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IN VITRO STUDIES ON YEAST CYTOCHROME *c* PEROXIDASE AND ITS POSSIBLE FUNCTION IN THE ELECTRON TRANSFER AND ENERGY COUPLING REACTIONS

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SUMMARY

1. Yeast cytochrome *c* peroxidase (ferrocytochrome *c*:H_2O_2 oxidoreductase, EC 1.11.1.5) located in the intermembrane space of the mitochondrion rapidly decomposes H_2O_2 generated during the operation of the respiratory chain and efficiently oxidizes ferrocytochrome *c*.

2. Rapid utilization of H_2O_2 is linked to the oxidation of ferrocytochrome *c* generated through the usual metabolic pathway in which phosphorylation Sites I and II are operative.

INTRODUCTION

Recent studies on the catalase (EC 1.11.1.6)–H_2O_2 intermediate in the rat liver mitochondrial fraction indicate that the respiratory system produces H_2O_2 at a constant rate and that the rate of H_2O_2 production depends on the metabolic state of the mitochondria[1]. This finding has been confirmed by direct measurements of the rate of H_2O_2 generation in rat liver and pigeon heart mitochondria using horse radish peroxidase (EC 1.11.1.7)[2] or cytochrome *c* peroxidase (ferrocytochrome *c*: H_2O_2 oxidoreductase, EC 1.11.1.5)[3].

Since H_2O_2 is highly toxic for biological systems aerobic organisms have developed various enzymes which couple the destruction of H_2O_2 to the oxidation of hydrogen donors. One of the better known of these is yeast cytochrome *c* peroxidase, located in the mitochondrial fraction[4] which decomposes H_2O_2 in the following sequence of reactions:

$$\text{Cytochrome } c \text{ peroxidase} + H_2O_2 \rightleftharpoons \text{cytochrome } c \text{ peroxidase} \cdot \text{complex } ES$$

$$\text{Cytochrome } c \text{ peroxidase} \cdot \text{complex } ES + 2 \text{ cytochrome } c^{2+} \rightleftharpoons \text{cytochrome } c \text{ peroxidase} + 2 \text{ cytochrome } c^{3+}$$

In this paper we shall present experimental data indicating that the amount of H_2O_2 which diffuses from the yeast mitochondria and can be measured in the incubation medium is very small in comparison with the rate of H_2O_2 generation observed in mammalian mitochondria. This can be taken as an indication for a role of cytochrome *c* peroxidase as one of the terminal oxidases which remove H_2O_2 from the cell. In addition, kinetic data on the oxidation of the respiratory chain

components *via* cytochrome *c* peroxidase indicate that electrons can be effectively removed from the substrate by this pathway and may participate in the energy-coupling reactions.

MATERIALS AND METHODS

Preparation of the mitochondria and their subfractions

Saccharomyces cerevisiae mitochondria were prepared from commercial bakers yeast by grinding the cells in a colloid mill[5] in 0.6 M mannitol, 0.5 mM EDTA (pH 6.8) medium containing bovine serum albumin (1 mg/ml). The mitochondrial fraction was obtained by differential centrifugation as described before[6] and washed twice in the same medium.

Pigeon heart mitochondria were isolated in 0.225 M mannitol–0.075 M sucrose–0.0002 M EDTA (pH 7.2) medium as described by Chance and Hagihara[7].

In the experiment designed to determine the subcellular distribution of the yeast cytochrome *c* peroxidase, mitochondria initially suspended in 0.6 M mannitol–0.5 mM EDTA were centrifuged down and the pellet resuspended in a "swelling medium" containing 0.010 M Tris–potassium phosphate buffer (pH 7.0)[8]. After 30 min incubation at 0 °C the mitochondrial suspension was again spun down (at $10000 \times g$ for 10 min). The supernatant was kept for further enzymic assays and the pellet suspended in 0.6 M mannitol–0.5 mM EDTA and sonicated for 45 s in a Branson sonifier. The sonicated sample was centrifuged for 1 h at $105000 \times g$ in a Spinco Model L ultracentrifuge. Cytochrome *c* peroxidase activity of the soluble fractions was measured in 0.1 M phosphate buffer, pH 7.0, using reduced cytochrome *c* as the substrate ($\Delta\varepsilon = 19.7\ \mathrm{l \cdot mmole^{-1} \cdot cm^{-1}}$). The reaction was started by the addition of ethyl hydrogen peroxide and the values were corrected for cytochrome *c* oxidation occurring in the absence of ethyl hydrogen peroxide.

Difference spectra and enzymatic assays

Difference spectra of the mitochondrial fraction with ethyl hydrogen peroxide in the presence and absence of methanol (to eliminate the interference due to catalase compound I) were obtained by plotting the absorption changes at each wavelength with reference to 418 nm. Antimycin A was added in order to minimize the changes induced by the differences in the redox state of the mitochondrial cytochromes absorbing in this wavelength region and to prevent the rapid decomposition of cytochrome *c* peroxidase complex *ES* by the mitochondrial respiratory chain.

Concentration of cytochrome *c* peroxidase was determined by the increase in $\Delta A_{425\ \mathrm{nm}} - A_{395\ \mathrm{nm}}$ due to the formation of cytochrome *c* peroxidase complex *ES* upon addition of different concentrations of ethyl hydrogen peroxide and extrapolating the results in a double reciprocal plot to infinite ethyl hydrogen peroxide concentration. The dual wavelength spectrophotometric technique as afforded either by the Aminco instrument or by that designed and constructed in the Johnson Foundation was employed. The millimolar extinction coefficient used for the calculation was 62 (ref. 9).

Malate dehydrogenase activity was determined by the method of Ochoa[10] and H_2O_2 generation was measured either fluorometrically using the exogenous horse radish peroxidase–scopoletin method[2] or by determining the steady-state level of the catalase–H_2O_2 intermediate[1].

Kinetic studies

Kinetic studies were carried out using the Johnson Foundation regenerative flow apparatus which has been described elsewhere[11]. Mitochondria suspended at an appropriate concentration (*cf.* legends to Figs 4–7) in 0.6 M mannitol–0.5 mM EDTA–20 mM phosphate buffer (pH 6.2) were placed in the main syringe of the instrument (15 ml) and made anaerobic by their respiration with the endogenous substrate. Cytochrome oxidase inhibited by carbon monoxide (added as CO-saturated buffer) and anaerobic (helium-saturated) ethyl hydrogen peroxide (used instead of H_2O_2 since it does not produce oxygen) was delivered from the side syringe giving a 1.25% dilution per discharge of the flow apparatus. Fast kinetic changes were recorded on a Tektronix storage oscilloscope and slow kinetic changes on an Esterline-Angus recorder. The optical path of the regenerative flow apparatus is 0.6 cm. The time scales are indicated on the abscissa of each figure and the top trace indicates the profile of flow velocity changes, particularly the time at which the flow stops. The instrument has a mixing time of a few tenths of a millisecond. The time measured during the flow at the position at which observations are made (1.2 ml downstream from the mixing point) is 20 ms and the stopping time is approximately 15 ms.

Cytochrome *c* spectral changes were followed at the wavelength pair 550–540 nm, cytochrome *a* at 605–630 nm, cytochrome *b* at 560–575 nm and 566–575 nm.

Incorporation of ^{32}P into [^{32}P]ATP

Participation of the mitochondrial respiratory carriers in the coupled electron flow during oxidation of cytochrome *c* by cytochrome *c* peroxidase was measured by the incorporation of ^{32}P into [^{32}P]ATP under anaerobic conditions. Yeast mitochondria were added to the main compartment of a Thunberg vessel containing 0.6 M mannitol–10 mM Tris–maleate–10 mM potassium phosphate (pH 6.8), 17 mM ethanol, 10 mM semicarbazide, 20 mM $MgSO_4$–0.5 mM EDTA, 32 mM glucose, 2.0 mg bovine serum albumin, 15 units of hexokinase and 10 μM azide. The side arm contained: ^{32}P in 1 mM potassium phosphate (pH 6.8), 1.0 mM ATP and ethyl hydrogen peroxide. In order to attain anaerobiosis and to inhibit cytochrome oxidase the vessels were flushed for 15 min with CO and the reaction was initiated by the addition of the contents of the side arms to the main compartments. Aliquots were withdrawn at times indicated in the legend of the figure, quenched in ice-cold $HClO_4$ and analyzed for ^{32}P incorporated into [^{32}P]ATP as described by Conover *et al.*[12].

Ethyl hydrogen peroxide used was obtained from Ferrosan (Malmo, Sweden) or synthesized according to the Williams and Moscher[13] procedures (courtesy of Dr G. R. Schonbaum).

RESULTS

Spectrum of peroxidase complex ES and determination of the cytochrome c peroxidase content in S. cerevisiae mitochondria

Addition of ethyl hydrogen peroxide to the suspension of yeast mitochondria supplemented with antimycin A induces a transient cycle of increase in absorbance at 428–418 nm whose duration depends on the amount of ethyl hydrogen peroxide added. When the trace returns to the original level subsequent addition of ethyl hydrogen peroxide causes an identical change in absorption. Plotting the extent of

the absorption change (with a constant amount of ethyl hydrogen peroxide) against the measuring wavelength results in the difference spectrum presented in Fig. 1. The spectrum is characterized by the absorption maximum at 425 nm and a trough at 405 nm and is identical to that reported by Chance[14] for the cytochrome *c* peroxidase complex *ES* of starved baker's yeast. In agreement with the previous data[14] it is found that in the presence of methanol catalase complex I does not contribute to the peroxidase complex *ES* absorbance peak at 425 nm. On the other hand, the 405-nm trough includes the contribution of both catalase complex I and peroxidase complex *ES*.

It is seen from the difference spectrum of Fig. 1 that the amount of cytochrome *c* peroxidase in the yeast can be conveniently measured in the presence of methanol and antimycin A at the wavelength pair 425–395 nm (Fig. 2). The absorbance change induced by the formation of the cytochrome *c* peroxidase complex *ES* increases with the increase in ethyl hydrogen peroxide concentration until the enzyme is quantitatively converted to its *ES* complex. The amount of cytochrome *c* peroxidase as determined by the double-reciprocal plot (lower part of Fig. 2) is 0.02–0.05 nmole/mg protein, for different mitochondrial preparations. (This value can be compared with 0.1–0.2 nmole of cytochrome *c* per mg protein present in these preparations.) Since the antimycin A leak establishes a certain steady-state concentration of reduced cytochrome *c* (*cf.* the upper curvature in the double reciprocal plot at high ethyl hydrogen peroxide concentration) the calculations were made for the values extrapolated to infinite ethyl hydrogen peroxide concentration.

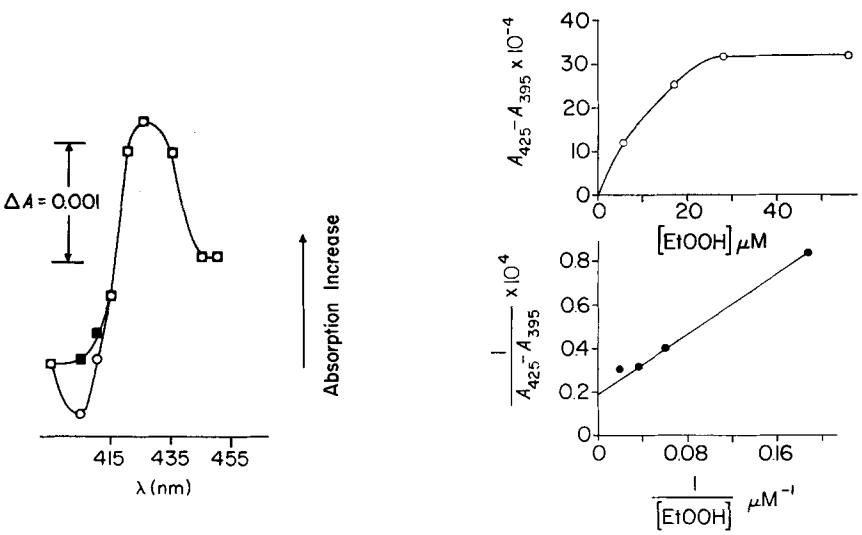

Fig. 1. Difference spectrum of yeast mitochondria with ethyl hydrogen peroxide in the presence (■) and absence (○) of methanol. The reaction mixture (3 ml) contained: 0.6 M mannitol–10 mM potassium phosphate–10 mM tris–maleate (pH 6.8), 0.5 mg antimycin A per mg protein and yeast mitochondria (1 mg protein per ml). The difference spectrum was obtained by plotting the extent of the absorbance change after the addition of 70 μM at each measuring wavelength with respect to 418 nm. The curve was repeated in the presence of 17 mM methanol.

Fig. 2. Determination of the cytochrome *c* peroxidase concentration in yeast mitochondria. The reaction mixture is described in Fig. 1; both antimycin A and methanol are present. Mitochondrial concentration 1.2 mg protein per ml. For details see the text. EtOOH, ethyl hydrogen peroxide.

Distribution of cytochrome c peroxidase within the mitochondrion

Since cytochrome *c* peroxidase serves as an alternative to the cytochrome oxidase pathway oxidizing mitochondrial cytochrome *c*, its localization within the mitochondrion is of primary importance for our understanding of the mechanism of action of this enzyme. Submitochondrial distribution of the enzymes in mammalian mitochondria has been tested by various independent techniques[15–17] but in the case of yeast mitochondria the difficulty arises that very little is known about the osmotic properties of the yeast mitochondrial membranes and the location of the various mitochondrial marking enzymes. Being aware of this difficulty, we compared the distribution pattern of cytochrome *c* peroxidase with that of malate dehydrogenase and succinate–cytochrome *c* reductase at various stages of treatment of the mitochondria. The assumption was made that malate dehydrogenase is located in the matrix space and succinate–cytochrome *c* reductase is tightly bound to the mitochondrial inner membrane. The results are presented in Table I. Total activity for cytochrome *c* peroxidase and malate dehydrogenase was assumed to be that found in the supernatant obtained after 2-fold sonication of the mitochondria (the pellet was devoid of these two enzymic activities). This supernatant did not contain, however, any succinate–cytochrome *c* reductase activity which at each stage of fractionation was connected with the particulate fraction.

TABLE I

COMPARATIVE DATA ON THE DISTRIBUTION OF CYTOCHROME *c* PEROXIDASE AND MALATE DEHYDROGENASE IN THE SOLUBLE FRACTIONS OF YEAST MITOCHONDRIA

Preparation of the soluble fractions is described in Results and the enzymatic assays were carried out as described under Methods. The activities are expressed as total activities (specific activity × total protein) and % refers to percent of total activity present in a particular soluble fraction.

	Protein		*Cytochrome c peroxidase activity*		*Malate dehydrogenase activity*	
	mg	%	*mmoles/min*	%	*mmoles/min*	%
Control						
1st washing	1.0	16	0.048	3.1	0.35	4.2
2 × sonicated	5.4	84	1.54	97.0	7.98	95.8
Experimental						
1st washing	0.6	8.9	0.050	3.2	0.36	5.5
Tris–phosphate swelling	0.3	4.8	0.355	23.3	0.59	9.0
Sonication	4.9	86.3	1.12	73.5	5.6	85.5

The results of Table I demonstrate that during hypotonic swelling in Tris–phosphate buffer, cytochrome *c* peroxidase and malate dehydrogenase are liberated to various degrees into the supernatant fraction. In the particular experiment presented in Table I about 23% of the peroxidase activity and 9% of the malate dehydrogenase

activity are recovered in this fraction (in other experiments up to 60 %of peroxidase and 35% of malate dehydrogenase activity were found in the supernatant after the swelling procedure).

Since cytochrome *c* peroxidase exhibits a greater tendency to "leak" out of the mitochondria than does the malate dehydrogenase a tentative conclusion can be drawn that at least part of the cytochrome *c* peroxidase is located in the intermembrane space between the outer and the inner membrane.

Generation of H_2O_2 by yeast mitochondria

As previously reported, H_2O_2 diffusing out of the mitochondria can be measured in the suspending medium. In Fig. 3 the rate of H_2O_2 generation occurring in yeast mitochondria is compared with that in pigeon heart mitochondria using the horse radish peroxidase–scopoletin method[2]. In confirmation of the results reported by Loschen *et al.*[2] addition of antimycin A in the presence of succinate enhances H_2O_2 generation in pigeon heart mitochondria as indicated by the rapid decrease in scopoletin fluorescence. The uncoupler, pentachlorophenol, diminishes antimycin A induced generation of H_2O_2. In contrast, yeast mitochondria do not seem to produce H_2O_2 significantly even in the presence of antimycin A.

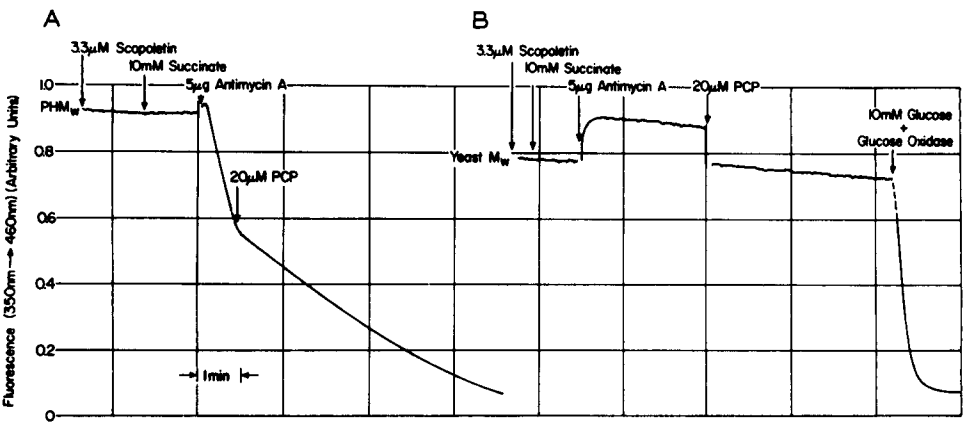

Fig. 3. Generation rate of H_2O_2 in pigeon heart and yeast mitochondria. The rate of H_2O_2 generation was measured fluorometrically using the scopoletin–horse radish peroxidase method of Loschen *et al.*[2]. Reaction mixture as in Fig. 1. 1 mg protein of pigeon heart mitochondria per ml (A) or 1.5 mg of yeast mitochondria per ml (B). PCP, pentachlorophenol.

This was further confirmed by the experiments in which the rate of H_2O_2 generation was followed by measuring the steady-state level of the catalase–H_2O_2 intermediate[1]. When 1.64 μM rat liver catalase was added to the suspension of yeast mitochondria, the steady-state concentration of the catalase–H_2O_2 intermediate was only 20% of its saturation value. This compares with 70–75% saturation observed in rat liver mitochondria[1]. From the 20% saturation one can estimate the rate of H_2O_2 production which corresponds at maximum to approx. 0.1 nmoles/min per mg protein[29]. Since, however, in the yeast mitochondria even in the presence of antimycin A and at high ethyl hydrogen peroxide concentration some decom-

position of cytochrome *c* peroxidase complex *ES* occurs (Fig. 2) our tentative explanation is that cytochrome *c* peroxidase is destroying the intramitochondrial H_2O_2 before it is able to diffuse out of the organelle.

Kinetics of the reaction of endogenous cytochrome c peroxidase with the mitochondrial cytochrome c

Soluble cytochrome *c* peroxidase reacts with soluble cytochrome *c* in one of the fastest reactions thus far described (second order velocity constant 10^8 $M^{-1} \cdot s^{-1}$). Yeast mitochondria afford a model system in which the reaction between the membrane bound cytochrome *c* and the mitochondrial peroxidase (most probably a soluble enzyme) can be studied *in situ*, in a much more physiological environment. In addition, the efficiency of cytochrome *c* peroxidase as an oxidant for cytochrome *c* can be directly compared with that of cytochrome oxidase, which is also present in the aerobic yeast mitochondria.

When anaerobic yeast mitochondria are rapidly mixed with oxygen, cytochrome *c* is oxidized as indicated by the rapid upward deflection of the trace (Fig. 4B). Most of the cytochrome *c* oxidation (85–90%) occurs during 20 ms of the flow time; the remaining 10–15% is oxidized much more slowly with a half-time of approx. 100–200 ms. In Fig. 4A, the anaerobic mitochondria are rapidly mixed with ethyl hydrogen peroxide. Cytochrome oxidase is in this case inhibited by previous addition of CO and the careful removal of traces of oxygen by passing helium gas through the solution of ethyl hydrogen peroxide. As is seen the oxidation trace of cytochrome *c* recorded under such conditions is biphasic: 50–60% of the absorbance change occurs in 20 ms of the flow time, the remaining 40% is oxidized in about 300–400 ms. This slow phase of the oxidation of cytochrome *c* is still faster than what would be expected for the oxidation of cytochrome *c* by CO-inhibited cytochrome

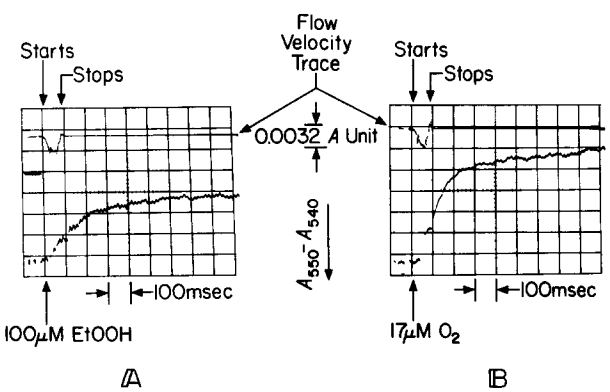

Fig. 4. Kinetics of oxidation of mitochondrial cytochrome *c* by cytochrome *c* peroxidase (A) and cytochrome oxidase (B). *S. cerevisiae* mitochondria were suspended in 0.6 M mannitol–0.5 mM EDTA–0.20 M potassium phosphate buffer (pH 6.9) at 5.1 mg protein per ml, 0.05% bovine serum albumin and 6.0 mM semicarbazide were present. The mitochondria were made anaerobic by the respiration of the endogenous substrate. 20 mM sodium malonate was then added to slow down the endogenous respiration and adjust the length of the cytochrome *c* oxidation cycle to about 10 s after the addition of 17 μM oxygen. In the experiments in which cytochrome *c* peroxidase was used as cytochrome *c* oxidant, 300 μM CO was present. Rapid kinetic technique was used as described in Methods. EtOOH, ethyl hydrogen peroxide.

oxidase. The extent of the initial rapid phase depends on the concentration of ethyl hydrogen peroxide and at saturating amounts of ethyl hydrogen peroxide, on the type of preparation. It is usually bigger in tightly coupled preparations. The rate of the slow phase can be substantially enhanced by the addition of antimycin A. This is to be expected if the rapid oxidation of the pool of carriers on the substrate side of the antimycin A block (in the absence of the inhibitor) tends to decrease the oxidized steady-state level of cytochrome *c*. Total amount of cytochrome *c* oxidized

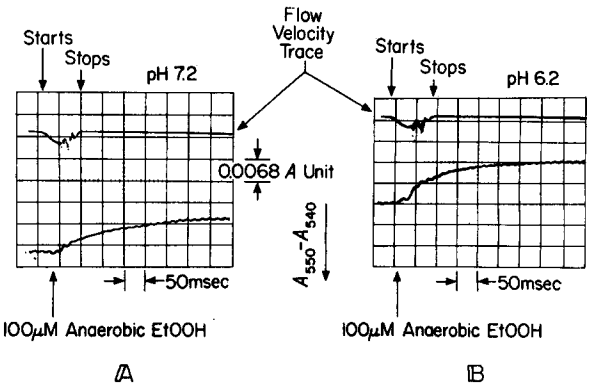

Fig. 5. The effect of pH on the rate of cytochrome *c* oxidation by cytochrome *c* peroxidase. Conditions were as those indicated in Fig. 4A. Protein concentration was 4.8 mg/ml.

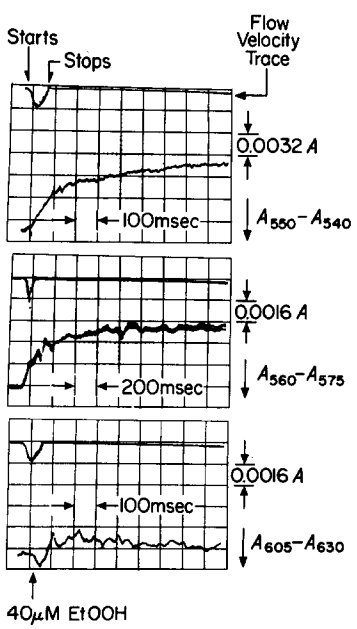

Fig. 6. Kinetics of oxidation of cytochrome *c*, b_{560} and a by cytochrome *c* peroxidase. Conditions were as those described in Fig. 4A. Protein concentration was 5.2 mg/ml.

in the presence of ethyl hydrogen peroxide is about 90% of the amount oxidized by oxygen. Thus most, if not all, of the endogenous cytochrome *c* is accessible to oxidation by cytochrome *c* peroxidase complex *ES*.

Oxidation of the soluble cytochrome *c* by the soluble cytochrome *c* peroxidase is strongly pH dependent[18]. Fig. 5 demonstrates that in the yeast mitochondria oxidation of cytochrome *c* induced by the addition of ethyl hydrogen peroxide also depends on the pH of the medium. It is slower when the pH is made more alkaline.

Oxidation of cytochrome *c* by cytochrome *c* complex *ES* is followed by the oxidation of the other respiratory chain carriers. Fig. 6 presents the time courses of oxidation of cytochrome b_{560} and cytochrome *a* obtained upon addition of ethyl hydrogen peroxide to the anaerobic suspension of the mitochondria. Closer correlation of the absorbance change at 605–630 and 445–458 nm (not shown) indicates that cytochrome a_3 is not oxidized under these conditions.

The role of cytochrome c peroxidase pathway in the energy coupling reactions

Since cytochrome *c* peroxidase complex *ES* is capable of rapid oxidation of the mitochondrial cytochrome *c* the question necessarily arises whether the operation of the pathway *in vivo* occurs through the "normal" sequence of the respiratory chain carriers and is coupled to the synthesis of ATP. Two types of experiments were specifically designed to test this suggestion: (1) by following the kinetic behavior of cytochrome b_{566} which as shown previously[19,20] can serve as a fairly sensitive test for the energy state of the membrane; (2) by direct measurement of the ^{32}P incorporation into [^{32}P]ATP upon addition of ethyl hydrogen peroxide to anaerobic yeast mitochondria. Fig. 7 compares the kinetic behavior of cytochrome b_{566} in the coupled and uncoupled yeast mitochondria using either oxygen (B) or ethyl hydrogen peroxide (A). The character of both traces is indistinguishable on the kinetic basis when either of the oxidants is used; the traces are biphasic in the coupled mitochondria with a small initial jump and slow second phase, and are monotonic and much more rapid in the presence of the uncoupler pentachlorophenol.

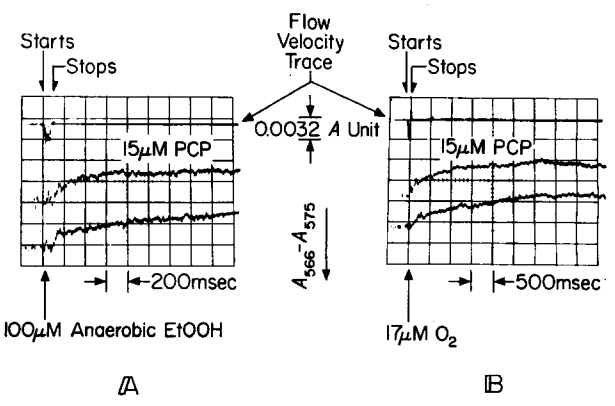

Fig. 7. Kinetics of oxidation of cytochrome b_{566} in coupled and uncoupled yeast mitochondria by cytochrome *c* peroxidase (A) and cytochrome oxidase (B). Conditions are those of Fig. 4A. Protein concentration was 4.5 mg/ml. The uncoupler was 15 μM pentachlorophenol (PCP).

Fig. 8 shows the time-dependent incorporation of ^{32}P into [^{32}P]ATP in the presence of ethyl hydrogen peroxide (analytical I and II) which suggests that the phosphorylation mechanisms are operative during anaerobic oxidation of cytochrome *c* by cytochrome *c* peroxidase. Since, however, the method can be subject to errors due to consumption of ethyl hydrogen peroxide by catalase any quantitative calculation of [^{32}P]ATP/ethyl hydrogen peroxide cannot be reliably made at present.

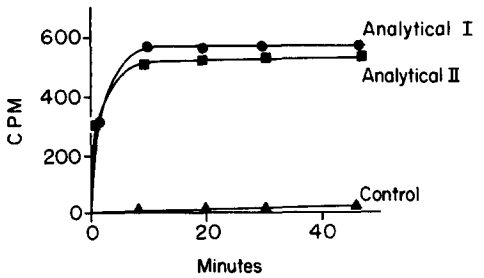

Fig. 8. Incorporation of ^{32}P in [^{32}P]ATP in anaerobic yeast mitochondria using cytochrome *c* peroxidase complex *ES* as cytochrome *c* oxidant. Conditions as specified in Methods. 70 μM ethyl hydrogen peroxide was used.

DISCUSSION

Cytochrome *c* peroxidase represents one of the better-known iron porphyrin proteins. Its mechanism of action *in vitro* has been studied extensively by the most sophisticated experimental techniques (for review, see Yonetani[21]), but the mechanism of cytochrome *c* oxidation *in vivo* has hardly been approached.

Cytochrome *c* peroxidase is found exclusively in aerobically grown yeast[4,22]. The biosynthesis of this enzyme is induced by oxygen in anaerobically grown cells which virtually lack the enzyme[23] though they contain apocytochrome *c* peroxidase[24]. Early direct spectroscopic studies of peroxidase in yeast cells[18] demonstrated rapid peroxide utilization and simultaneous oxidation of the intracellular ferrocytochrome *c*. The reaction was stimulated by the addition of glucose and inhibited by antimycin. Thus it was suggested that the rapid peroxide utilization was probably caused by the intracellular reaction of the peroxidase complex *ES* with ferrocytochrome *c* linked through the usual metabolic pathways to the utilization of glucose.

The object of this paper was to determine systematically structural–functional relationships of cytochrome *c* peroxidase *in vivo* in the intact yeast mitochondria. The two basic questions put forward were: (1) does the operation of the respiratory chain supply the mitochondrial peroxidase with peroxide; (2) does the peroxidase, when supplied adequately with peroxide, reduce the cytochrome at a rate comparable to that of the cytochrome oxidase.

It has been found previously[1–3] that the rate of the mitochondrial H_2O_2 generation in the mammalian mitochondria can be measured by the rate of its appearance in the suspending medium (it is explicitly assumed that the diffusion process is not rate limiting). We find, however, that in the intact yeast mitochondria the amount of H_2O_2 which diffuses out of the organelle is negligible. Since at least a part of cytochrome *c* peroxidase exists as a freely soluble enzyme in the intermembrane space (Table I) it can readily combine with H_2O_2 produced through the operation of

the respiratory chain and result in formation of cytochrome *c* peroxidase complex *ES*.

Detailed kinetic studies presented in this work lead to a number of interesting conclusions:

(1) Over 90% of the mitochondrial cytochrome *c* can be oxidized by cytochrome *c* peroxidase. Therefore, most, if not all, of the cytochrome *c* is accessible to this enzyme. It has been shown previously[25,26] that cytochrome *c* and the peroxidase form a reversible complex which can be detected by column chromatography. Employing chemically modified cytochrome *c* it was possible to demonstrate that electrostatic forces play a predominant role in the formation of the complex[25,26]. Our own data on the pH dependence of the rate of cytochrome *c* oxidation by the peroxidase (Fig. 5) and the effect of the ionic strength (not shown) of the medium seem to support such a suggestion.

(2) The apparent rate of oxidation of cytochrome *c* by the peroxidase is slower than that by the oxidase. Since the second order velocity constants for both isolated enzymes are about the same[9,27] this may suggest that either the mitochondrial cytochrome *c* is preferentially oriented towards the oxidase or that the equilibrium distribution of cytochrome *c* between the oxidase and the peroxidase is such that it favors the channeling of the reducing equivalents towards cytochrome oxidase.

(3) Cytochromes *b*, *c*, and *a* (as well as flavoproteins and pyridine nucleotides, see Chance[14]) can also be oxidized by cytochrome *c* peroxidase. The only respiratory chain component which does not undergo oxidation is cytochrome a_3. Since cytochrome a_3 under the experimental conditions used exists as cytochrome a_3–CO complex which has a very positive potential[28] it obviously does not equilibrate easily with cytochrome *a* and *c*.

(4) The behavior of cytochrome b_{566} with cytochrome *c* peroxidase as cytochrome *c* oxidant is indistinguishable on a kinetic basis from that observed with the oxidase. If the behavior of cytochrome b_{566} can be used as a reliable criterion for the energy state of the mitochondria (for rationale see refs 19 en 20) we must then accept that the mitochondrial membrane reaches the same energy state irrespective of whether the oxidase or the peroxidase is used to oxidize ferrocytochrome *c*. This suggestion has been confirmed by demonstrating that inorganic ^{32}P is incorporated into [^{32}P]ATP under anaerobic conditions upon addition of ethyl hydrogen peroxide.

In conclusion, mitochondrial cytochrome *c* peroxidase can rapidly react with H_2O_2 generated during the operation of the respiratory chain and efficiently oxidize ferrocytochrome *c*. Ferrocytochrome *c* is formed through the usual metabolic pathway coupled to the synthesis of ATP. Thus destruction of a very toxic by-product of the oxidative respiration is linked to an efficient system in which only one of the three operative phosphorylation sites is deleted.

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REFERENCES

1 Chance, B. and Oshino, N. (1971) *Biochem. J.* 122, 225–233
2 Loschen, G., Flohe, L. and Chance, B. (1971) *FEBS Lett.* 18, 261–266
3 Boveris, A., Oshino, N. and Chance, B. (1972) *Biochem. J.* 128, 617
4 Yonetani, T. and Ohnishi, T. (1966) *J. Biol. Chem.* 241, 2983–2984
5 Balcavage, W. X. and Mattoon, J. R. (1968) *Biochim. Biophys. Acta* 153, 521–525
6 Ohnishi, T., Kawaguchi, K. and Hagihara, B. (1966) *J. Biol. Chem.* 241, 1979–1806
7 Chance, B. and Hagihara, B. (1963) in *Proc. 5th Int. Congr. Biochem., Moscow* (Sissakian, N. N. M., ed.), Vol. 5, pp. 3–37, Pergamon Press, New York
8 Fukushima, K., Ito, A., Omura, T. and Sato, R. (1972) *J. Biochem. (Tokyo)* 71, 447–461
9 Yonetani, T. and Ray, G. S. (1966) *J. Biol. Chem.* 241, 700–706
10 Ochoa, S. (1955) *Methods Enzymol.* 1, 735–739
11 Chance, B., DeVault, D., Legallais, V., Mela, L. and Yonetani, T. (1967) in *Fast Reactions and Primary Processes in Chemical Kinetics, Nobel Symp. V* (Claesson, S., ed.), pp. 437–464, Interscience, New York
12 Conover, T. E., Prairie, R. L. and Racker, E. (1963) *J. Biol. Chem.* 238, 2831
13 Williams, H. R. and Moscher, H. S. (1954) *J. Am. Chem. Soc.* 76, 2984–2987
14 Chance, B. (1954) in *The Mechanism of Enzyme Action* (McElroy, W. D. and Glass, B., eds), pp. 399–453, Johns Hopkins Press, Baltimore, Md.
15 Sottocasa, G. L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967) *J. Cell Biol.* 32, 415–438
16 Parsons, D. F., Williams, G. R. and Chance, B. (1966) *Ann. N.Y. Acad. Sci.* 137, 643–656
17 Schnaitman, C. and Greenawalt, J. H. (1968) *J. Cell Biol.* 38, 158–175
18 Beetlestone, J. (1960) *Arch. Biochem. Biophys.* 89, 35–40
19 Chance, B., Wilson, D. F., Dutton, P. L. and Erecińska, M. (1970) *Proc. Natl. Acad. Sci. U.S.* 66, 1175–1182
20 Erecińska, M., Wagner, M. and Chance, B. (1973) in *Current Topics in Bioenergetics* (Sanadi, D. R., ed.), Vol. 5, in the press, Academic Press, New York
21 Yonetani, T. (1971) in *Advances in Enzymology* (Nord, F. F., ed.), Vol. 33, pp. 309–335, Interscience, New York
22 Altschul, A. M., Abrams, R. and Hogness, T. R. (1940) *J. Biol. Chem.* 136, 777
23 Chantrenne, H. (1955) *Biochim. Biophys. Acta*, 18, 58–62
24 Sels, A. A. and Cocriamont, C. (1968) *Biochem. Biophys. Res. Commun.* 32, 192–198
25 Mochan, E. (1970) *Biochim. Biophys. Acta* 216, 80–95
26 Nicholls, P. and Mochan, E. (1971) *Nature* 230, 276–277
27 Gibson, Q. H. and Greenwood, C. (1963) *Biochem. J.* 86, 541–554
28 Horie, S. and Morrison, M. (1963) *J. Biol. Chem.* 238, 1855–1860
29 Oshino, N., Chance, B., Sies, H. and Bücher, Th. (1973) *Arch. Biochem. Biophys.*, in the press