HYDROGEN PEROXIDE GENERATION BY THE ALTERNATE OXIDASE OF HIGHER PLANTS

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SUMMARY: Mitochondria isolated from mung bean hypocotyls, possessing a significant level of cyanide and antimycin A - resistant respiration via an alternate pathway, were assayed for hydrogen peroxide production by yeast cytochrome c peroxidase compound II formation. Rates of antimycin A - insensitive hydrogen peroxide production of 0.7-3 nmol/mg/min were observed which were too low to account for the observed oxygen consumption via the alternate pathway. However, further investigations revealed the presence of significant levels of catalase, peroxidase and hydrogen donor to peroxidase, even in gradient purified mitochondria and these could easily utilize any hydrogen peroxide produced by the alternate pathway. Similar experiments performed upon submitochondrial particles demonstrated a rate of H_2O_2 production which could easily account for the net electron flux through the alternate pathway. From these results, we postulate that the alternate pathway reduces oxygen only partially to hydrogen peroxide, and that the peroxidase and catalase activities of the mitochondria prevent its accumulation.

INTRODUCTION

Mung bean(Phaseolus aureus) mitochondria are known to possess an alternate pathway of electron transport to molecular oxygen which is insensitive to cyanide and antimycin A (1). Its branchpoint from the main respiratory pathway is thought to be at the level of ubiquinone (2). Although no significant accumulation of hydrogen peroxide (H_2O_2) due to the activity of the alternate pathway has been previously observed (3), a detailed investigation of the primary products of oxygen reduction by this pathway has been lacking.

The measured rates of H_2O_2 generation by whole mung bean mitochondria were found to account for no more than 5% of the observed oxygen uptake via the alternate pathway. Further investigations, however, revealed the presence of catalase and peroxidase (plus peroxidase hydrogen donor) assoc-

iated with both washed and gradient-purified mitochondria. These enzymes are capable of removing H_2O_2 produced, particularly since they would be concentrated close to the site of H_2O_2 production, and hence might lead to a serious underestimation of the real rate of H_2O_2 generation. In order to overcome these problems, measurements of H_2O_2 production and oxygen uptake *via* the alternate pathway were made with submitochondrial particles. In this case, it was found that H_2O_2 generation could easily account for the observed oxygen consumption *via* the alternate pathway, demonstrating that this oxidase only partially reduces oxygen, the observable product under appropriate conditions being H_2O_2.

MATERIALS AND METHODS

Preparation of Mitochondria and Submitochondrial Particles

Mitochondria from mung bean (*Phaseolus aureus*) hypocotyls were prepared according to the general method described by Bonner (4). Mung beans were grown for 5 days at 28°C in darkness and 60% relative humidity. Purified mitochondria were obtained by the method of Douce *et al* (5). Submitochondrial particles were prepared by sonication (2 x 25 s sonications at 40 watts power with a Branson Sonic Power Co. Sonifier, model W185) in a medium containing 250 mM sucrose, 15 mM $MgCl_2$, 1 mM ATP and 10 mM Tris-HCl, pH 7.5 at 10 mg/ml protein or by distilled water osmotic shock treatment of a mitochondrial pellet after the mitochondria had been suspended in 80% glycerol for 30 minutes. Submitochondrial particles were pelleted at 100 000 x g for 1 hour after an initial centifugation at 10 000 x g for 10 minutes to remove whole mitochondria.

H_2O_2, Catalase, Peroxidase and Hydrogen Donor Content Assays

H_2O_2 generation was determined by the yeast cytochrome *c* peroxidase method (6). Catalase activity was determined polarographically by measuring oxygen evolution with normal mitochondrial assay medium (4) which had been partially deoxygenated with argon. The reaction was started by addition of 185 µM H_2O_2. Alternatively H_2O_2 consumption was monitored directly at 240-280 nm and calculated with an extinction coefficient of 0.04 $mM^{-1}\ cm^{-1}$. Peroxidase activity was measured by the method of Gregory (7) or directly by monitoring ascorbate oxidation at 265-290 nm and calculated with an extinction coefficient of 15 $mM^{-1}\ cm^{-1}$. Stock solutions of ascorbate and H_2O_2 were quantitated spectrophotometrically both before and after the experiment. Internal hydrogen donor content was assayed by titration of scopoletin plus horseradish peroxidase with aliquots of H_2O_2 in the presence of varying amounts of mitochondria. A typical titration experiment is shown in figure 2, and the results were expressed in terms of nmols hydrogen donor per mg. of mitochondrial protein (table 2).

Oxygen Uptake Assays

Oxygen consumption was measured polarographically with a Clark-type oxygen electrode. The medium used was described by Bonner (4) of 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$ and 10 mM potassium phosphate buffer at pH 7.2. Additions were made as shown in the figures.

Protein

Protein was assayed by the method of Folin et al (8).

Materials and Chemicals

Cytochrome c peroxidase was prepared by the method of Yonetani and Ray (9). Scopoletin, antimycin A and horseradish peroxidase type VI were purchased from Sigma Chemical Company. Catalase was purchased from Boehringer-Mannheim Chemical Company and 1799 was obtained from Dr. P. Heytler, E.I. Dupont de Nemours Co., Wilmington, Delaware, U.S.A.

RESULTS

H_2O_2 Production and Oxygen Uptake by Whole Mitochondria

The rate of formation of H_2O_2 by isolated mung bean mitochondria in a variety of metabolic states as detected by yeast cytochrome c peroxidase Compound II formation is illustrated in figure 1. Upon addition of the mitochondria, a small but significant rate of H_2O_2 production was observed, probably due to the slow input of electrons into the respiratory chain from the endogenous reductants which are always present in plant mitochondria. The addition of succinate caused a marked stimulation of H_2O_2 production (figures 1 A, B). Upon transition to state 3, either by addition of ADP (figure 1A) or uncoupler (figure 1B), this rate decreased to a level just above the basal rate. The further addition of antimycin A under these conditions reactivated this rate, usually to near-maximal levels (figure 1B). When NADH was used instead of succinate as a source of reducing equivalents, a rather higher rate of H_2O_2 production was observed (figure 1C) and transition to state 3 did not markedly affect this rate. Similar rates were obtained with purified mung bean mitochondria in which state 4-3-4 transitions could be observed (figure 1D, E). The addition of 60 μM azide to the reaction medium did not significantly alter the observed rates of H_2O_2 release to the reaction medium.

Although these rates of H_2O_2 production were easily measurable by the CCP method, a comparison of the absolute rates with those of oxygen uptake by the mitochondria in various states (table 1) demonstrated that H_2O_2 production could not account for a significant part of the oxygen

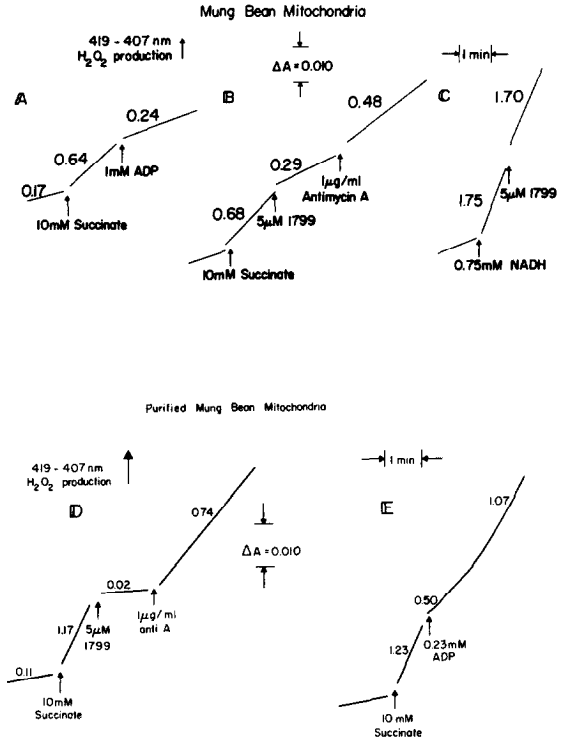

Fig. 1. H_2O_2 Generation in Intact Mung Bean Mitochondria as Measured by Cytochrome c Peroxidase Compound II Formation. Mitochondria (0.1-0.3 mg/ml protein) were suspended in a medium containing 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM potassium phosphate and 0.9 μM cytochrome c peroxidase, at pH 7.2 and 25°C. 0.3 mM ATP was also added when succinate was used as substrate. Additions were made as indicated. The numbers refer to H_2O_2 generation in nmol H_2O_2 produced/min/mg mitochondrial protein.

consumption by the alternate pathway. For example, in the presence of antimycin A and substrate under which condition the alternate pathway is fully operative, H_2O_2 production as measured by this method accounts for less than 5% of the observed oxygen consumption by intact mung bean mitochondria.

Endogenous Catalase, Peroxidase and Peroxidase Hydrogen Donor Levels in Plant Mitochondria.

Both peroxidase and catalase activities were assayed by two different methods (see methods section and table 2) and their rates were expressed both in internationally acceptable units and in terms of heme content per

Table 1. Oxygen Consumption and H_2O_2 Production by Intact Plant Mitochondria Under Various Conditions

Oxygen consumption was measured polarographically with a Clark-type oxygen electrode. H_2O_2 production was measured by the rate of CCP compound II formation. Mws, washed mitochondria, mps, gradient purified mitochondria.

Tissue and Conditions*	Oxygen Consumption	H_2O_2 Production
	nmol/mg/min @ 25°C	nmol/mg/min @ 25°C
Mung Bean		
Mws ± ATP	2.5	0.28
Mws + ATP + Succinate	58	0.71
Mws + ATP + Succinate + ADP	219	0.42
Mws + ATP + Succinate + 1799	219	0.31
Mws + ATP + Succinate + Anti A	37.7	0.68
Mws + NADH + 1799	230	1.82
Mps + ATP + Succinate + Anti A	111	0.74
Mps + NADH + Anti A	105	1.80

*ATP, 0.3 mM; succinate, 10 mM; ADP, 1 mM; 1799, 1 μM; Antimycin A, 1 μg/ml; NADH, 1 mM.

mg. of mitochondrial protein (table 2). Internal hydrogen donor was measured as described in the legend to figure 2. These measurements demonstrated that significant levels of catalase, peroxidase and endogenous hydrogen donor were associated with the mitochondria, even after density gradient purification.

H_2O_2 Production by Submitochondrial Particles

In order to test the possibility of a destruction of the H_2O_2 produced further, measurements of these parameters were made in mung bean

Table 2. Catalase, Peroxidase Hydrogen Donor Associated with Mung Bean Mitochondria and SMPs

SMPs were prepared as described in the text. Catalase activity [10] was measured (a) by measuring polarographically oxygen evolution in the reaction medium previously bubbled with argon, containing 185 μM H_2O_2 and approx. 0.2 mg. protein/ml and (b) by measuring H_2O_2 disappearance spectrophotometrically [11] at 240-280 nm (extn. coeff. = 0.04 $mM^{-1}cm^{-1}$) in the presence of 0.8 mM H_2O_2 and 0.02-0.3 mg protein/ml in 40 mM phosphate buffer pH 7.0. Acitivity is expressed in international units (μmol H_2O_2 evolved/mg. protein/min at 12.5 mM H_2O_2 and 25°C [12]) and in moles catalase heme/mg protein calculated according to K' = 1.16 x 10^7 $M^{-1}s^{-1}$ [13] and compared with beef liver catalase (Sigma C-100) which was found to have an activity of K' = 1.05 x $10^7 M^{-1}s^{-1}$.

The method of Gregory [7]was found unsuitable for quantitative work. Instead, peroxidase activity was determined by the oxidation of ascorbate at 265-290 nm (extn. coeff. = 15 $mM^{-1}cm^{-1}$) in a reaction medium containing 60 μM ascorbic acid, 100 μM H_2O_2, 1 mM EDTA, 40 mM phosphate pH 7.0 and 0.02-0.3 mg protein/ml. Under the conditions of the assay where $[H_2O_2]$ $[K_1]$ [ascorbate] $[K_4]$, then the peroxidase-H_2O_2 complex behaves as the rate limiting step [10] and allows the use of the K_4 to determine enzyme content. Activity is expressed in nmol ascorbate/min/mg protein in the conditions of the assay and as moles equivalent of horseradish peroxidase heme calculated according to the second order rate constant for ascorbate of K_4 = 1.2 x $10^4 M^{-1}s^{-1}$ [14].

Peroxidase H-donor was measured as described in the text.

* Numbers in brackets refer to the NADH supported antimycin A - insensitive oxygen uptake in the presence of 0.25 mM HRP.

	Respiration* (NADH + Anti-A)	H_2O_2 Prodn. (NADH + Anti-A)	Peroxidase		Catalase		Peroxidase H-Donor
	nmol O_2/mg/min @ 25°C	nmol H_2O_2/mg/min @ 25°C	nmol asc/mg/min @25 C & 60 μM asc	pmol HRP heme/mg p.	Int. units	pmol heme/mg. p.	nmol/mg. p.
Mws	39	1.5-3	0.23	6.30	37.4	6.22	3.5
Mps	105	1.80	0.13	3.61	26.0	4.34	3.3
SMPs(sonicated)	4.8 (16)*	17.5	1.87	51	14.8	2.5	---
SMPs (osmotic shock)	17.5 (26.5)*	25	1.5	41	22.6	3.73	---

asc - ascorbate p. - protein

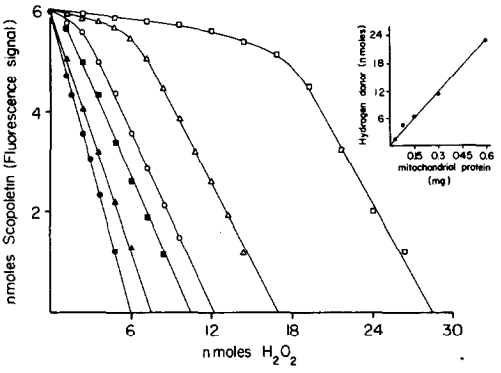

Fig. 2. The Titration of Endogenous Mitochondrial Hydrogen Donor to Peroxidase by a Modification of the Scopoletin-Horseradish Peroxidase Assay of H_2O_2 Production. Hydrogen donor content in skunk cabbage mitochondria was determined by titration of 2 μM Scopoletin with aliquots of H_2O_2 in the presence of 0.3 μM HRP and 0 (●), 0.01 (▲), 0.025 (■), 0.05 (○), 0.1 (△) or 0.2 mg (□) protein/ml in 20 mM phosphate buffer pH 7.2.

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submitochondrial particles (table 2). It can be seen that in this case, hydrogen peroxide production is able easily to account for the observed antimycin A - insensitive oxygen consumption via the alternate pathway. The residual catalase and peroxidase activities are presumably diluted into the whole reaction medium in these assays, under which conditions they do not interfere with the cytochrome c peroxidase assay, since under such conditions the cytochrome c peroxidase is able to efficiently compete with it for the H_2O_2 produced. In fact, the measured H_2O_2 produced usually exceeded the observed oxygen consumption by a factor of about two. This may be explained by the fact that the residual catalase, when not in competition for the H_2O_2 with CCP, was able to destroy the H_2O_2 and hence reduce the observed oxygen consumption by a factor of two. When the submitochondrial particle antimycin A - insensitive respiration was measured in the presence of 0.25 mM horseradish peroxidase to "trap" the H_2O_2, the observed rate did actually increase by approximately a factor of two.

These experiments could not be performed with succinate as substrate because of the low level of succinic dehydrogenase in submitochondrial particles.

DISCUSSION

Identification of the product of oxygen reduction by the alternate pathway of higher plants is of major importance when considering the possible nature and mechanism of the process. Reduction of molecular oxygen to water (i.e., a 4 electron process) would require a rather more sophisticated oxidase than one which partially reduces oxygen to H_2O_2 either directly or *via* the superoxide dismutase reaction: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. The data described in this paper has strongly suggested to us that the alternate oxidase of plant mitochondria does only partially reduce oxygen, the observable product under appropriate conditions being H_2O_2. Whether it is a one-electron process of reduction to superoxide working in conjunction with superoxide dismutase or a two-electron reduction to hydrogen peroxide is at present being investigated.

The fact that H_2O_2 generated by the alternate pathway never accumulates in the mitochondria explains why its production has been previously overlooked. It would appear that there are significant levels of both peroxidase and catalase associated with the mitochondria, even after density gradient purification. Although it has been previously thought these are merely contaminants (15), we would suggest that they may be functional and may have an intramitochondrial location.

In the light of these new findings, we would suggest that plant thermogenesis proceeds *via* the highly exothermic catalatic degradation of H_2O_2 produced by the alternate pathway. A possible biosynthetic role of the alternate pathway in supplying H_2O_2 for peroxidatic reactions is also being considered.

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REFERENCES

1. Henry, M.-F. and Nyns, E.-J. (1976) Sub.-Cell. Biochem. 4, 1-65.
2. Rich, P.R. and Moore, A.L. (1976) FEBS Letts., in press.
3. Bendall, D.S. and Bonner, W.D. Jr. (1971) Plant Physiol. 47, 236-245.
4. Bonner, W.D. Jr. (1967) in Methods in Enzymology (Estabrook, R.W. and Pullman, M.E.,eds.), Vol. X, pp. 126-133, Academic Press, New York.
5. Douce, R., Christensen, E.L. and Bonner, W.D. Jr. (1972) Biochim. Biophys. Acta 275, 148-160.
6. Boveris, A., Oshino, N. and Chance, B. (1972) Biochem. J. 128, 617-630.
7. Gregory, R.P.F. (1966) Biochem. J. 101, 582-583.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
9. Yonetani, T. and Ray, L. (1965) J. Biol. Chem. 240, 4503-4508.
10. Chance, B. and Mahely, A.C. (1965) in Methods in Enzymology 2 764-775.
11. Chance, B. (1954) in Methods of Biochemical Analysis (Glick, D., ed), pp. 408-424, Interscience, New York.
12. Luck, H. (1963) in Methods in Enzymatic Analysis (Bergmeyer, H.U., ed), p. 885, Academic Press, New York.
13. Sies, H., Bucher, T., Oshino, N. and Chance, B. (1973) Arch. Biochem. Biophys. 154, 106-116.
14. Chance, B. (1949) Arch. Biochem. 22, 224-252.
15. Plesnicar, M., Bonner, W.D. Jr. and Storey, B.T. (1967) Plant Physiol. 42, 366-370.