INHIBITION OF LIPID PEROXIDATION IN ISOLATED INNER MEMBRANE OF RAT LIVER MITOCHONDRIA BY SUPEROXIDE DISMUTASE

Rainer ZIMMERMANN, Leopold FLOHÉ*, Ulrich WESER and Hans-Jürgen HARTMANN
Physiologisch-Chemisches Institut der Universität Tübingen, Germany

Received 23 November 1972

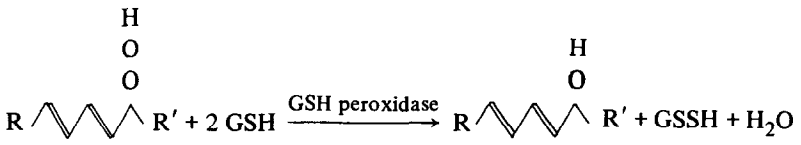
1. Introduction

Lipid peroxidation in mitochondrial membranes results in loss of respiratory control, lack of contractibility and final lysis of mitochondria [1–3]. It may be induced by incubation of isolated mitochondria in the presence of oxygen with either chaotropic agents [4] or autoxidizable substances like ascorbate [2] or thiol compounds [3]. Besides, formation of lipid peroxides in livers of tocopherol deficient rats has been observed under *in vivo* conditions [5] and may, therefore, have a pathophysiological significance.

Two enzymes, GSH peroxidase (C-factor I) [1] and catalase (C-factor II) [1], were found to prevent peroxidation of unsaturated lipids in suspensions of mitochondria or "high amplitude swelling" which is related to lipid peroxidation [2, 3, 6, 7]. These observations suggest an involvement of H_2O_2 which may for instance be built according to the equation

$$2\,GSH + O_2 \rightarrow GSSG + H_2O_2 \qquad (1)$$

The higher efficiency of GSH peroxidase may be explained by the additional ability of this enzyme to reduce hydroperoxides of unsaturated lipids according to equation (2) [8, 9]



(2)

On the other hand, the observations that minute amounts of tocopherol can stop lipid peroxidation completely [2] suggests that free radicals rather than H_2O_2 are mainly responsible for the oxidative destruction of the unsaturated lipids. A free radical chain would be initiated for instance by glutathione as follows:

$$GS^- + O_2 \rightarrow GS\cdot + \cdot O_2^- \qquad (3)$$

According to McCord and Fridovich, erythrocuprein dismutates the superoxide anion to oxygen and H_2O_2 [10] with a rate constant of about $2 \times 10^9\ M^{-1}\ sec^{-1}$ [11, 12] and may, therefore, be used as a sensitive tool to detect the participation of $\cdot O_2^-$ in the maintenance of a free radical chain [13]. In the present communication we describe that superoxide dismutase substantially inhibits GSH induced lipid peroxide formation in isolated inner membrane of rat liver mitochondria.

2. Materials and methods

2.1. *Materials*

Liver mitochondria of male wistar rats (230–250 g)

* Where reprint requests should be addressed.

were prepared according to [14]. The inner membrane fraction was isolated according to the method of Parsons et al. [15] and further purified as described by Whereat et al. [16]. Superoxide dismutase (erythrocuprein) was isolated from bovine blood using the procedures reported in [10] and [17]. The preparation was chromatographically and electrophoretically homogeneous. The visible and UV absorption properties were:

$$\frac{\epsilon_{280}}{\epsilon_{259}} = 0.590 \text{ and } \frac{\epsilon_{259}}{\epsilon_{680}} = 31.5$$

2.2. *Incubation procedure*

Suspensions of mitochondrial membranes (0.27 mg protein/ml) in 18 ml medium (0.02 M Tris-HCl, pH 7.4, 0.12 M KCl) containing 20 nmoles GSH/ℓ were agitated at 30° with plastic stirrers at 240 rpm in open beakers (50 ml) to guarantee a sufficient oxygen supply. Controls were run by omission of GSH. Catalase (beef liver) and/or superoxide dismutase, respectively, were added immediately at the beginning of the incubation. The concentrations of enzymes are given in the figure. At suitable intervals probes were removed for analytical purposes.

2.3. *Analytical procedures*

The protein concentration was estimated by the method of Lowry et al. [18]. The molarity of catalase was evaluated by determination of the absorption in the Sorret region [19]. The concentration of superoxide dismutase was calculated by use of the molar extinction coefficient $\epsilon_{259} = 9840$ [17]. Lipid peroxides were estimated as malone dialdehyde by the thiobarbiturate method as described [2].

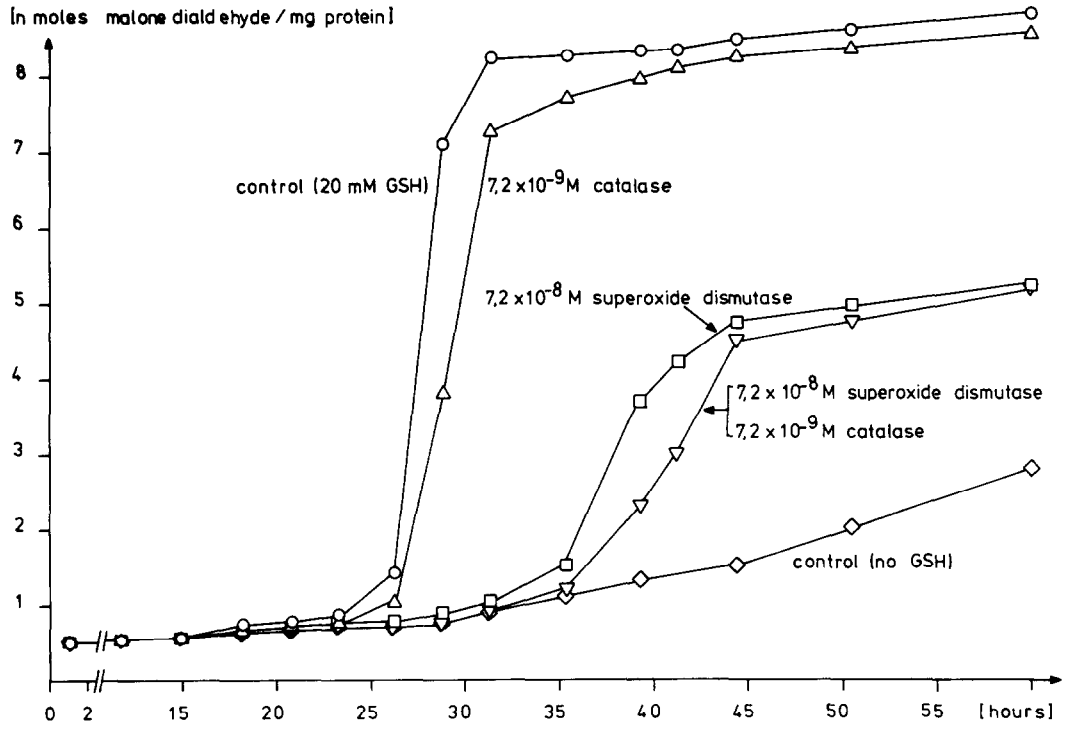


Fig. 1. Inhibition of GSH-induced lipid peroxide formation in isolated inner membrane of rat liver mitochondria by catalase and/or superoxide dismutase. Conditions of incubation: 0.02 M Tris-HCl, pH 7.4, 0.12 M KCl, 0.27 mg protein/ml, 30°. (◇—◇—◇) No additions; (○—○—○) 20 mM GSH; (△—△—△) 20 mM GSH, 7.2 $\times 10^{-9}$ M catalase; (□—□—□) 20 mM GSH, 7.2 $\times 10^{-8}$ M superoxide dismutase; (▽—▽—▽) 20 mM GSH, 7.2 $\times 10^{-9}$ M catalase and 7.2 $\times 10^{-8}$ M superoxide dismutase. Lipid peroxide formation was estimated by the thiobarbituric acid procedure [2]. The degree of lipid peroxide formation is calculated as nmoles of malone dialdehyde per mg of mitochondrial protein.

3. Results

In purified inner membranes of rat liver mitochondria GSH-induced peroxidation of lipids occurs only after a considerable lag phase of several hours, whereas it reaches a maximum value after about 5 hr in intact mitochondria [6]. The absence of additional oxidation reduction catalysts like non heme iron proteins or soluble cytochromes in the purified membrane fraction may account for this delay [4, 20]. The rate of peroxide formation and the length of the lag phase largely varied with different membrane preparations. In the experiment shown in fig. 1 GSH-induced lipid peroxide formation in the absence of any protective enzyme starts significantly after about 25 hr of incubation. Within a relative short period of further 5–6 hr a maximum level of thiobarbituric acid positive material is achieved. In the absence of GSH only a slow increase of lipid peroxides is observed starting after 35–40 hr of incubation.

The presence of catalase at a concentration of 7.2×10^{-9} M causes a delay of the initial burst of peroxide formation of about 2 hr but does not effect the final level of peroxidized lipids. As can be seen from table 1, inhibition of lipid peroxide formation by catalase does not depend on the concentration of the enzyme in a linear sense.

In the presence of 7.2×10^{-8} M superoxide dismutase the lag phase of lipid peroxide formation was prolonged by about 10 hr and the maximum level of lipid peroxides was not reached within 60 hr. Experiments at lower concentrations of superoxide dismutase (not shown) revealed an inhibitory effect comparable to that of catalase. As can be seen from the data of table 1, a further increase of catalase concentration does not result in a proportional decrease of peroxidized lipids if a certain degree of inhibition by catalase is achieved. In contrast, the addition of catalase to the superoxide dismutase protected membranes further inhibits the production of malone dialdehyde to an extent comparable to that observed with unprotected membranes. This roughly additive inhibition of lipid peroxide formation by catalase and superoxide dismutase was established in three independent experiments with different membrane preparations.

Table 1
Inhibition of lipid peroxide formation in inner membrane of rat liver mitochondria by catalase.

Catalase [M]	Delay of half maximum lipid peroxidation with respect to control [hr]
3.12×10^{-9}	3
1.5×10^{-8}	6.3
3.12×10^{-8}	7.1

4. Discussion

The additive inhibitory effect of lipid peroxidation by catalase and superoxide dismutase might be interpreted by the assumption of an oxidative attack of the unsaturated lipids by the substrates of either enzymes i.e. H_2O_2 and $\cdot O_2^-$. The following considerations, however, reveal that this obvious explanation may not be valid: The spontaneous dismutation of the superoxide radical

$$HO_2\cdot + HO_2\cdot \rightarrow H_2O_2 + O_2^* \qquad (4)$$

may yield highly reactive singlet oxygen [21], whereas in the enzymic dismutation the low energy triplet state of oxygen is produced. Besides, Agrò [22] and coworkers supplied some evidence for the ability of superoxide dismutase to scavenge singlet oxygen even under experimental conditions in which no free superoxide radicals could be detected. For these reasons we have to take into account that singlet oxygen might be the actual oxidant in GSH-induced lipid peroxidation.

For several reasons, the immediate reaction of H_2O_2 with the unsaturated lipids apparently does not play a major role in the GSH-induced autoxidation process.

i) "High amplitude swelling" of mitochondria which is related to lipid peroxidation can not be induced by H_2O_2 [1].

ii) When H_2O_2 is built as a product of the superoxide dismutase reaction, a higher rate of lipid peroxide formation is to be expected, if H_2O_2 is more reactive than $\cdot O_2^-$ or the products of its spontaneous dismutation. This is consistently not observed.

iii) The inhibition of lipid peroxide formation by

catalase may be explained as follows: H_2O_2 may produce •OH radicals via the cycle of Haber and Weiss [23] (eq. (5) and (6)) and finally singlet oxygen [24] according to equation (7):

$$\cdot O_2^- + H_2O_2 + H^+ \rightarrow O_2 + H_2O + \cdot OH \quad (5)$$

$$\cdot OH + H_2O_2 \rightarrow H_2O + \cdot O_2^- + H^+ \quad (6)$$

$$\cdot O_2^- + \cdot OH \rightarrow OH^- + O_2^* \quad (7)$$

Both singlet oxygen and the •OH radical may act as initiators of free radical chains and thereby induce lipid peroxidation. The only conclusion which can be drawn at the moment is that H_2O_2 and $\cdot O_2^-$ or another substrate of superoxide dismutase are involved in a free radical mechanism which finally results in the formation of malone dialdehyde arising from the peroxidized lipids.

Regarding the physiological significance of our results we have to consider that erythrocuprein apparently is not present in mitochondria. A superoxide dismutase containing manganese instead of zinc and copper, however, has been found in chicken liver mitochondria [25]. This enzyme is assumed to be functionally equivalent to erythrocuprein [25]. Erythrocuprein may protect biological membranes of other compartments of the cell in a manner similar to the model reaction described in the present communication. Very recently, Fee and Teitelbaum [26] reported that erythrocuprein can prevent dialuric acid-induced lipid peroxide formation in erythrocytes. The latter phenomenon is closely related to drug-induced hemolysis [27]. Thus, superoxide dismutase most probably has to be added to the list of enzymes like catalase and glutathione peroxidase which protect biological membranes against oxidative damage.

References

[1] D. Neubert, A.B. Wojtczak and A.L. Lehninger, Proc. Natl. Acad. Sci. U.S. 48 (1962) 1651–1658.

[2] F.E. Hunter, Jr., A. Scott, P.E. Hoffsten, F. Guerra, J. Weinstein, A. Schneider, B. Schutz, J. Fink, L. Ford and E. Smith, J. Biol. Chem. 239 (1964) 604–613.

[3] F.E. Hunter, Jr., A. Scott, P.E. Hoffsten, J.M. Gebicki, J. Weinstein and A. Schneider, J. Biol. Chem. 239 (1964) 614–621.

[4] Y. Hatefi and W.G. Hanstein, Arch. Biochem. 138 (1970) 73–86.

[5] L.M. Corwin, Arch. Biochem. Biophys. 97 (1962) 51–58.

[6] L. Flohé and R. Zimmermann, Biochim. Biophys. Acta 223 (1970) 210–213.

[7] L. Flohé, W. Schlegel, A. Wendel and R. Zimmerman, in: Energy Transduction in Respiration and Photosynthesis, eds. E. Quagliariello, S. Papa and C.S. Rossi (Adriatica Editrice, Bari, 1971) p. 595–601.

[8] B.O. Christophersen, Biochim. Biophys. Acta 164 (1968) 35–46.

[9] L. Flohé, Klin. Wschr. 49 (1971) 669–683.

[10] J.M. McCord and I. Fridovich, J. Biol. Chem. 244 (1969) 6049–6055.

[11] G. Rotilio, R.C. Bray and M. Fielden, Biochim. Biophys. Acta 268 (1972) 605–609.

[12] D. Klug, J. Rabani and I. Fridovich, J. Biol. Chem. 247 (1972) 4839–4842.

[13] J.M. McCord and I. Fridovich, J. Biol. Chem. 244 (1969) 6056–6063.

[14] L. Flohé and W. Schlegel, Hoppe-Seyl. Z. Physiol. Chem. 352 (1971) 1401–1410.

[15] D.F. Parsons, G.R. Williams, W. Thompson, D. Wilson and B. Chance, in: Mitochondrial Structure and Compart., eds. E. Quagliariello, S. Papa, E.C. Slater and J.M. Tager (Adriatica Editrice, Bari, 1967) p. 29–70.

[16] A.F. Whereat, M.W. Orishimo, J. Nelson and S.J. Phillips, J. Biol. Chem. 244 (1969) 6498–6506.

[17] U. Weser, E. Bunnenberg, R. Cammack, C. Djerassi, L. Flohé, G. Thomas and W. Voelter, Biochim. Biophys. Acta 243 (1971) 203–213.

[18] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.

[19] A.S. Brill, in: Comprehensive Biochemistry, eds. M. Florkin and E.H. Stotz (Elsevier Publishing Company, Amsterdam, London, New York, 1966) p. 447–479.

[20] W.G. Hanstein and Y. Hatefi, Arch. Biochem. 138 (1970) 87–95.

[21] A.U. Khan, Science 168 (1970) 476–477.

[22] F. Agrò, C. Giovagnoli, P. de Sole, L. Calabrese, G. Rotilio and B. Mondovì, FEBS Letters 21 (1972) 183–185.

[23] F. Haber and J. Weiss, Proc. Roy. Soc., London A 147 (1934) 332–351.

[24] R.M. Arneson, Arch. Biochem. Biophys. 136 (1970) 352–360.

[25] I. Fridovich. Joint Meeting of the Biochemical Society Molecular Enzymoloy Group and the British Biophysical Society Magdalen College, Oxford, 4–7 September 1972, Abstracts p. 22–24.

[26] J.A. Fee and H.D. Teitelbaum, Biochem. Biophys. Res. Commun. 49 (1972) 150–158.

[27] L. Flohé, G. Niebch and H. Reiber, Z. Klin. Chem. Klin. Biochem. 9 (1971) 431–437.