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Glycerophosphate-Dependent Hydrogen Peroxide Production by Brown Adipose Tissue Mitochondria and Its Activation by Ferricyanide

Zdeněk Drahota,[1,4] Subir K. R. Chowdhury,[1] Daniel Floryk,[1] Tomáš Mráček,[1] Jiří Wilhelm,[2] Hana Rauchová,[1] Giorgio Lenaz,[3] and Josef Houštěk[1]

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Oxidation of glycerophosphate (GP) by brown adipose tissue mitochondria in the presence of antimycin A was found to be accompanied by significant production of hydrogen peroxide. GP-dependent hydrogen peroxide production could be detected by *p*-hydroxyphenylacetate fluorescence changes or as an antimycin A-insensitive oxygen consumption. One-electron acceptor, potassium ferricyanide, highly stimulated the rate of GP-dependent antimycin A-insensitive oxygen uptake, which was prevented by inhibitors of mitochondrial GP dehydrogenase (mGPDH) or by coenzyme Q(CoQ). GP-dependent ferricyanide-induced peroxide production was also determined luminometrically, using mitochondria or partially purified mGPDH. Ferricyanide-induced peroxide production was negligible, when succinate or NADH was used as a substrate. These results indicate that hydrogen peroxide is produced directly by mGPDH and reflect the differences in the transport of reducing equivalents from mGPDH and succinate dehydrogenase to the CoQ pool. The data suggest that more intensive production of reactive oxygen species may be present in mammalian cells with active mGPDH.

KEY WORDS: Brown adipose tissue; mitochondrial glycerophosphate dehydrogenase; ferricyanide; hydrogen peroxide.

INTRODUCTION

Reactive oxygen species (ROS) are proposed as mediators of tissue injury in several human pathologies (Halliwell, 1987; Richter, 1997; Schapira, 1994), in acceleration of degenerative processes in ageing (Barja, 1999; Barja and Herrero, 1998; Sohal, 1993), and in initiation of necrotic and apoptotic processes (Kroemer *et al.*, 1998; Mignotte and Vayssiere, 1998; Pedersen, 1999). Most of ROS are produced in mitochondria because of interaction of oxygen with free electrons released by the respiratory chain. The leak of electrons from the mitochondrial respiratory chain has been localized in Complex I (NADH-CoQ reductase) and Complex III (CoQ-cytochrome *c* reductase) (Herrero and Barja, 2000; Kwong and Sohal, 1998; Sohal, 1991; Turrens *et al.*, 1986). Under physiological conditions, toxic effects of ROS formed from released electrons are compensated for by the cell antioxidative defense mechanisms, which decompose the ROS produced. As suggested recently, another defense mechanism may involve the function of uncoupling proteins, which decrease ROS production by lowering the inner mitochondrial membrane potential (Negre-Salvayre *et al.*, 1997). However, formation of ROS may be highly activated

[1] Institute of Physiology and Center for Integrated Genomics, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic.
[2] Institute of Medical Chemistry and Biochemistry, 2nd Medical Faculty, Charles University, 150 00 Prague, Czech Republic.
[3] Department of Biochemistry, Medical School, University of Bologna, 40126 Bologna, Italy.
[4] To whom correspondence should be addressed; e-mail: drahota@biomed.cas.cz.

Key to abbreviations: BAT – brown adipose tissue, CoQ – coenzyme Q, SUC – succinate, GP – glycerophosphate, mGPDH – mitochondrial glycerophosphate dehydrogenase, ROS – reactive oxygen species, HRP – horse-radish peroxidase.

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under pathological situations, e.g., during hypoxic injury or because of the respiratory chain defects in mitochondrial encephalomyopathies (Wallace, 1999) which increase the reducing potential of the electron carriers of the respiratory chain towards the NADH.

The high rate of ROS production has been also detected in insect fly muscle mitochondria (Bolter and Chefurka, 1990; Sohal, 1991) and in brown adipose tissue (BAT) mitochondria (Sekhar *et al.*, 1987), when glycerophosphate (GP) was used as a respiratory substrate. It was suggested that mitochondrial GP dehydrogenase (mGPDH) could be another complex of the mitochondrial respiratory chain where the leak of electrons may occur.

mGPDH is involved in oxidation of cytosolic NADH by GP shuttle which bypasses Complex I (Bucher and Klingenberg, 1958), in regulation of triglyceride synthesis (Kornacker and Ball, 1968) and possibly also in uncoupling protein-independent energy dissipation which involves also malic enzyme (Bobyleva *et al.*, 1993, 2000). The highest activity of mGPDH in equimolar proportion with cytosolic GPDH was found in BAT (Houstek *et al.*, 1975). However, it is quite evident that this enzyme plays an important role also in other tissues and cells. mGPDH was studied in pancreatic beta cells (Ishihara *et al.*, 1996; Meglasson *et al.*, 1989), brain (Cottingham and Ragan, 1980a,b), heart muscle (Scholz *et al.*, 1997), placenta (Swierczynski *et al.*, 1976), testis (MacDonald and Brown, 1996), fibroblasts (Chretien *et al.*, 1994), and after hormonal induction also in liver (Bobyleva *et al.*, 2000). However, the multiple roles of mGPDH in cellular metabolism are not yet fully clarified, and production of ROS could be another important feature of this enzyme.

In this paper we present the evidence that mGPDH is very potent producer of hydrogen peroxide in BAT cells and that this hydrogen peroxide production may be highly increased when the electron flow from the enzyme to CoQ pool is affected by the one-electron acceptor, potassium ferricyanide. This also indicates differences in electron transport from mGPDH and succinate dehydrogenase to the CoQ pool.

MATERIALS AND METHODS

Isolation of Brown Adipose Tissue Mitochondria

Mitochondria were isolated from BAT of adult male Syrian hamsters, cold adapted at 4°C for 3 weeks as described by Hittelman *et al*. (1969). Isolation medium was 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4. For experiments freshly isolated mitochondria or mitochondria frozen at −70°C were used.

Fluorometric, Polarographic, and Luminometric Detection of Hydrogen Peroxide Production

Hydrogen peroxide formation was detected fluorometrically according to Hyslop and Sklar (1984) by following the oxidation of *p*-hydroxyphenylacetate into a fluorescent form during the enzymatic reduction of hydrogen peroxide by horse-radish peroxidase (HRP). The reaction mixture contained 154 mM KCl, 5 mM K_3PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4 and 160 μg *p*-hydroxyphenylacetate/mL, 80 units of HRP/mL, and 80 μg of mitochondrial protein/mL. The rate of hydrogen peroxide production was followed using Perkin Elmer LS-5 spectrofluorometer (excitation 317 nm, emission 400 nm). Known concentrations of hydrogen peroxide were used to establish the standard concentration curve.

Oxygen consumption was measured using High Resolution Oxygraph from OROBOROS, Austria (Gnaiger *et al.*, 1995). Measurements were performed at 30°C in 1.5 mL of incubation medium containing 100 mM KCl, 10 mM Tris-HCl, 4 mM K_3PO_4, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.2, using 0.1–0.5 mg/mL of the mitochondrial protein. OROBOROS software was used for calculations of oxygen production and for graphic presentation of experimental data. Oxygraphic curves represent the first derivation of oxygen tension changes, the area of peaks represents the total oxygen consumption, and the height of the peak represents the rate of reaction.

Luminescence was measured by Luminometer 1250 from Bioorbit (Finland) as described earlier (Wilhelm and Vilim, 1986). Measurements were performed at room temperature in 1 mL of medium containing 50 mM KCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.4, and 0.1–0.3 mg of mitochondrial protein. Luminol (5-amino-2,3-dihydro-1,4-phtalazionedione) concentration was l mM, and HRP was 2.5 units/mL. The reaction was started by addition of 100 μl of 5 mM potassium ferricyanide. A calibration curve of hydrogen-peroxide-induced luminescence was determined under the same experimental conditions. The luminescence peak reached maximum values within 2/3 s after addition of potassium ferricyanide. For evaluation of peroxide production the maximum value (peak) was used. The peak value was found to be fully proportional to luminescence peak area.

Solubilization and Purification of mGPDH

Frozen–thawed mitochondria were washed twice in 10 mM *K*-phosphate buffer, pH 7.4 by 10 min centrifugation at 10,000 g, and the protein was adjusted to 10 mg/mL. Mitochondria were solubilized by digitonin treatment as

described by Klement *et al.* (1995). An equal volume of 10 mg digitonin/mL dissolved in 10 mM *K*-phosphate buffer was added to the mitochondrial suspension and the mixture was incubated for 15 min on ice. Unsolubilized material was removed by centrifugation for 30 min at 30,000 g at 4°C. Supernatant (maximum 20 mg protein) was applied on the hydroxylapatite column (Biogel HTP, BioRad, 10 cm × 1 cm) equilibrated with 10 mM *K*-phosphate buffer pH 7.4. The same buffer was used for elution of unbound proteins. Fractions containing the highest activity of mGPDH were collected and concentrated with Amicon PM-30 membrane. This partially purified enzyme was used for enzyme activity measurements and for detection of hydrogen peroxide production. Enzyme activity was measured spectrophotometrically according to Garrib and McMurray (1984). The SDS polyacrylamide gel electrophoresis (Schagger and von Jagow, 1987) and the Blue-Native electrophoresis (Schagger and von Jagow, 1991) were used for determination of purity of the soluble enzyme preparation.

Chemicals

Mercaptodicarbanonaborate (Na.5-SH, 7, 8-$C_2B_9H_{11}$) was synthesized by Dr J. Plesek in the Institute of Inorganic Chemistry, Academy of Sciences of the Czech Republic, Prague. D,L-glycerol-3-phosphate, *p*-hydroxymercuribenzoate, *p*-hydroxyphenylacetate, and antimycin A were from Sigma, and catalase was from Boehringer (Germany). All other chemicals were from Lachema (Czech Republic).

RESULTS

Glycerophosphate-Dependent Hydrogen Peroxide Production by Brown Adipose Tissue Mitochondria

In agreement with the study of Sekhar *et al.* (1987), we found, using fluorometric detection, that BAT mitochondria generate 3.1 ± 0.05 ($n = 10$) nmol of H_2O_2/min/mg protein when GP was used as a substrate and 1.74 nmol with succinate (Fig. 1). GP-dependent hydrogen peroxide formation was completely abolished by 0.1 mM mercaptodicarbanonaborate (Fig. 1) which strongly inhibits mGPDH activity (Drahota *et al.*, 1995) or with 0.5 mM *p*-hydroxymercuribenzoate (not shown), another inhibitor of mGPDH (Rauchova *et al.*, 1985).

Hydrogen peroxide formation could be also detected by polarographic determination of GP-dependent oxygen consumption insensitive to antimycin A or KCN. Similarly as in fluorometric measurements we found

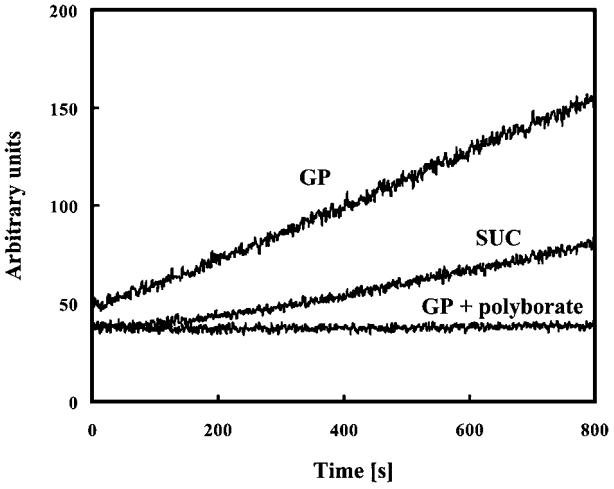

Fig. 1. Fluorometric detection of hydrogen peroxide formation by BAT mitochondria. Measurements were performed in the presence of 2 μM antimycin A and 25 mM glycerophosphate (GP) or 25 mM succinate (SUC) using frozen–thawed BAT mitochondria. Where indicated, 0.1 mM mercaptodicarbanonaborate was added 30 s prior to addition of glycerophosphate (GP + polyborate).

almost twofold higher rate of hydrogen peroxide production when GP was used as a substrate than in the presence of succinate (Fig. 2). In accordance with previous studies (Houstek *et al.*, 1978) the activity of succinate cytochrome *c* reductase was even higher than that of GP cytochrome *c* reductase in BAT mitochondria (0.365 ± 0.034 and 0.15 ± 0.016 μmol/min/mg protein, respectively). This indicates, that in the presence of antimycin A more hydrogen peroxide is produced during oxidation of GP than during oxidation of succinate.

Ferricyanide-Induced Hydrogen Peroxide Production

The rate of antimycin A-insensitive oxygen consumption was highly accelerated by the addition of potassium ferricyanide (Fig. 3), a one-electron acceptor of mitochondrial dehydrogenases (Klingenberg, 1970). Ferricyanide-induced GP-dependent oxygen consumption could be demonstrated with freshly isolated (Fig. 3) as well as with frozen–thawed mitochondria.

As shown in Fig. 3, when the added potassium ferricyanide was reduced by mGPDH, the rate of oxygen consumption returned to the original low values. Subsequent additions of potassium ferricyanide again activated the rate of oxygen consumption. In contrast, succinate- or NADH-induced antimycin A-insensitive oxygen consumption measured in frozen–thawed mitochondria was lower than that with GP and was not further enhanced by ferricyanide.

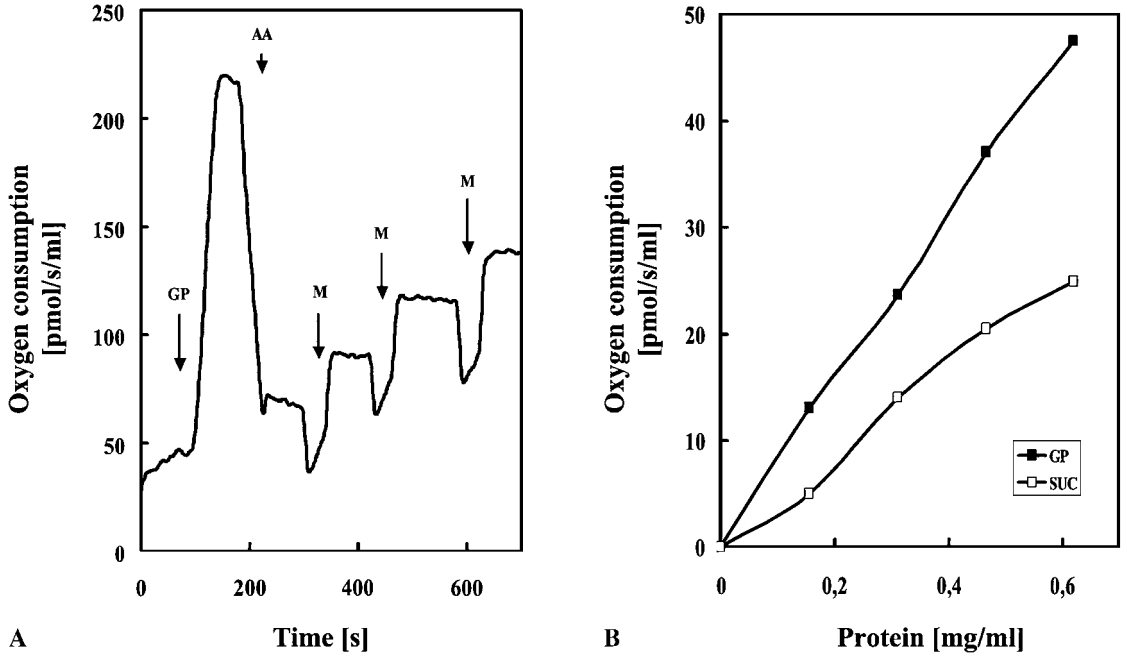

Fig. 2. Antimycin A-insensitive oxygen uptake in the presence of glycerophosphate (GP) and succinate (SUC). (A) Representative recording of oxygen consumption in the presence of glycerophosphate. (B) Calculated antimycin A-insensitive oxygen consumption in the presence of glycerophosphate and succinate. Frozen BAT mitochondria (0.15 mg/mL) were suspended in KCl medium (see Methods). Where indicated, 10 mM glycerol phosphate or succinate was added. Oxygen consumption was inhibited by 2 μM antimycin A (AA) followed by three subsequent additions of 0.15 mg/mL of mitochondria (M).

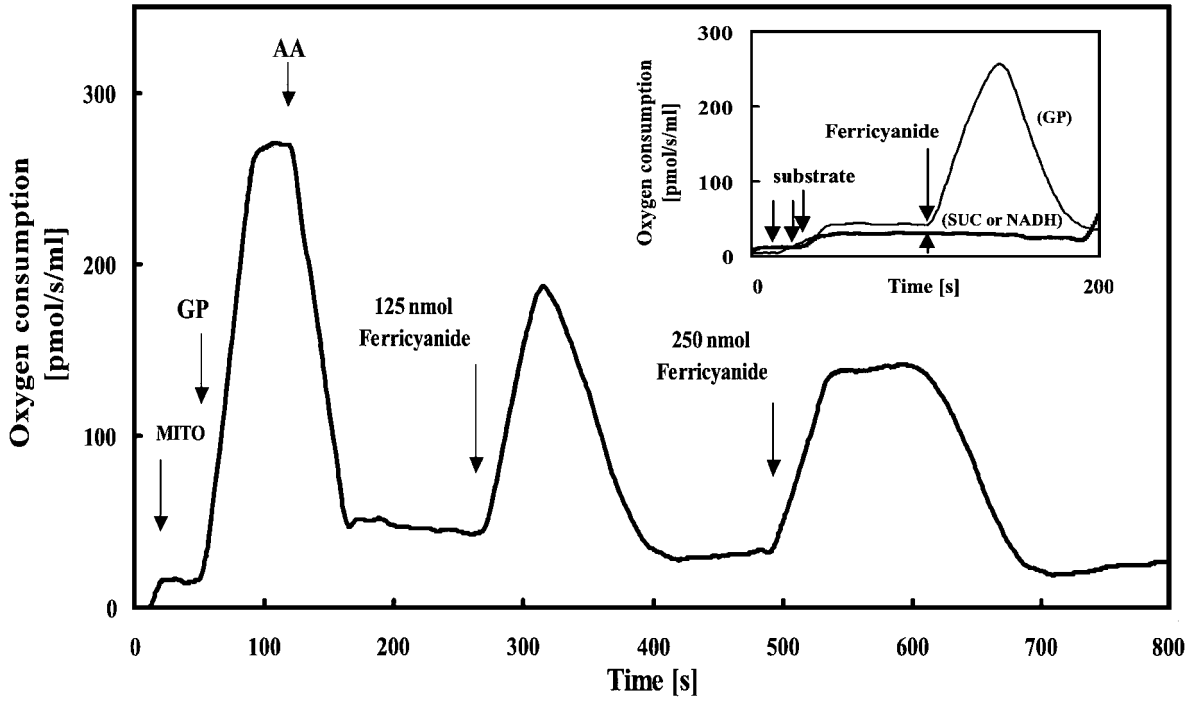

Fig. 3. Ferricyanide-induced, glycerophosphate-dependent, antimycin A-insensitive oxygen consumption by BAT mitochondria. For experiment, 0.3 mg protein/mL of freshly isolated BAT mitochondria (MITO), 10 mM glycerophosphate (GP), 2 μM antimycin A (AA), and 125 and 250 nmol of ferricyanide were used. Insert shows the effect of ferricyanide on antimycin A-insensitive oxygen consumption in the presence of 10 mM glycerophosphate or 10 mM succinate or 0.1 mM NADH using frozen–thawed BAT mitochondria.

The rate of the ferricyanide-induced oxygen consumption (height of the peak) was dependent on the amount of mitochondria used, and the total amount of oxygen consumed (area of the peak) was proportional to the amount of ferricyanide added. In Fig. 3 the total oxygen consumption was 42.3 nAt O and 61.2 nAt O, respectively using 125 and 250 nmol ferricyanide. The stoichiometry between the added potassium ferricyanide and the oxygen consumed at low ferricyanide concentrations (80–150 μM) was about 0.3 nAt O/nmol ferricyanide. At higher ferricyanide concentrations (500–1000 μM) or after repeated additions of potassium ferricyanide the ratio decreased to values about 0.2 and lower (Table I). This indicates that during the transfer of electrons from mGPDH to the one-electron acceptor, hydrogen peroxide was produced.

Table I. GP-Dependent, Potassium Ferricyanide-Induced Hydrogen Peroxide Production: Ratio Between Added Potassium Ferricyanide and Oxygen Consumed

Ferricyanide added (nmol)	Oxygen consumed (nAt)	Oxygen/ferricyanide ratio (nAt O/nmol ferricyanide)
125	37 ± 1.7	0.30 ± 0.012
250	71 ± 1.5	0.28 ± 0.010
500	105 ± 1.8	0.21 ± 0.019
1000	162 ± 2.2	0.16 ± 0.023

Note. Oxygen uptake was measured in the presence of 20 mM glycerophosphate and 2 μM antimycin A. Frozen–thawed mitochondria were used. Data indicate mean $\pm$ SD, $n = 3$.

Inhibition of Hydrogen Peroxide Production by Catalase, mGPDH Inhibitors, and CoQ

Hydrogen peroxide production by BAT mitochondria oxidizing GP in the presence of antimycin A and ferricyanide could be also confirmed by the effect of catalase. As shown in Fig. 4, the total oxygen consumption after addition of ferricyanide was 54.7 nAt O. The release of oxygen after addition of catalase was 20.5 nAt O. Subsequent addition of potassium ferricyanide in the presence of catalase induced oxygen consumption that was also about 50% lower than that in the absence of catalase (28.0 n At O).

Similarly, as in fluorometric measurements, potassium-ferricyanide-induced hydrogen peroxide production was completely abolished by the inhibition of mGPDH by mercaptodicarbanonaborate or oleate, another inhibitor of mGPDH (Houstek and Drahota,

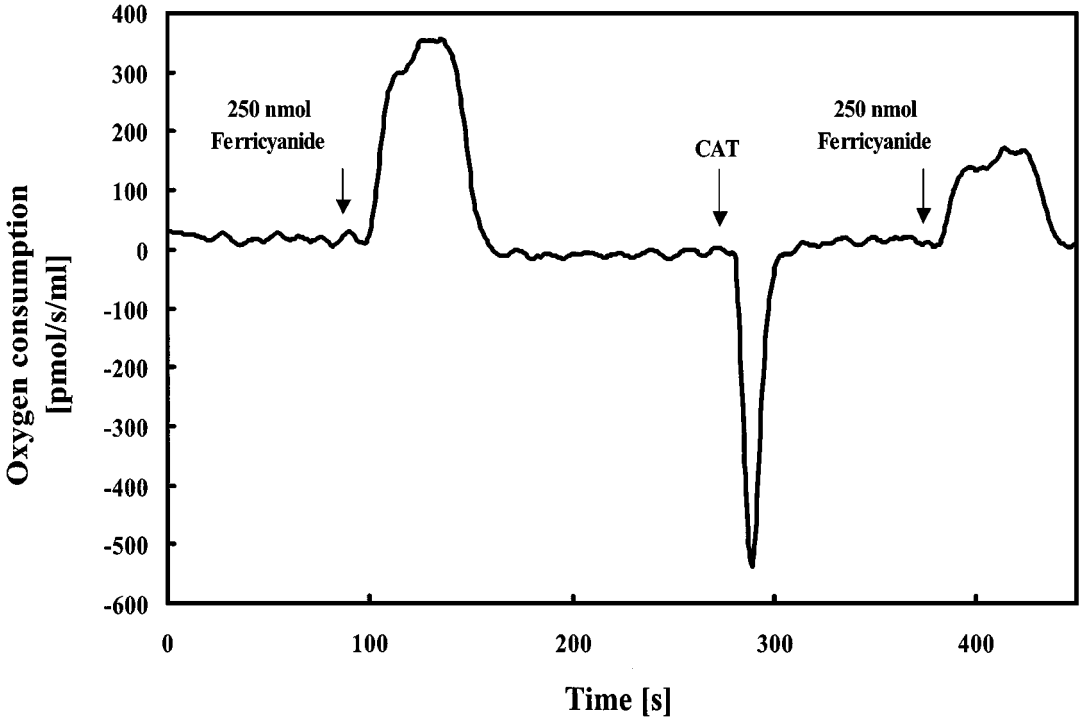

Fig. 4. The effect of catalase on ferricyanide-induced hydrogen peroxide production. Measurements were performed in the presence of 10 mM glycerophosphate and 2 μM antimycin A using frozen–thawed BAT mitochondria. Where indicated, 250 nmol of ferricyanide and 4300 U/mL of catalase (CAT) were added.

Table II. Effect of mGPDH Inhibitors on GP-Dependent, Potassium Ferricyanide-Induced Oxygen Consumption

Additions	Oxygen consumed (nAt/min/mg protein)	%
GP + KCN	5.20 ± 0.06	9
GP + KCN + 125 nmol ferricyanide	59.26 ± 0.15	100
GP + KCN + 40 μM C_2B_9SH + 125 nmol ferricyanide	7.04 ± 0.11	12
GP + KCN + 2 μmol oleate/mg protein + 125 nmol ferricyanide	14.46 ± 0.05	24

Note. Oxygen consumption was measured in frozen–thawed mitochondria (0.3 mg protein/mL) in the presence of 10 mM GP and 0.5 mM KCN. Where indicated potassium ferricyanide, sodium mercaptodicarbanonaborate (C_2B_9SH) or sodium oleate were added. Data indicate mean ± SD, $n = 4$.

1975) (see Table II). GP-dependent ferricyanide-induced hydrogen peroxide production was also abolished by addition of CoQ_3 (Fig. 5). At 10 μM CoQ_3 the total oxygen consumption was decreased from 40.0 to 9.4 nAt O, respectively. As shown in Fig. 5, the half-maximal inhibition was obtained at 5 μM CoQ_3.

Luminometric Detection of Glycerophosphate-Dependent Hydrogen Peroxide Production by Mitochondria and Partially Purified mGPDH

As demonstrated in Fig. 6, in the presence of GP, intensive ferricyanide-induced peroxide production can be also detected by luminometry. Luminometric detection showed the same kinetics and the same substrate dependency as polarographic measurements. We obtained a small, but significant luminescence signal when succinate was used (Fig. 6). Evidently, luminometry is more sensitive in detection of small changes in hydrogen peroxide production than polarography. With NADH no luminescence signal was obtained (not shown). Similarly as in polarographic measurement, mGPDH inhibitors and CoQ_3 inhibited hydrogen peroxide formation (not shown).

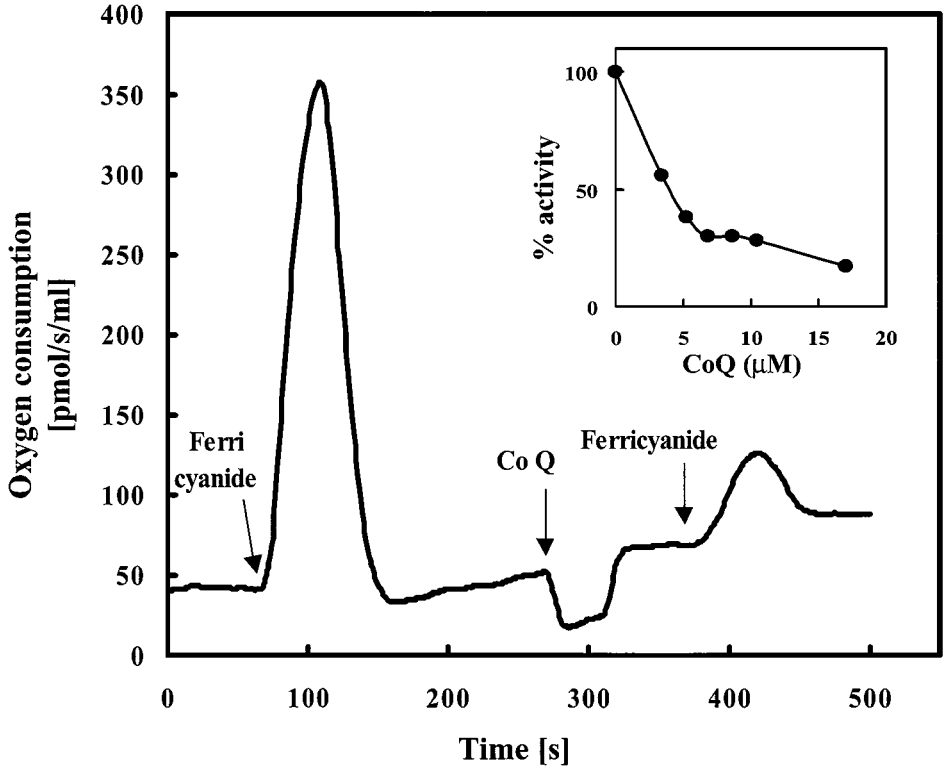

Fig. 5. Inhibition of ferricyanide-induced, GP-dependent hydrogen peroxide production by Coenzyme Q. Measurements were performed in the presence of 10 mM glycerophosphate and 2 μM antimycin A using frozen–thawed BAT mitochondria. Where indicated, 125 nmol of ferricyanide and 10 μM Coenzyme Q_3 (CoQ) were added. Insert shows the inhibitory effect of 2.5–17.5 μM concentrations of CoQ.

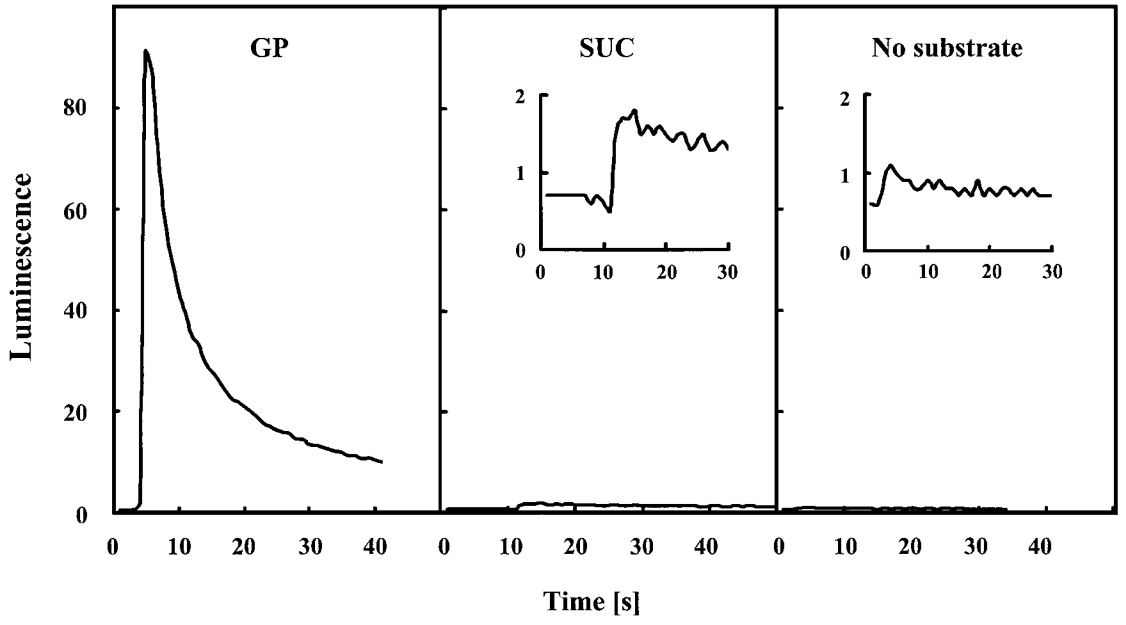

Fig. 6. Luminescence detection of ferricyanide-induced hydrogen peroxide production by BAT mitochondria in the presence of 10 mM glycerophosphate or 10 mM succinate (SUC). A total of 0.2 mg of mitochondrial protein was used. The same results were obtained with freshly isolated and with frozen–thawed mitochondria.

In further experiments we tested the ability of mGPDH to catalyze hydrogen peroxide production, using partially purified mGPDH from BAT mitochondria. Based on electrophoretic analysis and enzyme activity measurements, the enzyme was purified 20-fold. The enzyme preparation was free of contamination by succinate and NADH dehydrogenases, cytochrome *c* oxidase, and bc_1 complex. Further purification was accompanied by rapid loss of the enzyme activity, as also described by Cole *et al.* (1978).

As shown in Fig. 7, isolated mGPDH was able to catalyze the ferricyanide-induced, GP-dependent production of hydrogen peroxide. In comparison with hydrogen peroxide production by BAT mitochondria, luminescence signal elicited by the isolated mGPDH was about 10-fold higher when calculated per mg protein. Hydrogen peroxide production by the isolated mGPDH was also proportional to the amount of protein added in the range up to 10 μg/mL. Similarly as in isolated mitochondria, the hydrogen peroxide production was completely inhibited by mGPDH inhibitors and by CoQ_3 (not shown).

DISCUSSION

Our results clearly showed that mGPDH represents an additional site of ROS generation in the mammalian mitochondrial respiratory chain. Our data on isolated enzyme further proved previous suggestions (Bolter and Chefurka, 1990) that mGPDH can act as ROS generator. This could be especially important for mammalian cells and tissues with high activity of this FAD-dependent dehydrogenase, namely in BAT of newborn mammals and hibernators (Houstek *et al.*, 1975, 1978), pancreatic beta cells (MacDonald *et al.*, 1996), or brain (Cottingham and Ragan, 1980a,b). However, the mechanism of GP-dependent hydrogen peroxide formation by mGPDH is not yet quite clear.

Hydrogen peroxide may be formed in analogy with mechanism proposed for ROS production by Complex I and III from ubisemiquinone radical III (Barja and Herrero, 1998; Kwong and Sohal, 1998; Turrens *et al.*, 1986). Our data, however, indicate that there exist differences in the transport of reducing equivalents from mGPDH and succinate dehydrogenase to the CoQ pool. GP-induced ROS production is higher than that induced by succinate. This difference could be due to the fact that whereas a specific CoQ-binding protein was detected in succinate dehydrogenase (succinate CoQ-reductase) (Yu *et al.*, 1978), it appears to be missing in mGPDH (Cottingham and Ragan, 1980a,b; Rauchova *et al.*, 1992). This CoQ-binding protein evidently represents a natural protection of ubisemiquinone formed during CoQ reduction by succinate dehydrogenase. Only in situations when reduced flavoprotein in succinate dehydrogenase has no accessible electron transfer partner, higher portion of ubisemiquinone may react under specific conditions

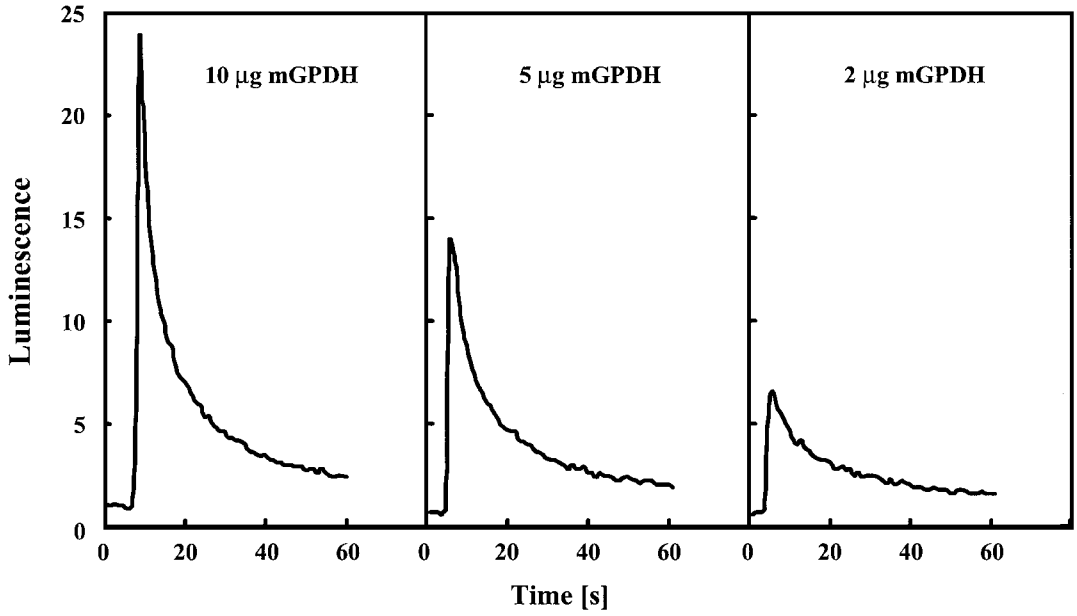

Fig. 7. Glycerophosphate-dependent ferricyanide-induced hydrogen peroxide formation by isolated mGPDH. Luminescence was measured as described in Methods. A total of 2–10 μg protein of isolated enzyme and 10 mM glycerophosphate were used.

with oxygen (Zhang *et al.*, 1998). Because of the absence of CoQ-binding protein, ubisemiquinone formed by mGPDH may be more accessible to oxygen. This should be even more pronounced when the mGPDH reacts with one-electron acceptor ferricyanide (see Fig. 8). Similar activation of ROS production by ferricyanide was also found for isolated succinate dehydrogenase (Zhang *et al.*, 1998).

Absence of the protective effect of CoQ-binding protein thus could explain both the higher ROS production induced by GP than by succinate in the presence of antimycin A (Figs. 1 and 2) and the higher activatory effect of ferricyanide (Figs. 3 and 6). The inhibitory effect of CoQ (Fig. 5) is also fully in agreement with this proposed mechanism (Fig. 8). Increased exogenous concentration of CoQ may evidently substitute the protective effect of CoQ-binding protein. However we cannot exclude that the hydrogen peroxide is formed directly by the interaction of molecular oxygen with reduced flavoprotein moiety of the mGPDH similarly as proposed for the acetyl CoA dehydrogenase (Osumi and Hashimoto, 1978).

Further studies are required to clarify the significance of mGPDH as a ROS producer. It should be clarified whether high activity of mGPDH in BAT, brain, muscle, or pancreas, represents a potential risk, which must be controlled by various defense mechanisms, or whether the peroxide production by mGPDH may have some regulatory significance. For example, in BAT it could participate in activation of apoptotic process, which starts in BAT when the thermogenic function of BAT is no more required (Lindquist and Rehnmark, 1998) and mitochondria maintain high membrane potential (low respiratory rate) because of absence of hormonal thermogenic stimuli.

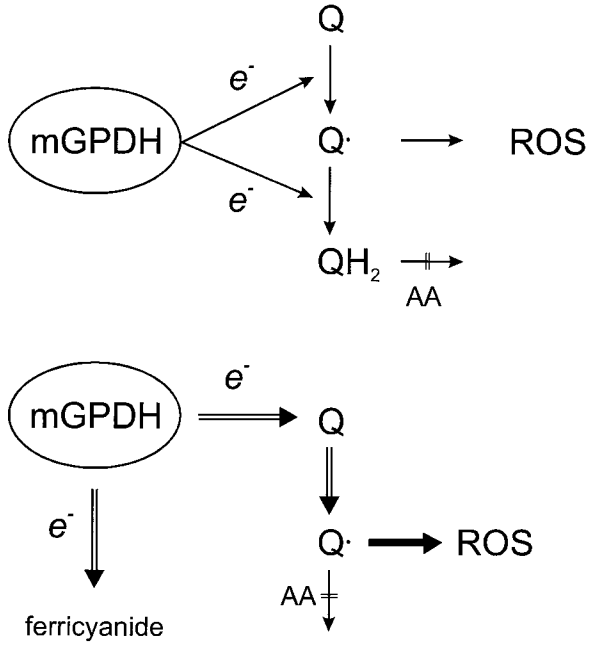

Fig. 8. Proposed mechanism of the activatory effect of potassium ferricyanide on glycerophosphate induced ROS production.

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