Glycerophosphate-Dependent Peroxide Production by Brown Fat Mitochondria from Newborn Rats

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Abstract. Glycerophosphate (GP)-dependent, ferricyanide-induced hydrogen peroxide production was studied in brown adipose tissue mitochondria from newborn rats. Relations between the rate of hydrogen peroxide production and total amount of hydrogen peroxide produced at different GP and ferricyanide concentrations were determined. It was found that the rate of hydrogen peroxide production increases with increasing GP concentration and decreases with increasing ferricyanide concentration. Total amount of hydrogen peroxide produced increases with increasing ferricyanide concentration, however, not proportionally, and the efficiency of this process (oxygen/ferricyanide ratio) strongly declines. Data presented provide further information on the character and kinetics of hydrogen peroxide production by mammalian mitochondrial glycerophosphate dehydrogenase.

Key words: Brown fat — Mitochondria — Glycerophosphate dehydrogenase — Hydrogen peroxide

Introduction

Mitochondrial, flavoprotein-dependent glycerophosphate dehydrogenase (mGPDH) together with the cytosolic, NADH-dependent glycerophosphate dehydrogenase (cGPDH) forms the glycerophosphate shuttle (Bucher and Klingenberg 1958) through which cytosolic NADH may be oxidized by mitochondrial respiratory chain (Werner and Berry 1987). Activity of this cycle is dependent on quantity of the mGPDH, which is in most cells very low, contrary to the cGPDH. Up to now only in two cell populations, i.e. in insect muscle (Bucher and Klingenberg 1958) and mammalian brown adipose tissue (Houštěk et al. 1975) mGPDH was found in equimolar proportions to cGPDH, required for the high functional activity of the glycerophosphate shuttle. However, sufficiently high mGPDH activity was also

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detected in rabbit brain, skeletal (Cole et al. 1978) and heart muscle (Scholz et al. 1997), human placenta (Swierczynski et al. 1976a,b), testes (MacDonald and Brown 1996) and in insulin secreting cells in pancreas (MacDonald et al. 1996).

The mGPDH is under control of many endogenous factors like Ca^{2+} and Mg^{2+} ions (MacDonald and Brown 1996), acyl CoA esters (Bukowiecki and Lindberg 1974), fatty acids (Houštěk and Drahota 1975) and intermediates of glycolysis (Swierczynski et al. 1976c). In the liver cells biogenesis of mGPDH is under hormonal control (Muller and Seitz 1994).

The role of the mGPDH and glycerophosphate shuttle in regulation of cell metabolism is not yet fully clarified. It is proposed that the glycerophosphate shuttle participates in regulation of glycolysis by reoxidation of NADH (Werner and Berry 1987), in regulation of triglyceride synthesis (Kornacker and Ball 1968) and as energy dissipating system due to bypassing one phosphorylation site at Complex I (Bobyleva et al. 1993, 2000).

In our previous studies (Drahota et al. 2002) we have found that mGPDH participates in mitochondrial production of reactive oxygen species (ROS), which are proposed as mediators of degenerative process in aging (Barja 1999) and in initiation of apoptotic and necrotic processes (Pedersen 1999). The leak of electrons from mitochondrial respiratory chain responsible for ROS generation was localized at Complex I and III (Herrero and Barja 2000). Our data showed, that significant glycerophosphate (GP)-dependent ROS production could also be detected in mammalian mitochondria. This fact may explain why expression of mGPDH is repressed in most mammalian tissues. We also found, that GP-dependent, but not succinate- or NADH-dependent hydrogen peroxide production could be highly stimulated by one electron acceptor potassium ferricyanide (FeCN). These data showed, that transport of two electrons from mGPDH to coenzyme Q (CoQ) was less protected, evidently due to the absence of CoQ-binding protein that was found in Complex I and II (Cottingham and Ragan 1980a,b). Activatory effect of FeCN, found only for GP-dependent oxygen uptake, further demonstrates different mechanism of reducing equivalent transfer between mGPDH and succinate or NADH dehydrogenase.

In this paper we present data that extend our previous findings obtained on brown fat mitochondria from cold adapted hamsters (Drahota et al. 2002) also for brown fat of neonatal rat. We confirmed the finding of GP-dependent FeCN-induced hydrogen peroxide production in brown fat of neonatal rats and we present detail information about relations between peroxide production and GP and FeCN concentration. We also analyzed in more detail kinetics of this process and relations between the rate of peroxide production and O/FeCN ratio under various conditions especially at various rates of mGPDH activity.

Materials and Methods

Newborn 5-day-old rats *(Rattus norwegicus)* of Wistar strain were used for experiments. Interscapular brown adipose tissue from 5–10 animals was used for isola-

tion of mitochondria as described by Hittelmann et al. (1969). Isolation medium was 0.25 mol/l sucrose, 10 mol/l Tris-HCl, 1 mol/l EDTA, pH 7.4. Twice washed mitochondria were stored at −70°C. Frozen-thawed mitochondria were used for measurements.

Oxygen consumption of mitochondria was measured with High Resolution Oxygraph from Oroboros, Austria (Gnaiger et al. 1995) in a medium containing 80 mmol/l KCl, 10 mmol/l Tris-HCl, 4 mmol/l K-Phosphate, 3 mmol/l $MgCl_2$, 1 mmol/l EDTA, pH 7.2 at 30°C. Total volume was 1.5 ml. Oroboros software was used for calculation of oxygen consumption and for graphic presentation of experimental data. Oxygraphic curves presented in figures are the first derivation of oxygen tension changes and oxygen uptake is expressed as picomol oxygen *per* second *per* mg of mitochondrial protein. The height of the peak of the curve represents the rate of the reaction; the area of the peak represents the total amount of oxygen consumed. From these data the reaction rates (pmol oxygen/s/mg mitochondrial protein) and the total amount of oxygen consumed during the reduction of added FeCN were calculated. The efficiency of the reaction was expressed as the ratio between the total amount of oxygen consumed and amount of FeCN added (nAt oxygen/nmol FeCN). In our previous paper (Drahota et al. 2002) we have found that GP-dependent, FeCN-induced oxygen uptake

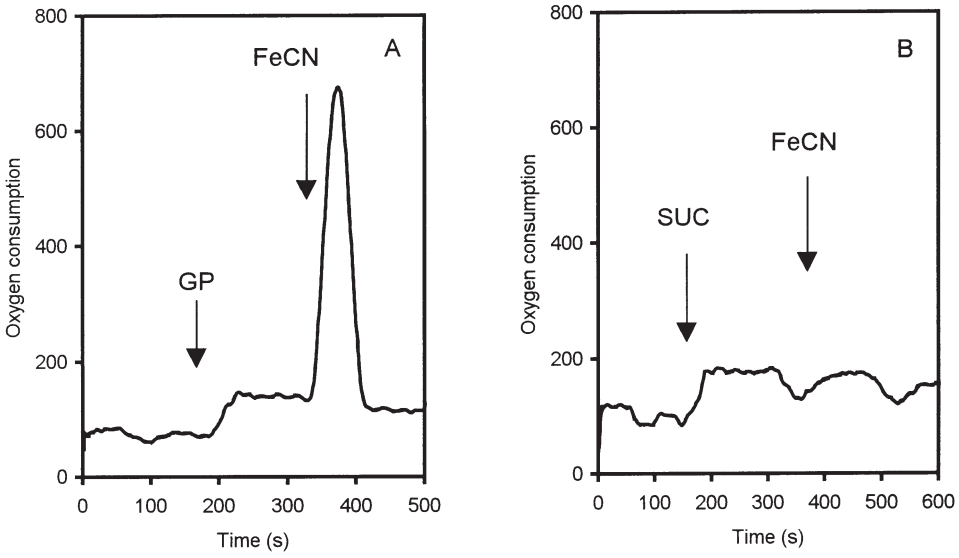


Figure 1. GP-dependent FeCN-induced oxygen consumption (pmol oxygen/s/mg protein). Measurements were performed in the presence of 0.3 mmol/l KCN and 10 mmol/l GP – (A) or 10 mmol/l succinate – (B). FeCN concentration was 0.083 mmol/l and mitochondrial protein concentration was 0.43 mg/ml. The same results were obtained using three preparations of brown fat mitochondria isolated from 8 newborn rats.

in the presence of KCN was directly connected with hydrogen peroxide generation.

Results

As demonstrated in Fig. 1, we found that in brown adipose tissue mitochondria from neonatal rats GP-dependent, KCN insensitive oxygen uptake can be detected, which indicates hydrogen peroxide production. The data confirm our previous findings on brown fat mitochondria from cold adapted hamster (Drahota et al. 2002). The rate of GP-dependent peroxide production can be highly activated (approximately 6 times) by one-electron acceptor, potassium ferricyanide (FeCN). This effect is specific for mGPDH and cannot be observed in the presence of succinate and KCN (Fig. 1B) or NADH (not shown). When all added FeCN was reduced, as was confirmed by parallel spectrophotometric measurements (not shown), the rate of the oxygen consumption returned to values obtained before the addition of FeCN (Fig. 1A). In this experiment we used the same GP concentration – 10 mmol/l as in previous experiments on hamster mitochondria (Drahota et al. 2002). As demonstrated in Table 1 in further experiments we used also 30–40 mol/l GP; the total amount of oxygen consumed and O/FeCN ratio was the same as at 10 mol/l, however, maximum rate of oxygen consumption was increased.

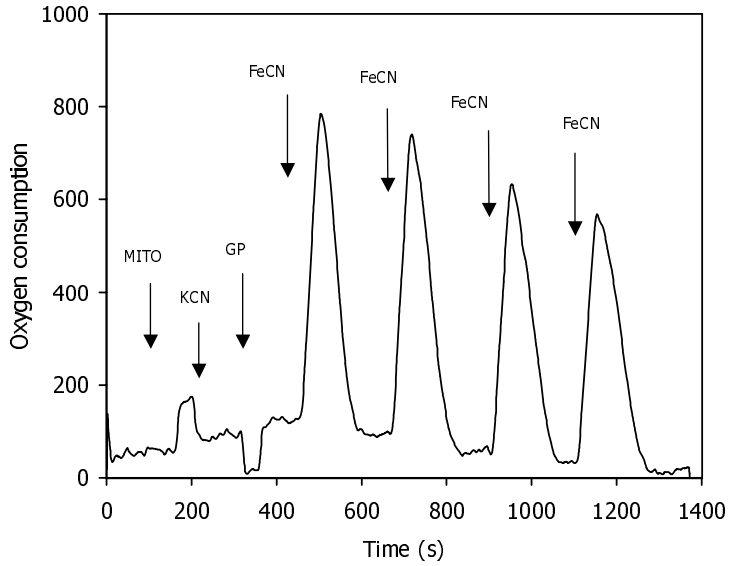


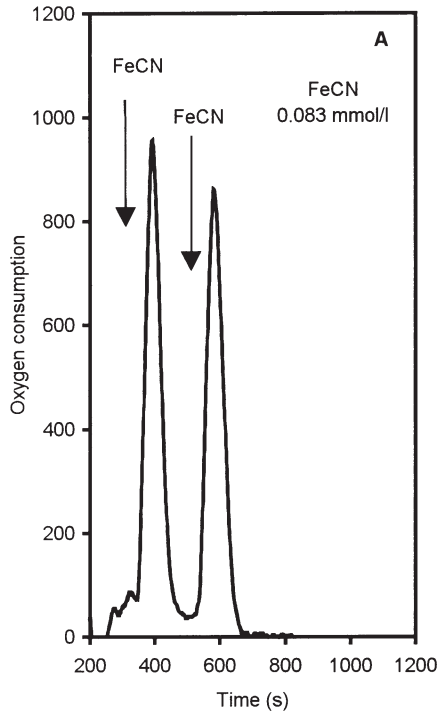
Figure 2. GP-dependent FeCN-induced oxygen consumption (pmol oxygen/s/mg protein). Measurements were performed in the presence of 0.3 mmol/l KCN, 30 mmol/l GP, 0.083 mmol/l FeCN. Mitochondrial protein (MITO) was 0.2 mg/ml. The same results were obtained using three preparations of brown fat mitochondria isolated from 8 newborn rats.

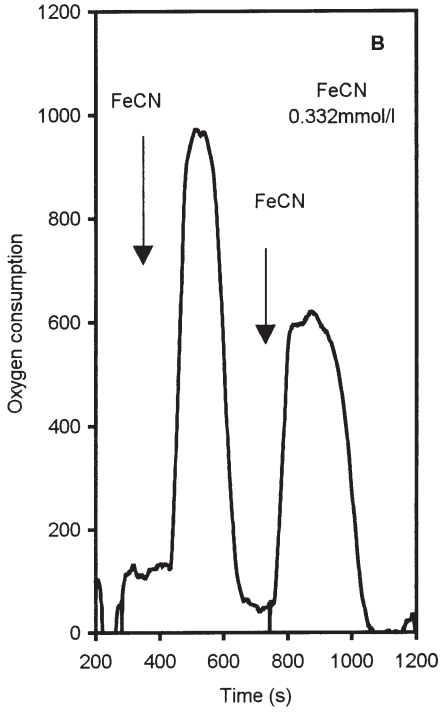
Another addition of the same amount of FeCN gives the identical response in oxygen uptake as the first one. In Fig. 2 four additions of 0.083 mmol/l FeCN are shown. As calculated in Tab. 1, the rate of oxygen uptake induced by the first two additions of FeCN was practically the same, 756 and 730 pmol oxygen/s/mg protein, respectively, but it decreased after the third and fourth addition of FeCN to 618 and 568 pmol oxygen/s/mg protein, respectively. Both, the total amount of oxygen consumed and the molar ratio of oxygen consumed to FeCN added (O/FeCN), however, remained the same in all additions, i.e. 98–125 nAt O/mg protein and 0.24–0.30 nAt O/nmol FeCN, respectively.

Table 1. KCN-insensitive, GP-dependent, FeCN-induced oxygen consumption by rat brown adipose tissue mitochondria

Additions	Oxygen consumption (pmol oxygen/s/ mg protein)	Total oxygen consumption (nAt O/mg prot.)	Efficiency of FeCN-induced oxygen uptake (nAt O/nmol FeCN)
FeCN (415 nmol/mg prot. and 30 mmol/l GP)			
1^{st}	756	125	0.30
2^{nd}	730	123	0.30
3^{rd}	618	104	0.25
4^{th}	568	98	0.24
mmol/l GP (using 415 nmol FeCN/mg prot.)			
10	550	98	0.24
30	722	100	0.24
50	739	112	0.27
mmol/l FeCN (using 40 mmol/l GP)			
0.083 (286 nmol/mg prot.)	950	90	0.31
0.332 (1145 nmol/mg prot.)	912	232	0.20
0.664 (2290 nmol/mg prot.)	685	299	0.13
1.328 (4597 nmol/mg prot.)	419	405	0.09
Oleate inhibition (using 40 mmol/l GP and 415 nmol FeCN/mg prot.)			
Control	625	105	0.25
+0.1 mmol/l oleate	200	132	0.32
Polyborate inhibition (using 40 mmol/l GP and 415 nmol FeCN/mg prot.)			
Control	690	105	0.25
+0.05 mmol/l polyborate	325	132	0.32

All values are average of three preparations of brown fat mitochondria. In each experiment mitochondria were isolated from 8 newborn rats.


A
FeCN
FeCN
FeCN
0.083 mmol/l
Oxygen consumption
Time (s)
0
200
400
600
800
1000
1200


B
FeCN
FeCN
0.332mmol/l
FeCN
Oxygen consumption
Time (s)
0
200
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1000
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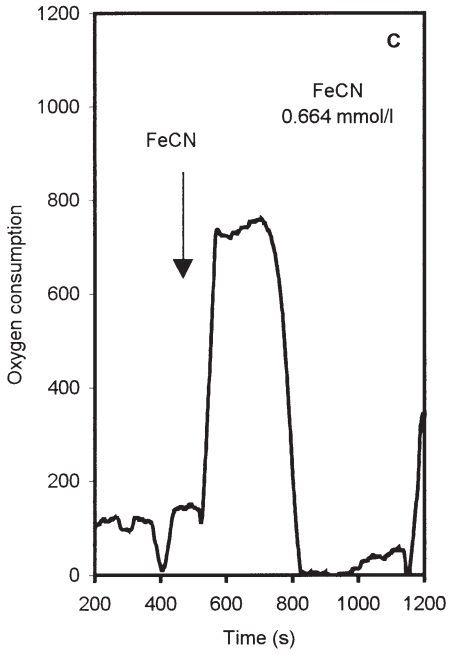
C
FeCN
0.664 mmol/l
FeCN
Oxygen consumption
Time (s)
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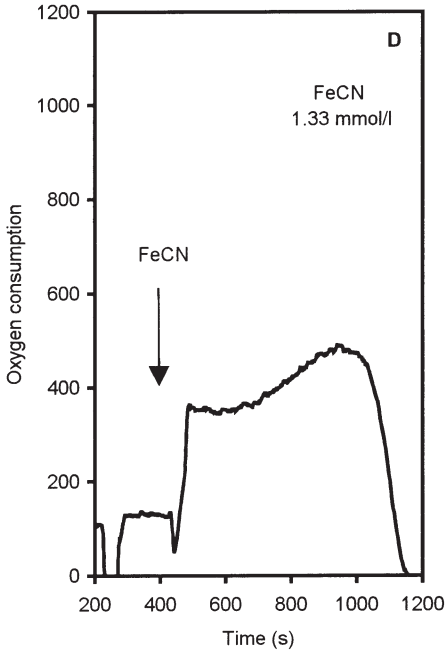
D
FeCN
1.33 mmol/l
FeCN
Oxygen consumption
Time (s)
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600
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Figure 3. FeCN-induced oxygen consumption (pmol oxygen/s/mg protein) at various FeCN concentrations. Measurements were performed in the presence of 0.3 mmol/l KCN, 30 mmol/l GP and 0.3 mg of mitochondrial protein/ml. Additions of FeCN were 0.083 mmol/l – (A), 0.332 mmol/l – (B), 0.664 mmol/l – (C) and 1.33 mmol/l – (D). The same results were obtained using three preparations of brown fat mitochondria isolated from 8 newborn rats.

As can be seen in Tab. 1, the rate of FeCN-induced, GP-dependent oxygen consumption increased from 550 to 722 pmol oxygen/s/mg protein at 10 and 30 mmol/l of GP, respectively, and remained unchanged at higher GP concentrations. The ratio O/FeCN was not changed at increasing GP concentrations.

The highest rate of FeCN-induced, GP-dependent oxygen uptake was obtained at low FeCN concentrations (0.083–0.30 mmol/l). At concentrations above 0.50 mmol/l the rate of the oxygen consumption decreased (Tab. 1, Fig. 3). At different FeCN concentrations used, the kinetics of the reaction, indicating the hydrogen peroxide production, was modified as well (see Fig. 3). The rate of the reaction

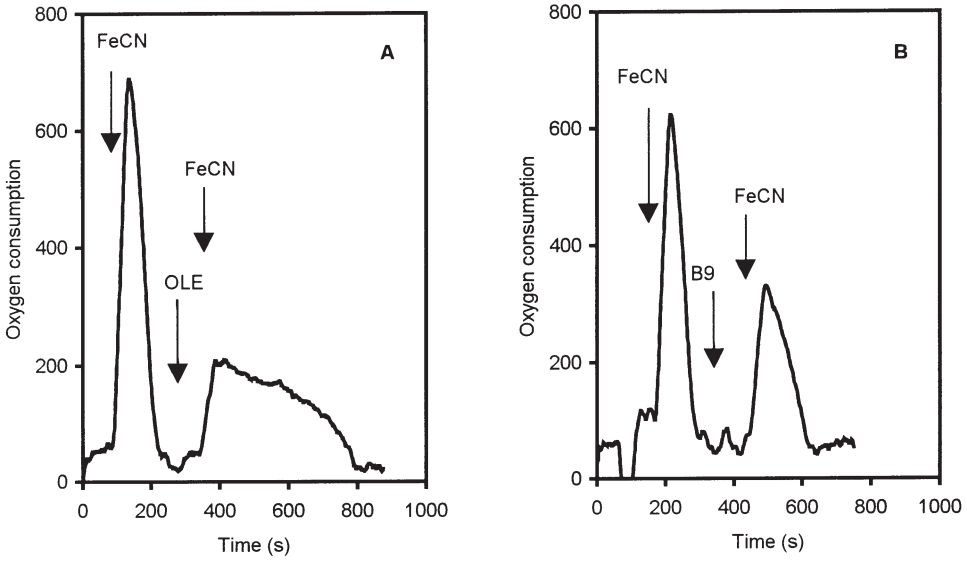

Figure 4. FeCN-induced oxygen consumption (pmol oxygen/s/mg protein) in the presence of mGPDH inhibitors. Measurements were performed in the presence of 0.3 mmol/l KCN, 40 mmol/l GP, and 0.33 mg of mitochondrial protein/ml. Oleate (OLE) 0.1 mmol/l – (A) and mercapto nonaborate (B9) 0.05 mmol/l – (B) were added between two additions of 0.083 mmol/l FeCN. The same results were obtained using three preparations of brown fat mitochondria isolated from 8 newborn rats.

decreased. The total amount of oxygen consumed increased, but this increase was not proportional to the increasing concentration of added FeCN. Therefore the efficiency of FeCN-induced oxygen uptake (nAt of total oxygen consumed *per* nmol of FeCN added) strongly decreased from 0.31 to 0.09 (Tab. 1).

In last experiments we decreased mGPDH activity by specific inhibitors and we compared the rate of oxygen consumption, total amount of oxygen consumed and O/FeCN ratio. We used 0.05 mmol/l mercapto dicarbanonaborate (Drahota et al. 1995) and 0.1 mmol/l oleate (Houštěk and Drahota 1975), which inhibited mGPDH approximately by 50 %. As shown in Fig. 4 and Tab. 1, both inhibitors depressed the rate of FeCN-induced oxygen uptake, but the total oxygen uptake and the O/FeCN ratio remained unchanged. When the enzyme activity of mGPDH was completely inhibited no ferricyanide-induced oxygen consumption was found (not shown).

Discussion

Our previous study (Drahota et al. 2002) showed that besides already described sites of mitochondrial ROS production localized at Complex I (NADH-CoQ reductase) and III (CoQ-cytochrome c reductase) the mGPDH represents an additional source of ROS generation. In this communication on brown fat mitochondria from neonatal rat we confirmed our previous findings obtained on mitochondria from brown adipose tissue of cold adapted hamsters, that one electron acceptor, namely FeCN, highly accelerates hydrogen peroxide production and that FeCN-induced hydrogen peroxide production is specific for mGPDH. It can be detected when GP is oxidized, but not when succinate or NADH are used as substrates. In this paper we studied in more detail various aspects of this phenomenon, namely kinetics of hydrogen peroxide production at various FeCN concentrations and correlation between the rate of peroxide production and total amount of peroxide produced at various FeCN concentrations or at various rates modified by specific mGPDH inhibitors.

Data presented in Fig. 2 demonstrate that when small amounts of FeCN (83 nmol/ml) are added several times, the rate of hydrogen peroxide production slowly decreases (see Tab. 1). However, the total amount of hydrogen peroxide produced remained constant as well as the O/FeCN ratio. The decrease of the oxygen uptake rate after each FeCN addition in accordance with spectrophotometric analysis indicates, that all FeCN added was reduced. Our data presented also showed that the total amount of hydrogen peroxide produced could be highly increased using elevated concentrations of FeCN (Tab. 1). Changes of kinetics of this reaction (Fig. 3), however, indicate that the decrease of the rate of oxygen uptake is due to higher concentration of FeCN used.

The declining rate of the reaction may be caused by the inhibitory effect of accumulation of the reduced FeCN. The total amount of peroxide produced increased with increasing FeCN concentrations in the medium, however, O/FeCN ratio strongly decreased. From those data we may also conclude that even at

the highest FeCN concentration used (1.3 mmol/l), which already saturated the mGPDH activity, a certain fraction of KCN insensitive oxygen consumption (peroxide production) still remains. FeCN at lower concentrations thus strongly induced hydrogen peroxide production, but at higher concentrations (mmol/l) ability of mGPDH to produce ROS has declined.

Oleate (Houštěk and Drahota 1975) and dicarbanonaborate (Drahota et al. 1995) reversibly decrease the mGPDH activity. In the presence of these inhibitors the rate of hydrogen peroxide production was proportionally decreased, however the total amount of oxygen consumed remained the same as well as the O/FeCN ratio (Fig. 4, Tab. 1).

All these findings presented bring evidence in support of our hypothesis (Drahota et al. 2002) that FeCN-induced GP-dependent peroxide production is due to different mechanism of electron transfer from mGPDH and succinate dehydrogenase or from NADH dehydrogenase to the CoQ. It is evidently due to the absence of CoQ binding protein in mGPDH enzyme complex (Cottingham and Ragan 1980a,b). Under these conditions, when the reduced flavoprotein reacts with one electron acceptor, the second electron is more accessible for interaction with oxygen.

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