EVIDENCE FOR THE EXISTENCE OF CATALASE IN THE MATRIX SPACE OF RAT-HEART MITOCHONDRIA

Hans NOHL and Dietmar HEGNER

Institute of Pharmacology, Toxicology and Pharmacy, Faculty of Veterinary Medicine, University Munich, 13, Veterinärstraße D-8000 Munich 22, FRG

Received 16 February 1978

1. Introduction

Despite the ability of isolated rat-heart mitochondria to produce H_2O_2 under state 4 conditions [1,2], it was concluded that state 4 respiration cannot occur in vivo on account of the apparent absence of H_2O_2-destroying enzyme systems [1–5]. In addition, H_2O_2 per se was shown to be without effect on such sensitive mitochondrial functions as reversed electron flow and energy conservation [6]. However, the participation of this compound as an essential intermediate in the initiation of lipid peroxidation appears to be widely established [7–10]. This concept is in agreement with the findings [11], namely that lipid peroxides are formed in vivo by a radical-generating system involving H_2O_2. The existence of these compounds in rat-heart mitochondria of living cells poses the question as to whether or not mitochondria from rat-heart are equipped with enzymes for metabolizing H_2O_2. This paper reports on the presence of catalase in the matrix of rat-heart mitochondria by various techniques.

2. Materials and methods

Catalase, antimycin A and FCCP were purchased from Boehringer, Mannheim; 3-amino-1,2,4 triazole from Fluka AG, Switzerland and 3,3′-diaminobenzidine tetrahydrochloride from Schuchardt GmbH, Munich. Rat-heart mitochondria were prepared as in [12]. Disruption of mitochondrial membranes was carried out by sonicating batches of 0.7 ml in Netheler cups (10 mg/ml) at 40 W with a Branson sonifier. Sonication for 40 s was performed in bursts of 3 s, with cooling to 0°C in the intervals. Submitochondrial particles (SMP) and supernatants from high-speed centrifugation were prepared as in [11]. Protein was determined by a modified biuret method [13]. Oxygen was determined polarographically using a micro Clark-type electrode.

Abbreviations: FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; HEPES, N-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SMP, submitochondrial particles

3. Results and discussion

3.1. *Polarographic measurement of catalase activity*

The addition of freshly isolated rat-heart mitochondria to an anaerobic solution containing 5 mM H_2O_2 results in a slow rate of O_2 formation (fig.1A). Ultrasonic disruption of the mitochondrial membrane stimulated O_2 production 3–5-fold, suggesting the existence of diffusion barriers, as already known from studies with peroxisomes [14]. The identity of the enzyme was tested by its sensitivity towards KCN and HN_3. EDTA had no effect on hydrogen peroxide metabolism, indicating that the presence of iron or similar metallic ions is not critical. The protein nature of the catalyst has been demonstrated by the destruction of H_2O_2-decomposing activity by heating mitochondria for 1 h at 70°C (fig.1B). Mitochondria isolated from rats pretreated with 3-amino-1,2,4 triazole (0.13 g/100 g body wt), which is a

Elsevier/North-Holland Biomedical Press

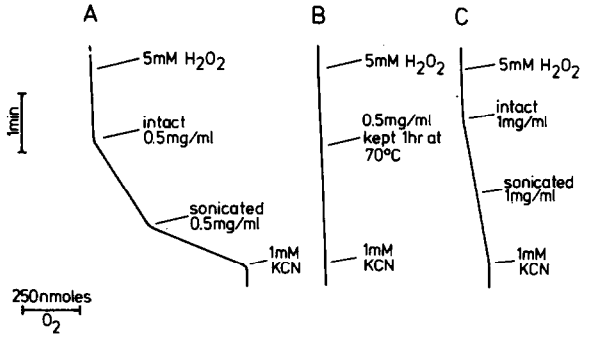

Fig.1. Existence and characterization of mitochondrial H_2O_2 decomposition. H_2O_2 was added to a reaction medium (0.25 M sucrose, 50 mM HEPES, 2 mM EDTA, pH 7.4) equilibrated with oxygen-free nitrogen at 25°C. Rates of H_2O_2 decomposition following the addition of intact or sonicated mitochondria were followed by the rate of O_2 accumulation in the buffer. The same inhibitory effect of CN^- was also obtained with HN_3. All solutions added were in equilibrium with N_2. Other details are given in the text.

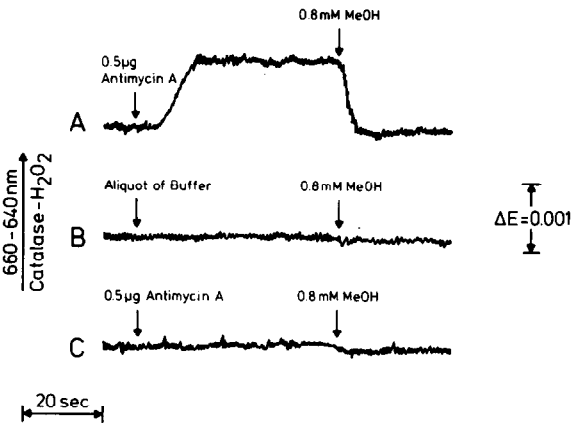

Fig.2. Steady state of catalase–H_2O_2 intermediate. (A) 0.8 mg mitochondria were suspended in a buffer (0.25 M sucrose, 50 mM HEPES, 2 mM EDTA, pH 7.4) saturated with oxygen at 25°C. H_2O_2 formation was initiated on addition of 0.5 µg antimycin A, after mitochondria were supplemented with 6 mM succinate and 10 µg FCCP. The formation and steady state of compound I was followed at 660–640 nm in a dual-wavelength spectrophotometer (American Instrument Company, MD). (B) Conditions were those of (A), except for the lack of antimycin A, which was replaced by an aliquot of the buffer. (C) Rat-heart mitochondria were prepared 2 h after intraperitonal injection of 3-amino-1,2,4 triazole; other conditions are given under (A). The traces are representative of 16 (A), 6 (B) and 5 (C) experiments.

specific inhibitor of catalase [15], were 95% less active in producing O_2 from H_2O_2. Ultrasonic treatment of this mitochondrial preparation failed to stimulate H_2O_2 decomposition (fig.1C). These observations strongly support the assumption that the catalyst under study is identical with catalase.

3.2. *Spectrophotometric detection of the catalase-H_2O_2-intermediate*

When a constant rate of H_2O_2 generation is maintained in microorganisms [16,17] or in perfused rat liver [18,19], a steady state concentration of the catalase–H_2O_2 intermediate (compound I) is established and can be measured at $\Delta A_{660-640}$ nm. The ability to form this compound was taken as evidence for the existence of catalase in the preparation. Generation of H_2O_2 was initiated in FCCP-uncoupled succinate-respiring rat-heart mitochondria following the addition of antimycin A [11]. A small absorbance increase indicated the formation of compound I (fig.2A). Addition of 0.8 mM methanol revealed the existence of the catalase–H_2O_2 complex by causing the absorbance to return to its original level [20]. The catalase–H_2O_2 intermediate could not be generated in the absence of antimycin A because no H_2O_2 is produced (fig.2B). Additional evidence indicating that the $\Delta A_{660-640}$ results from the change in steady state concentration of the catalase–H_2O_2 intermediate comes from the use of 3-amino-1,2,4 triazole. Mitochondria prepared 2 h after an intraperitoneal injection of the substance exhibited no detectable antimycin A- or methanol-induced absorbance change (fig.2C). The capacity of the preparation for H_2O_2-formation however, was not affected by 3-amino-1,2,4 triazole pretreatment. This was tested in a separate experiment by following the rate of fluorescence decrease of reduced scopoletin in the presence of horseradish peroxidase [4,21].

3.3. *Localization of catalase within the mitochondrion*

From the evident increase in catalase activity after ultrasonic disruption of the membrane (fig.1), one might conclude that the enzyme is located in the matrix space of the mitochondria. If this were the case,

then either H_2O_2 diffusion from the solution to the enzyme or oxygen diffusion from the enzyme into the solution would be limited as long as the enzyme is embedded within the membrane. The following experiments were performed to investigate the permeability of the mitochondrial membrane with regard to H_2O_2. The cytochromes of freshly isolated rat-heart mitochondria were reduced by the addition of succinate; oxygen was removed by saturating the suspension with oxygen-free nitrogen. Introduction of H_2O_2 into the reference cuvette in the dual-wavelength spectrophotometer resulted in an immediate oxidation of the cytochromes, which was indicated by the appearance of difference spectra (fig.3). The increase of cytochrome oxidation was found to depend on the H_2O_2 concentration in the medium. In the presence of 110 μM H_2O_2, the increase of cytochrome $c+c_1$ oxidation did not exceed 40% of the maxima which were obtained after having bubbled the suspension with oxygen or after the addition of a small amount of exogeneous catalase.

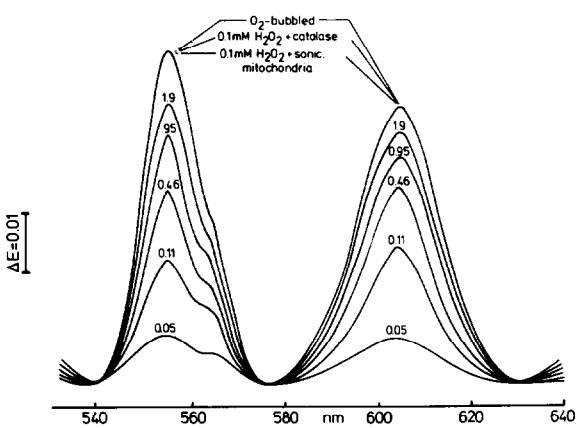

Fig.3. Oxidation of the cytochromes at different concentrations of H_2O_2. Freshly-prepared mitochondria, 0.65 mg, were suspended in 0.5 ml anaerobic sucrose buffer and allowed to react with 6 mM succinate 2 min prior to the addition of an anaerobic solution of H_2O_2. The difference spectra were measured immediately after the introduction of H_2O_2 into the reference cuvette of a dual-wavelength spectrophotometer. The cuvettes were closed with gas-tight covers. The spectra at different concentrations of H_2O_2 (the numbers associated with the curves represent mM H_2O_2) were each obtained with another mitochondrial suspension. The spectra are representative of 12 experiments. Further details are given in the text.

Difference spectra became maximal at this concentration range of H_2O_2, as when ultrasonically-treated rat-heart mitochondria were used. Additional amounts of catalase were in this case without effect. The increase in cytochrome oxidation as a function of H_2O_2 concentration is consistent with the supposition that the mitochondrial membrane presents a barrier for H_2O_2 diffusion to catalase and, second, that this barrier is no longer in existence when the enzyme is released into the solution after sonic disruption of the membrane. Further support for this concept comes from the fact that the cytochromes of intact rat-heart mitochondria do not become fully oxidized in the presence of relatively high concentrations of H_2O_2. For example, 1.9 mM H_2O_2 should yield 0.95 mM oxygen on total decomposition, assuming that the accessibility of catalase for H_2O_2 is not critical. This oxygen concentration is about 4 times higher than that of a solution in equilibrium with air at 25°C and exceeds the K_m of the electron transport chain for O_2 by a factor of 10^3 [22,23]. Oxygen diffusion across the mitochondrial membrane from the matrix into solution (using intact rat-heart mitochondria) and vice-versa (using ultrasonically-treated rat-heart mitochondria) was tested as a function of mitochondrial respiration in an anaerobic solution containing H_2O_2 as the only source of oxygen (fig. not shown). Addition of succinate (state 1 – state 4 transition) and ADP (state 4 – state 3), as well as uncoupling of the respiration, was each accompanied by a check to the accumulation of oxygen in the solution. Furthermore, a decrease in oxygen concentration was seen when ultrasonically-treated rat-heart mitochondria were supplemented with succinate. Oxygen consumption was measured in another set of experiments under the above conditions in an aerobic suspension. The amounts of oxygen used for state 4 and state 3 respiration, respectively, were found to be in the same range as those calculated from the decrease of oxygen production from H_2O_2 in the anaerobic buffer system. One may conclude from these data that the oxygen flux through the mitochondrial membrane is under the control of oxygen-consuming processes, and not limited by the membrane itself. The apparently low activity of catalase as measured in intact mitochondria may therefore be explained as a result of the spatial separation of the enzyme from its substrate by the inner membrane, which appears

to impose an imperfect permeability barrier to H_2O_2. The existence of catalase in the matrix of mitochondria was further established by the change of catalase activity in following repeated sonication and washing (SMP). As demonstrated in fig.4 the specific activity of catalase in SMP decreases after each wash, while its activity in the supernatant after high-speed centrifugation increases markedly. Further evidence for the existence and localization of catalase comes from cytochemical investigations with crude mitochondrial fractions, using the alkaline diaminobenzidine technique for visualization of peroxidatic activity of catalase [24]. Figure 5 shows the electron micrograph of a mitochondrial preparation incubated in diaminobenzidine–H_2O_2 medium at pH 10.5. Alkaline conditions were chosen to prevent a reaction with cytochrome oxidase [25,26]. The preparation revealed no peroxisomal contamination; the reaction products of oxidation of diaminobenzidine (dark spots) are localized exclusively within the mitochondrial matrix. The cytochemical detection

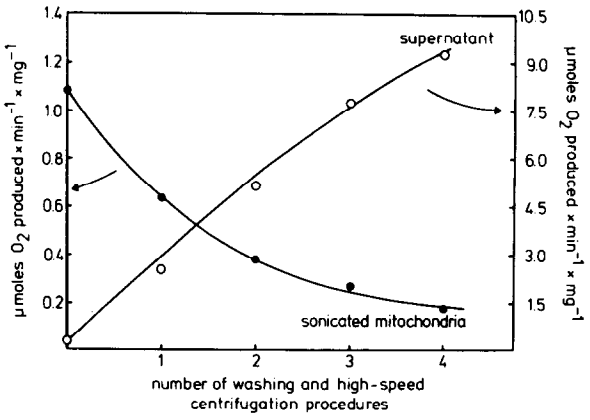

Fig.4. Release of catalase from rat-heart mitochondria into the supernatant from high-speed centrifugation after repeated sonication and washing. Specific activities of catalase were measured in the amount of supernatant which was recovered from corresponding mitochondrial particles after high-speed centrifugation. The total release of catalase into the supernatant after consecutive washings was calculated as the sum of the actual and preceding activities.

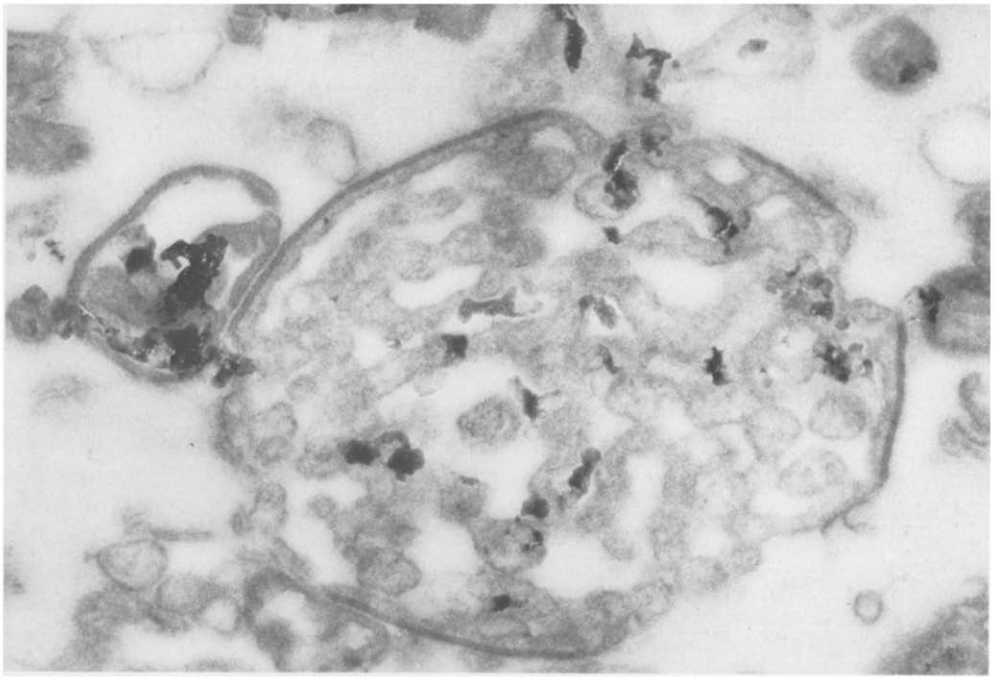

Fig.5. Electron micrograph of a section of a glutaraldehyde-fixed mitochondrial fraction in 10^{-4} M 3,3′-diaminobenzidine and 10^{-1} M H_2O_2, pH 10.5 (× 50 000). For more methodical details see [26].

of catalase not only supports the biochemical findings but also the interpretations that catalase is indeed present in the matrix of rat-heart mitochondria, and not as a contaminant by peroxisomes.

Acknowledgements

The excellent technical assistence of P. Hauptmann is gratefully acknowledged. We are greatly indebted to Dr H. G. Liebich who performed the electron microscopy. We thank Dr V. Herzog for helpful discussions.

References

[1] Loschen, G., Flohé, L. and Chance, B. (1971) FEBS Lett. 18, 261–264.
[2] Loschen, G., Azzi, A. and Flohé, L. (1973) FEBS Lett. 33, 84–88.
[3] Loschen, G., Azzi, A. and Flohé, L. (1973) in: Glutathione, Proc. 16th Conf. Germ. Soc. Biol. Chem., Tübingen (Flohé, L. Benöhr, H. Ch., Sies, H., Waller, H. D. and Wendel, A. eds) pp. 228–236, Georg Thieme, Stuttgart.
[4] Loschen, G. (1975) Thesis, Univ. Tübingen.
[5] Boveris, A. and Chance, B. (1973) Biochem. J. 134, 707–716.
[6] Azzi, A., Loschen, G. and Flohé, L. (1973) in: Glutathione, Proc. 16th Conf. Germ. Soc. Biol. Chem., Tübingen (Flohé, L., Benöhr, H. Ch., Sies, H., Waller, H. D. and Wendel, A. eds) pp. 237–242, Georg Thieme, Stuttgart.
[7] Beauchamp, C. and Fridovich, I. (1970) J. Biol. Chem. 245, 4641–4646.
[8] Fong, K. L., McCay, P. B., Poyer, J. L., Keele, B. B. and Misra, H. (1973) J. Biol. Chem. 248, 7792–7797.
[9] Bors, W., Saran, M., Lengfelder, E., Spöttl, R. and Michel, C. (1974) Curr. Top. Radiat. Res. Q. 9, 247–309.
[10] Fridovich, I. (1975) Ann. Rev. Biochem. 44, 147–159.
[11] Nohl, H. and Hegner, D. (1978) Eur. J. Biochem. 82, 563–567.
[12] Szarkowska, L. and Klingenberg, M. (1963) Biochem. Z. 338, 674–697.
[13] Bode, Ch., Goebell, H. and Stähler, E. (1968) Z. Klin. Biochem. 6, 418–422.
[14] DeDuve, Ch. and Baudhuin, P. (1966) Physiol. Rev. 46, 323–357.
[15] Price, V. E., Sterling, W. R., Tarantola, V. A., Hartley, R. W. and Richcigl, M. (1962) J. Biol. Chem. 237, 3468–3475.
[16] Chance, B. (1952) Science 116, 202–203.
[17] Chance, B. (1954) Science 120, 767–775.
[18] Sies, H. and Chance, B. (1970) FEBS Lett. 11, 172–176.
[19] Sies, H., Bücher, Th., Oshino, N. and Chance, B. (1973) Arch. Biochem. Biophys. 154, 106–116.
[20] Oshino, N., Chance, B., Sies, H. and Bücher, Th. (1973) Arch. Biochem. Biophys. 154, 117–131.
[21] Andreae, W. A. (1955) Nature 175, 859–860.
[22] Degn, H. and Wohlrab, H. (1971) Biochim. Biophys. Acta 245, 347–335.
[23] Bienfait, H. F., Jacobs, M. C. and Slater, E. C. (1975) Biochim. Biophys. Acta 376, 446–457.
[24] Fahimi, H. D. (1969) J. Cell. Biol. 43, 275–288.
[25] Seligman, A. M., Karnovsky, M. J., Wasserkrug, H. L. and Hanker, J. S. (1968) J. Cell. Biol. 38, 1–14.
[26] Herzog, V. and Fahimi, H. D. (1976) Histochemistry 46, 273–286.