemostat cultures of a catalase-negative mutant of *H. polymorpha* as a function of the methanol/glucose ratio in the medium feed. The dilution rate was 0.1 · h^{-1}. (B) Cell yield, specific acitivity of CCP, catalase, and in situ rate of H_2O_2 destruction in wild-type *H. polymorpha* under similar conditions as in panel A.

the mutant and the wild type during growth on a fixed concentration of glucose increased with increasing concentrations of methanol in the medium feed (Fig. 1A and B). An enzymic analysis of cells growing on mixtures of glucose and methanol revealed that catalase was not detectable in the mutant. In the wild type, the specific activity of this enzyme increased from 60 U · mg of $protein^{-1}$ during growth on glucose to 140 U · mg of $protein^{-1}$ during growth on a mixture of methanol and glucose at a molar ratio of 2.

A search for the alternative H_2O_2-decomposing system in the catalase-negative mutant showed that glutathione peroxidase was absent in this organism. However, CCP markedly increased with increasing amounts of methanol in the medium feed (Fig. 1A). The activity of this enzyme at a molar methanol/glucose ratio of 2 was as high as 12 U · mg of $protein^{-1}$. In the wild type, however, CCP remained at the basic level of 2.5 to 3.0 U · mg of $protein^{-1}$, independent of the concentration of methanol in the feed (Fig. 1B).

Metabolism of exogenous H_2O_2 by *H. polymorpha*. The H_2O_2-destroying capacity of the catalase-negative mutant is also clearly illustrated by experiments in which the organism was cultured on mixtures of glucose and H_2O_2. The yeast could be grown on a fixed concentration of glucose with increasing concentrations of H_2O_2 in the medium feed up to a molar H_2O_2/glucose ratio of 4. Upon transition to higher molar ratios, H_2O_2 became detectable in the culture fluid and excessive foaming occurred, which is indicative of cell lysis. This phenomenon was associated with a rapid wash out of the culture. Thus, the organism was able to grow in chemostat culture on a medium feed consisting of 30 mM glucose and up to 130 mM H_2O_2 at a dilution rate of 0.10 · h^{-1}. Under these conditions, H_2O_2 could not be detected in the culture fluid and the rate of H_2O_2 consumption by the catalase-negative mutant was 8 mmol · g of $cells^{-1}$ · h^{-1}. In contrast to utilization of methanol by glucose-limited cultures, consumption of H_2O_2 resulted in a decrease in the cell yield (Fig. 2), but cell lysis was not observed. The amount of CCP in the mutant rapidly increased with increasing amounts of H_2O_2 in the medium feed up to a molar H_2O_2/glucose ratio of 2 and then slightly decreased (Fig. 2).

The wild type was also able to destroy exogenous H_2O_2. It was anticipated that this would result from the presence of catalase in this organism. Indeed, the amount of catalase in the cells increased with increasing concentrations of H_2O_2, a

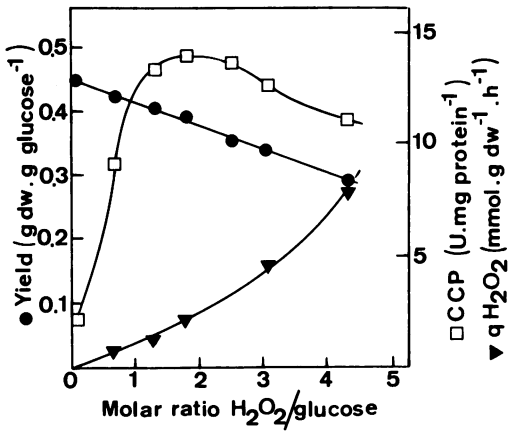

FIG. 2. Cell yield, specific activity of CCP, and in situ rate of H_2O_2 destruction in chemostat cultures of a catalase-negative mutant of *H. polymorpha* as a function of the H_2O_2/glucose ratio in the medium feed. The dilution rate was 0.1 · h^{-1}.

situation similar to that with growth on glucose-methanol mixtures. However, the levels not only of catalase but also of CCP were markedly enhanced during growth in the presence of H_2O_2 (Fig. 3).

Metabolism of exogenous H_2O_2 by *S. cerevisiae*. Since the results presented above indicate that CCP also may play a role in H_2O_2 metabolism by wild-type (i.e., catalase-containing) *H. polymorpha*, it was of interest to study the level of this enzyme in other yeasts. *S. cerevisiae* was chosen as a model organism, particularly since it has been reported that growth of this organism in chemostat cultures in the presence of H_2O_2 does not result in enhancement of catalase activity (23). The ability of *S. cerevisiae* CBS 8066 to decompose exogenous H_2O_2 was tested in glucose-limited cultures up to a molar H_2O_2/glucose ratio of 4. An enzymatic analysis of catalase activities under these growth conditions confirmed the observations of Veenhuis et al. (23). The specific activity of the enzyme remained at a low level of 4.5 to 7 U · mg of protein^{-1}. CCP activity, however, was almost linearly proportional to the H_2O_2 concentration in the medium feed (Fig. 4). In contrast, when *S. cerevisiae* is grown on oleic acid, catalase activities are strongly enhanced (23). This serves a physiological function, since the metabolism of oleic acid requires the action of a peroxisomal H_2O_2-producing fatty acid oxidase (22). From the fact that catalase activity remains low during growth of *S. cerevisiae* in the presence of exogenous H_2O_2 despite a high rate of cellular H_2O_2 destruction, as during growth on oleic acid, it can be concluded that catalase does not play a significant role in the destruction of exogenous H_2O_2.

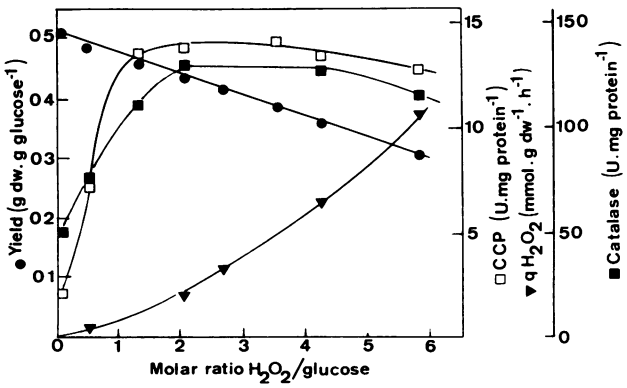

FIG. 3. Cell yield, specific activity of CCP, catalase, and in situ rate of H_2O_2 destruction in wild-type *H. polymorpha* under similar conditions as in Fig. 2.

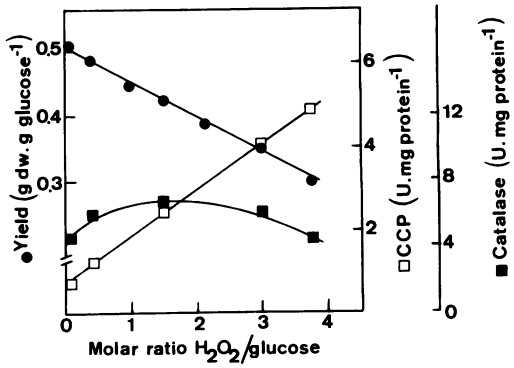

FIG. 4. Cell yield, specific activity of CCP, and catalase in *S. cerevisiae* under similar conditions as in Fig. 2.

DISCUSSION

H_2O_2 scavenging by CCP. The effective destruction of both exogenous and endogenous H_2O_2 by the catalase-negative mutant of *H. polymorpha* clearly establishes the potential of CCP as an H_2O_2 scavenger (Fig. 1A and 2). Although it cannot be excluded that other H_2O_2-destroying enzymes may be present in this mutant, the strong correlation between CCP activity and the rate of in vivo H_2O_2 destruction indicates that at least a major part of this toxic compound is decomposed by this enzyme. We were unable to detect glutathione peroxidase in this yeast. This confirms the results of Smith and Shrift (20), who reported the absence of glutathione peroxidase in yeast, and of Aisaka et al. (2), who examined a large number of microorganisms for the presence of this enzyme. Glutathione peroxidase is an important H_2O_2-destroying enzyme system in mammalian cells. It acts in concert with NADPH-linked glutathione reductase and the hexose monophosphate pathway enzymes (for a review, see reference 5). Apart from the fact that glutathione peroxidase was not detectable in *H. polymorpha*, it was also found that the levels of glutathione reductase and glucose-6-phosphate dehydrogenase did not change significantly upon feeding of H_2O_2 to cells (results not shown).

CCP is located in the intracristate space of the mitochondria (27). As a result, H_2O_2 metabolism during consumption of methanol by the catalase-negative mutant is highly compartmentalized (Fig. 5). The H_2O_2 produced by alcohol oxidase must diffuse out of the peroxisomes, pass the cytoplasm, and cross the outer mitochondrial membrane in order to be available as a substrate for CCP. Apparently the high affinity of CCP for H_2O_2 (on the order of 1 to 5 μM; 11, 30) and its high activity can keep the intracellular H_2O_2 concentration sufficiently low to allow growth under the conditions employed. In apparently similar fashion, the mutant is able to cope with exogenous H_2O_2.

Competition between CCP and catalase. Not only in the catalase-negative mutant of *H. polymorpha*, but also in *S. cerevisiae*, CCP plays an important, if not unique, role in destruction of exogenous H_2O_2. Although *S. cerevisiae* is capable of enhancing its catalase level under growth condi-

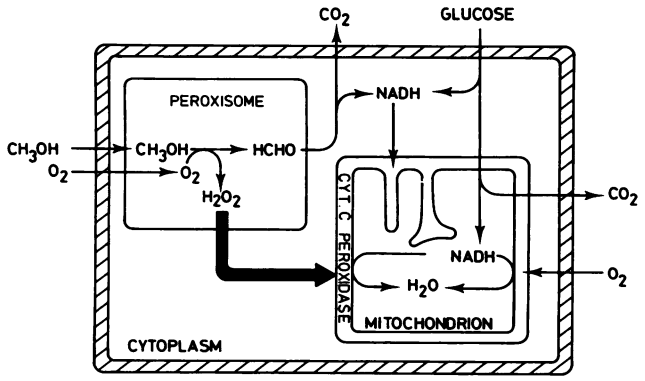

FIG. 5. Schematic representation of H_2O_2 metabolism in the catalase-negative mutant of *H. polymorpha* during growth on a mixture of methanol and glucose.

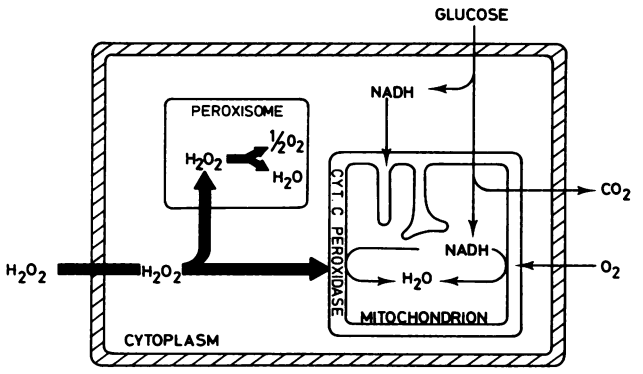

FIG. 6. Schematic representation of H_2O_2 metabolism in wild-type *H. polymorpha* during growth on a mixture of H_2O_2 and glucose.

tions in which H_2O_2 must be destroyed, the activity of this enzyme remained very low when cells were grown on H_2O_2-glucose mixtures (Fig. 3). Apparently the scavenging of H_2O_2 by CCP is so efficient that the regulatory events triggering catalase synthesis are circumvented.

With respect to destruction of exogenous H_2O_2, the situation in *H. polymorpha* is more complex. In the wild type, both catalase and CCP levels were enhanced in response to environmental conditions (Fig. 3). It can be argued that in this case only catalase is involved in H_2O_2 destruction and that the synthesis of extra CCP would be gratuitous. This seems unlikely for the following reasons. First, in the catalase-negative mutant an enhancement of the CCP level is a physiological necessity. In the wild type, the pattern of CCP synthesis was identical to that of the catalase-negative mutant (compare Fig. 2 and 3) and therefore points to a physiological role in this case as well. Second, it is well known that CCP has an affinity for H_2O_2 which is at least 3 orders of magnitude higher than the affinity of catalase for H_2O_2 (1, 11, 19, 30). This fact and the very high activity of CCP (up to 14 U · mg of protein^{-1}) are in line with the hypothesis that CCP decomposes at least a major part of the H_2O_2 provided extracellularly in the wild type also. Third, a further observation that supports the role of CCP in the destruction of exogenous H_2O_2 in wild-type *H. polymorpha* is the decrease in cell yield in the presence of H_2O_2. A decrease in cell yield is to be expected since, as a result of cytochrome *c* oxidation by CCP, ATP generation at the third proton-translocating loop (site III) of the electron transport chain is circumvented. This decrease in cell yield occurred to approximately the same extent in both wild-type and mutant *H. polymorpha*. The number of proton-translocating loops in *H. polymorpha* is not known. If the first proton-translocating loop (site I) were absent, as it is in *S. cerevisiae* (17), the action of CCP would result in the reduction of the P/O ratio by a factor of two. If only catalase were responsible for H_2O_2 decomposition during growth of the wild type with exogenous H_2O_2, the efficiency of oxidative phosphorylation would not be affected, since in the case of decomposition of H_2O_2 by catalase, neither consumption nor production of biologically useful energy occurs. Quantitative aspects of H_2O_2 destruction by CCP on the bioenergetics of growth, the possible damage of cell structures under the growth conditions employed, and the inability of the catalase-negative mutant to grow on methanol as a sole carbon and energy source will be dealt with in a forthcoming paper.

In summary, it can be concluded that CCP probably plays an important role in the destruction of exogenous H_2O_2 in wild-type *H. polymorpha* also (Fig. 6).

Role of peroxisomes in H_2O_2 destruction. The only case in which CCP levels were not affected by growth conditions that required H_2O_2 destruction was when wild-type *H. polymorpha* was grown on mixtures of glucose and methanol (Fig. 1B). Under these conditions, the CCP level remained constant, whereas that of catalase increased. This situation is opposite to that which occurred in *S. cerevisiae* growing on mixtures of glucose and H_2O_2. In the latter case, catalase was constant and CCP increased (Fig. 4). These two examples illustrate the crucial role of subcellular compartmentation in H_2O_2 metabolism. In the case of *S. cerevisiae*, one could argue that peroxisomes (containing catalase) and mitochondria (containing CCP) would both be equivalent candidates for the decomposition of H_2O_2. However, scavenging of H_2O_2 by CCP is apparently so efficient that triggering of catalase synthesis is avoided. On the other hand, during growth of wild-type *H. polymorpha* on glucose and methanol, scavenging of H_2O_2 by catalase is apparently so efficient that triggering of CCP synthesis does not occur. In the latter case, H_2O_2 destruction seems entirely restricted to the peroxisomes. Thus, despite its poor affinity for H_2O_2, catalase is an efficient detoxifying enzyme for peroxisomal H_2O_2 because it has the advantage of being located where its substrate is produced. However, when H_2O_2 is of extraperoxisomal origin, peroxisomes would provide little protection against H_2O_2, which is exactly what was proposed by de Duve more than 20 years ago (7).

Physiological function of CCP. CCP has been the subject of many genetic and biochemical studies. It is a model enzyme for the study of transport of proteins into the mitochondrial compartment (6, 12, 18). Much progress has also been made on the biochemical properties of this heme protein, especially by Yonetani and co-workers (28–30). The physiological function of the enzyme seemed less clear. With respect to this problem, Yonetani remarked (quotation from 28): "From the physiological viewpoint, the role and function of CCP in the metabolism of yeast are not at all understood at the present time. Since this enzyme is present in aerobically grown yeasts in a concentration comparable to that of cytochrome oxidase and since its molecular activity is at least 10 times greater than that of cytochrome oxidase, CCP would be a much more efficient system for accepting the flow of electrons from the cytochrome chain than cytochrome oxidase, provided that H_2O_2 is abundant in yeast cells." Our results confirm this assumption of Yonetani. By acting as an

H_2O_2 scavenger under conditions in which H_2O_2 is abundant in cells, CCP can replace cytochrome oxidase in the electron transport chain. Although this process is apparently efficient in kinetic terms, it is not so in energetic terms, since the action of CCP leads to a bypass of site III phosphorylation.

The conditions under which CCP seems to play a major role in the cases studied here are evidently artificial but merely serve to illustrate the important role the enzyme can play in H_2O_2 removal. Under more physiological conditions, the enzyme may act as a scavenger of the H_2O_2 produced in the electron transport chain (9, 16). However, the fact that yeasts possess a regulatory mechanism which can come into action under conditions of H_2O_2 stress suggests that such conditions may also occur in nature. In this respect, it has to be mentioned that most aerobic organisms possess superoxide dismutase (EC 1.15.1.1) as a protection against oxygen radicals. Since the action of this enzyme leads to H_2O_2 formation, a possible role of CCP in the overall process of detoxification of oxygen radicals should be reinvestigated. In this respect, it is worth mentioning that in studies on the effect of radical-generating agents, such as paraquat and adriamycin, a possible role of CCP in H_2O_2 destruction has so far been neglected (13, 15).

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