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SUCCINATE MODULATION OF H₂O₂ RELEASE AT NADH:UBIQUINONE OXIDOREDUCTASE (Complex I) IN BRAIN MITOCHONDRIA

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Complex I is responsible for most of the mitochondrial H₂O₂ release, both during the oxidation of NAD-linked substrates and during succinate oxidation. The much faster succinate-dependent H₂O₂ production is ascribed to Complex I, being rotenone sensitive. We report high affinity succinate - supported H₂O₂ generation in the absence as well as in the presence of glutamate/malate (G/M) 1 or 2 mM each. In brain mitochondria, their only effect was to increase from 0.35 to 0.5 or to 0.65 mM the succinate concentration evoking the semi-maximal H₂O₂ release. G/M are still oxidized in the presence of succinate, as indicated by the oxygen consumption rates, which are intermediate between those of G/M and of succinate alone when all substrates are present together. This effect is removed by rotenone, showing that it is not due to inhibition of succinate influx. Moreover, α -ketoglutarate production from G/M, a measure of the activity of Complex I, is decreased but not stopped by succinate. It is concluded that succinate-induced H₂O₂ production occurs in conditions of regular downward electron flow in Complex I. Succinate concentration appears to modulate the rate of H₂O₂ release probably by controlling the QH₂/Q ratio.

Short title : H₂O₂ release at mitochondrial NADH:ubiquinone oxidoreductase in brain

Keywords : Brain mitochondria, NADH:ubiquinone oxidoreductase (Complex I), H₂O₂ production, Succinate.

Abbreviations used : Complex I, NADH:ubiquinone oxidoreductase; ROS, reactive oxygen species; G/M, glutamate/malate; α -KG, α -ketoglutarate; OAA, oxaloacetate; SOD, superoxide dismutase; HRP, horseradish peroxidase; PCA, perchloric acid; $\Delta\Psi$, mitochondrial membrane potential; Δ pH, mitochondrial pH gradient; Δ p, mitochondrial protonmotive force.

INTRODUCTION

Mitochondria are the major site of reactive oxygen species (ROS) production in mammalian cells and superoxide (O_2^-) appears to be the primary ROS produced as the result of single electron reduction of O_2 .

Mitochondrial Complex I is the most complicated and least understood electron carrier in the respiratory chain. Its primary function is to pump protons across the inner membrane. A second property of Complex I is a highly modulated O_2^- production. O_2^- from Complex I is generated on the matrix side of the inner membrane [1] and is transformed into H_2O_2 by mitochondrial Mn-SOD. The highly permeable H_2O_2 is the measured species in the extramitochondrial space. The importance of ROS removal from the mitochondrial matrix is demonstrated by the poor survival of Mn-SOD null mice [2] and by the increased life span of mice expressing mitochondrial catalase [3]. Complex III also is known to produce O_2^- (mainly on the cytosolic space). However this O_2^- release is largely dependent on the presence of the electron transfer inhibitor antimycin A [4].

ROS have been considered important in various pathologies [5] and aging [6]. More recently however it has been recognized that ROS have important physiological roles as signalling molecules, whose functions are just emerging [7]. Complex I is now considered the major site of O_2^-/H_2O_2 production.

H_2O_2 is generated during coupled respiration of NAD-linked substrates (the release is increased by the electron transfer inhibitor rotenone); it is also generated, at a much faster rate, during coupled succinate respiration (production is strongly inhibited by rotenone and by decreasing membrane potential). These properties are taken as evidence that succinate-dependent peroxide generation occurs in Complex I uphill of the rotenone inhibition site (i.e. via energy-dependent reverse electron transfer from Complex II to Complex I [8-13]). We have shown that the Parkinson disease toxin dopaminochrome (derived from dopamine) promotes a strong increase of H_2O_2 production both with glutamate/malate (G/M) and in the further presence of succinate [14], and that H_2O_2 is removed by GSH peroxidase [15]. The succinate-dependent peroxide production is controlled by ΔpH across the inner membrane and is depressed when such gradient is abolished [16]. The physiological importance of succinate-sustained H_2O_2 generation, being linked to a situation of reverse electron transfer, has been considered dubious [9-13,16,17].

In this study we analysed the production of H₂O₂ in brain mitochondria in the presence of both the NAD-linked substrates G/M (or pyruvate/malate, which gave the same results) and succinate, i.e. close to *in vivo* conditions. We show that succinate-dependent peroxide production initiates at low physiological concentrations and is only marginally modified by the presence of G/M. Furthermore, G/M oxidation is progressively decreased, but not abolished by succinate and the two substrates appear to compete for electron transfer.

We propose that the sharp dependence of the H₂O₂ generation rate on succinate concentration is an important modulator in the overall cellular ROS production.

MATERIALS AND METHODS

Reagents

Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) was from Molecular Probes, Inc. Horseradish peroxidase (HRP) (grade I ; EC 1.11.1.7) , catalase from bovine liver (EC 1.11.1.6) and α -ketoglutarate (α -KG) dehydrogenase from pig heart (EC 1.2.4.2) were supplied from Sigma. All other reagents were of analytical grade.

Preparation of Rat Brain Mitochondria

Brain mitochondria were isolated from cerebral cortices of 6-7 weeks old rats as described [15].

Standard Incubation Method

Mitochondria (0.4 – 0.6 mg/ml) were incubated at 30 °C in 125 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgCl₂, 500 μ g/ml defatted bovine serum albumin, 20 mM MOPS, pH 7.2 (adjusted with KOH), and 100 μ M EGTA. Further additions were as specified in the figure legends.

Hydrogen Peroxide Measurements

Hydrogen peroxide was measured with 7 μ M Amplex Red and 15 μ g/ml HRP (3.75 Units) included in the incubations. H₂O₂ was detected by the formation of the fluorescent Amplex Red oxidation product resorufin using excitation and emission wavelengths of 563 and 587 nm, respectively, on a Shimadzu RL-5000 spectrofluorometer in a stirred cuvette [14]. H₂O₂ release was linear for at least 6 – 8 minutes. The H₂O₂ calibration scale is linear in the 0-5 μ M range, and at the end of each assay, traces were calibrated by the addition of H₂O₂ (500 pmol).

Assay of α -Ketoglutarate

The α -KG assay was performed as in [18,19]. In brief mitochondria (4 mg/ml) were incubated in the standard incubation medium under agitation for 10 minutes with the

indicated amount of substrates. During incubation substrate concentrations of glutamate, malate and succinate were maintained constant by addition of 0,3 mM glutamate and malate and 0,5 mM succinate at 5 minutes. Reactions were stopped by addition of cold PCA (12 %) and samples left in ice 20 minutes before centrifugation. Enzymatic fluorimetric titration of NADH formed by α -KG oxidation was carried out in 100 μ l aliquots of the supernatant neutralized with KOH in 200 μ l (final volume) of medium containing 100 mM Na-phosphate (pH 7.3), CaCl_2 5 mM, MgCl_2 1 mM, EGTA 5 mM, NAD^+ 1 mM, and 0.2 mg/ml (160 mU) of α -KG dehydrogenase in a plate reader Fluoroskan at 355/460 nm ex/em. The reaction was started by addition of 100 μ M CoASH. α -KG concentrations were calculated from the titration curve of the rates obtained with known comparable amounts of standard α -KG. Values are reported as n moles /min/mg protein. Similar results were obtained by spinning down mitochondria at the end of incubation and measuring α -KG in the PCA-treated supernatant. Also, including catalase (5 μ g/ml) in the incubation medium was without significant effect on α -KG accumulation.

Other assays

Mitochondrial Membrane potential ($\Delta\psi$) was measured using fluorescence quenching of the cationic dye safranin (3 μ M) at 495 nm (excitation) and 586 nm (emission) as in [15]. NAD(P) reduction was monitored fluorimetrically at 348 nm (excitation) and 464 nm (emission) as in [15]. Mitochondrial Respiration was monitored in the presence of HRP and Amplex Red with a Clark-type oxygen electrode in a 1.6 ml closed chamber with continuous stirring.

RESULTS AND DISCUSSION

Complex I dependent H_2O_2 release with succinate and G/M

Fig.1A shows the fast H_2O_2 production rate in brain mitochondria oxidizing succinate (trace a) and the much slower peroxide generation with G/M as the substrates (trace b). The production of H_2O_2 as a function of succinate concentration, in the absence and presence of G/M, is reported in Fig.1B. The maximal H_2O_2 release (with 3 mM succinate) was unaffected by G/M. The half maximal peroxide production with succinate alone was at approximately 0.35 mM. With G/M (1 mM each), a concentration which produced maximal Δp , the H_2O_2 release at zero succinate was about 0.15 nmol/mg/min, and the further addition of succinate promoted a sharp increase of peroxide production, with a half maximal stimulation at about 0.5 mM. A similar behaviour was obtained at higher (2 mM) G/M. In this situation the half

maximal stimulation of peroxide release by succinate was increased to 0.6 - 0.65 mM. The rotenone-inhibited H_2O_2 production rates with succinate are also reported in Fig. 1B. It is likely that this residual H_2O_2 release also originated from Complex I, since succinate promoted a large NAD(P) reduction in the presence of rotenone, likely via malate dehydrogenase. Similar results were obtained with piericidin (not shown). Anyway, in the hypothesis that the peroxide generation with succinate and rotenone was not originating at Complex I, these rates were subtracted from the succinate and succinate-G/M values of Fig.1B. The results are reported in Fig.1C. They represent certainly Complex I-dependent H_2O_2 releases with succinate and G/M.

Succinate does not prevent electron flow from G/M in Complex I

The generally accepted view is that succinate promotes H_2O_2 release by pushing electrons back into Complex I, in a reverse mode of electron transfer. With NAD-linked substrates and in the absence of succinate, H_2O_2 release is slow and can be increased in a non-physiological situation, i.e. in the presence of rotenone-type electron transfer inhibitors and high ΔpH [17]. Alternatively peroxide release was reported to be increased in cytochrome c depleted mitochondria [20]. The findings reported in the present study show that low succinate promotes H_2O_2 release also in the presence of high NAD-linked substrates. It can be questioned if succinate in these conditions operates a “reverse” mode of electron flux, i.e. if the oxidation of G/M is prevented. To solve this problem first of all we analysed the rates of O_2 consumption with G/M (1 mM each), succinate and the mixture. Typical results are reported in Table 1 showing the experiment with 0.5 mM succinate (a concentration that promotes a large H_2O_2 release). The oxidation rate was higher with succinate than with G/M, likely reflecting the lower number of protons pumped by succinate, which does not feed electrons to Complex I. However, the O_2 consumption rate with the mixture was intermediate between that of the two constituents alone, a possible indication that both succinate and G/M were contributing to O_2 consumption when they were present together. In the presence of rotenone, the O_2 consumption rate with succinate was not significantly affected by G/M, showing that there was no limitation to succinate availability. The overall titration of O_2 consumption with increasing succinate, in the absence and presence of constant (1 mM) G/M is reported in Fig. 2. Above 0.2 mM the succinate oxidation rate was faster than that of G/M and the addition of G/M induced a decrease of O_2 consumption to intermediate values. Only above about 1.5 mM succinate, G/M became incapable to decrease the succinate oxidation rate. These results confirm that a fraction of the overall oxidation rate was indeed dependent on G/M when both G/M and succinate were supplied to mitochondria.

Although the results just presented are a strong evidence in favour of the hypothesis that electrons actually flow downward in Complex I also during the succinate-promoted H_2O_2 release in the presence of G/M, they are not a direct demonstration that this really occurs. As a second approach to prove this point we measured the generation of α -KG with G/M, in the absence and presence of succinate. The accumulation of α -KG is directly connected to the oxidation of G/M, via glutamate dehydrogenase, or via glutamate-oxaloacetate (OAA) transaminase, where OAA originates from malate oxidation. As shown in Fig. 3, α -KG was accumulated during the oxidation of G/M, but not during succinate oxidation. With G/M, the further presence of succinate progressively decreased, but did not abolish, α -KG production. At 1 mM succinate, α -KG production was still 30% of the control without succinate. These results are in agreement with the data on respiration presented above.

These experiments demonstrate conclusively that electrons keep flowing down from Complex I in the presence of succinate and during the process of succinate-supported H_2O_2 production.

H_2O_2 release is controlled by ΔpH

The succinate-induced H_2O_2 release is dependent on Δp across the inner mitochondrial membrane. However, Δp is the sum of two components, the difference in electrical potential $\Delta\psi$ and the difference in pH (ΔpH). It has been shown recently that ΔpH is a major controller of succinate-dependent peroxide production [16]. In fact, the inclusion of the K^+/H^+ ionophore nigericin (in a high K^+ medium) allows K^+ equilibration and hence H^+ equilibration ($\Delta\text{pH} = \text{zero}$): this results in a corresponding equivalent increase of $\Delta\psi$ to maintain Δp constant (ref 16, and results not shown). In these conditions, the succinate promoted H_2O_2 release is at its lowest. The succinate titration of peroxide release in the presence of nigericin and G/M (Fig. 4) shows that the overall production rate was depressed relative to Fig. 1B (dotted line) but that the pattern was unchanged. On the contrary, increasing ΔpH by omitting phosphate (whose influx via the P_i/H^+ symporter decreases ΔpH), promoted a higher succinate-dependent H_2O_2 release [16], without changing the general behaviour in the presence of G/M (Fig.4).

Conclusions

The results presented show that the succinate stimulation of mitochondrial H_2O_2 release occurs at low concentrations and that the presence of G/M modifies only marginally the

succinate promotion of peroxide release. Furthermore, G/M oxidation is decreased but not prevented by succinate.

When both NADH and succinate are present (the *in vivo* situation) it appears that they compete for Complex I (or a part of it), the first pushing electrons down and the second pushing electrons up into the complex. The two processes appear to occur at the same time, possibly due to competition between regular quinone reduction from NADH and re-binding of the reduced species due to its higher concentration during the operation of Complex II. Electron backflow with succinate could mean just binding of QH₂ originating from Complex II to the quinone binding site on Complex I with O₂⁻ generation occurring right there (as speculated in ref.17) instead of electrons going uphill to a different site of O₂⁻ production. In this view, the fact that G/M are still oxidized in the presence of succinate does not contradict any previously drawn conclusions.

The high mitochondrial QH₂/Q ratio induced by succinate appears the major determinant of H₂O₂ release. Similarly, G/M alone induce high H₂O₂ release rates in cytochrome c depleted mitochondria [20]. In the latter situation the removal of cytochrome c decreases the rate of electron flow and consequently increases QH₂/Q. A similar effect could occur following NO[·] inhibition of cytochrome oxidase [21,22].

Succinate concentration *in vivo* is controlled by the activity of the other dehydrogenases, which are NAD-dependent. It is significant that *in vivo* succinate concentration oscillates between 0.3 and 1 mM, as determined by Sato et al. [23] in perfused heart.

Preliminary results appear to indicate a similar organization of H₂O₂ release in heart mitochondria.

REFERENCES

1. St-Pierre, J., Buckingham, J.A., Roebuck, S.J. and Brand, M.D. (2002) Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J.Biol.Chem.* **277**, 44784-44790.
2. Melov, S., Coskun, P., Patel, M., Tuinstra, R., Cottrell, B., Jun, A.S., Zastawny, T.H., Dizdaroglu, M., Goodman, S.I., Huang, T.T., Mizioro, H., Epstein, C.J. and Wallace, D.C. (1999) Mitochondrial disease in superoxide dismutase 2 mutant mice. *Proc.Natl.Acad.Sci.USA.* **96**, 846-851.
3. Schriener, S.E., Linford, N.J., Martin, G.M., Treuting, P., Ogburn, C.E., Emond, M., Coskun, P.E., Ladiges, W., Wolf, N., Van Remmen, H., Wallace, D.C., Rabinovitch, P.S. (2005) Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science* **308**, 1909-1911.
4. Turrens, J.F., Alexandre, A. & Lehninger, A.L. (1985) Ubisemiquinone is the electron donor for superoxide formation by Complex III of heart mitochondria. *Arch.Biochem.Biophys.* **237**, 408-414.
5. Halliwell, B. and Gutteridge, J.M.C. (1999) in *Free radicals in biology and medicine*, Third Edition. Oxford University Press Inc. NY.
6. Cadenas, E. and Davies, K.J. (2000) Mitochondrial free radical generation, oxidative stress, and aging. *Free Radical Biol.Med.* **29**, 222-230.
7. Allen, R.G. and Tresini, M. (2000) Oxidative stress and gene regulation. *Free Radical Biol.Med.* **28**, 463-499.
8. Turrens, J.F. & Boveris, A. (1980) Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem.J.* **191**, 421-427.
9. Hansford, R.G., Hogue, B.A. and Mildaziene, V. (1997) Dependence of H₂O₂ formation by rat heart mitochondria on substrate availability and donor age. *J.Bioenerg.Biomembr.* **29**, 89-95.
10. Liu, Y., Fiskum, G. and Schubert, D. (2002) Generation of reactive oxygen species by the mitochondrial electron transport chain. *J.Neurochem.* **80**, 780-787.
11. Korshunov, S.S., Skulachev, V.P. and Starkov, A.A. (1997) High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett.* **416**, 15-18.

12. Votyakova, T.V. and Reynolds, I.J. (2001) $\Delta\Psi_m$ -dependent and independent production of reactive oxygen species by brain mitochondria. *J.Neurochem.* **79**, 266-277.
13. Han, D., Canali, R., Rettori, D. and Kaplowitz, N. (2003) Effect of glutathione depletion on sites and topology of superoxide and hydrogen peroxide production in mitochondria. *Mol.Pharmacol.* **64**, 1136-1144.
14. Zoccarato, F., Toscano, P. and Alexandre, A. (2005) Dopamine-derived dopaminochrome promotes H_2O_2 release at mitochondrial Complex I. Stimulation by rotenone, control by Ca^{2+} , and relevance to Parkinson Disease. *J.Biol.Chem.* **280**, 15587-15594.
15. Zoccarato, F., Cavallini, L. and Alexandre, A. (2004) Respiration-dependent removal of exogenous H_2O_2 in brain mitochondria. Inhibition by Ca^{2+} . *J.Biol.Chem.* **279**, 4166-4174.
16. Lambert, A.J. and Brand, M.D. (2004) Superoxide production by NADH:ubiquinone oxidoreductase (Complex I) depends on the pH gradient across the mitochondrial inner membrane. *Biochem.J.* **382**, 511-517.
17. Lambert, A.J. and Brand, M.D. (2004) Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (Complex I). *J.Biol.Chem.* **279**, 39414-39420.
18. Sanadi, D.R. (1969) α -Ketoglutarate dehydrogenase from pig heart. In *Methods in Enzymology* **13**, pp. 52-55.
19. McCormack, J.G. and Denton, R.M. (1979) The effects of calcium ions and adenine nucleotides on the activity of pig heart 2-oxoglutarate dehydrogenase complex. *Biochem.J.* **189**, 533-544.
20. Kushnareva, Y., Murphy, A.N. and Andreyev, A. (2002) Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)⁺ oxidation-reduction state. *Biochem.J.* **368**, 545-553.
21. Mason, M.G., Nicholls, P., Wilson, M.T. and Cooper, C.E. (2006) Nitric oxide inhibition of respiration involves both competitive (heme) and noncompetitive (copper) binding to cytochrome c oxidase. *Proc.Natl.Acad.Sci. U.S.A.* **103**, 708-713.
22. Antunes, F., Boveris, A. and Cadenas, E. (2004) On the mechanism and biology of cytochrome oxidase inhibition by nitric oxide. *Proc.Natl.Acad.Sci. U.S.A.* **101**, 16774-16779.

23. Sato, K., Kashiwaya, Y., Keon, C.A., Tsuchiya, N., King, M.T., Radda, G.K., Chance, B., Clarke, K. and Veech, R.L. (1995) Insulin, ketone bodies, and mitochondrial energy transduction. *FASEB J.* **9**, 651-658.

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Table 1 O₂ consumption rate measured in mitochondria oxidizing different respiratory substrates.

Mitochondria (0.9 mg/1.6 ml) were incubated in standard medium supplemented with amplex red and HRP and respiration rates were measured. Glutamate/malate (1 mM each), succinate (0.5 mM) and rotenone (1 μ M) were present or added as indicated. Data are means \pm SD from at least seven independent experiments.

Substrate	Rate of Oxygen consumption (n atoms x min ⁻¹ x mg ⁻¹)
Glutamate/malate	10.5 \pm 2.5
+ succinate	16.8 \pm 2.0
Succinate	28.0 \pm 3.0
+ glutamate/malate	17.5 \pm 1.2
Rotenone/glutamate/malate/succinate	27.5 \pm 2.0

FIGURE LEGENDS

Fig. 1. Titration of succinate-promoted H₂O₂ generation. A) Different rates of H₂O₂ production are elicited by G/M and succinate. Succinate (trace a) and glutamate plus malate (trace b) were 1 mM. Mitochondria (0.75 mg/1.6 ml) were incubated in standard incubation medium containing HRP. Amplex Red was added at the arrow. B) H₂O₂ production was measured in the absence (○) and presence of G/M, 1 mM (△) or 2 mM each (▲). Where present, rotenone was 2.5 μM (●) without G/M. C) Net H₂O₂ releases calculated by subtracting the rotenone rates from the no rotenone rates in B. Traces in A are representative of duplicate traces from at least seven independent experiments. Data in B are means ± SD from at least five independent experiments.

Fig. 2. Succinate does not prevent G/M oxidation. Mitochondria (0.9 mg/1.6 ml) were incubated as in Table 1 with variable succinate and constant G/M (1mM each). Succinate alone (○) and in the presence of G/M (□). Data are means ± SD from at least five independent experiments.

Fig. 3. α-KG is produced during coupled oxidation of G/M and succinate. Mitochondria (4 mg/ml) were incubated 10 min with 1 mM G/M and succinate as indicated. Extra G/M (0.3 mM each) and succinate (0.5 mM) were added at 5 min. The values are means ± SD from duplicate incubations from five preparations.

Fig. 4. The H₂O₂ release rates are controlled by ΔpH. Conditions as in Fig. 1 with 1 mM G/M. The titration was performed in the presence of 100 nM nigericin (□) or in the absence of phosphate (○). The dotted line represent values from Fig. 1, B (trace in the presence of G/M 1 mM). Data are means ± SD from at least four independent experiments.

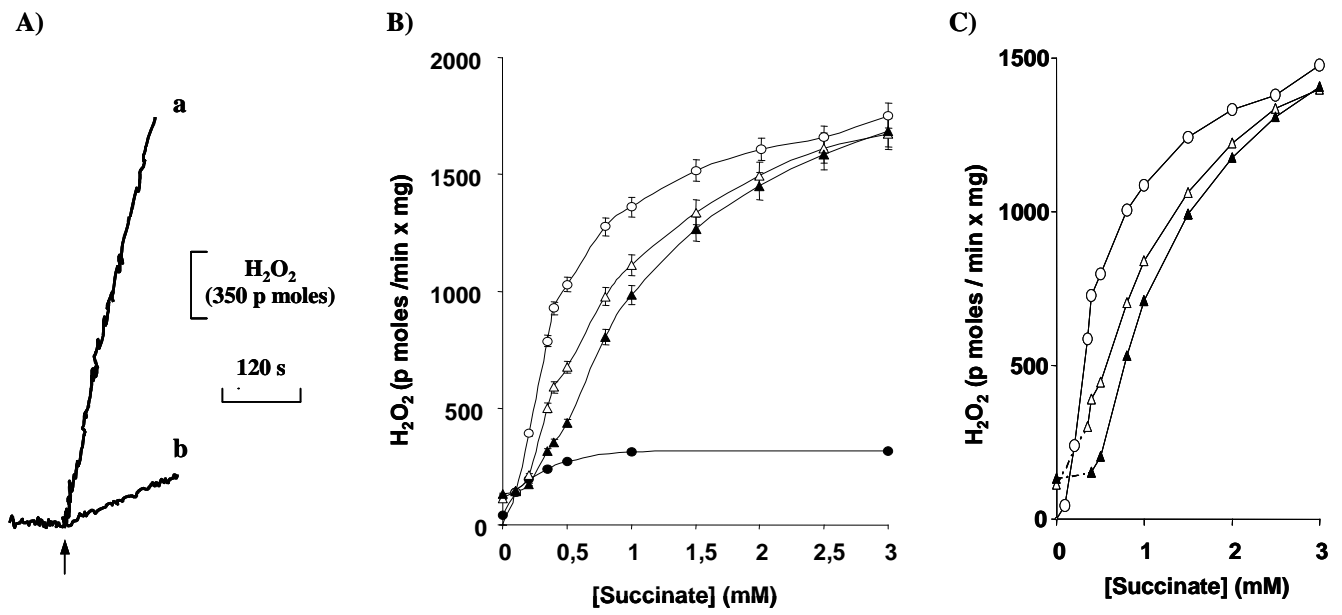


Fig.1

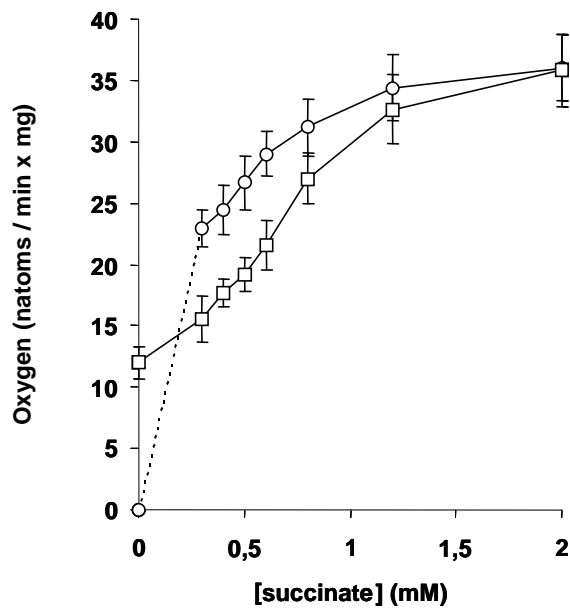


Fig. 2

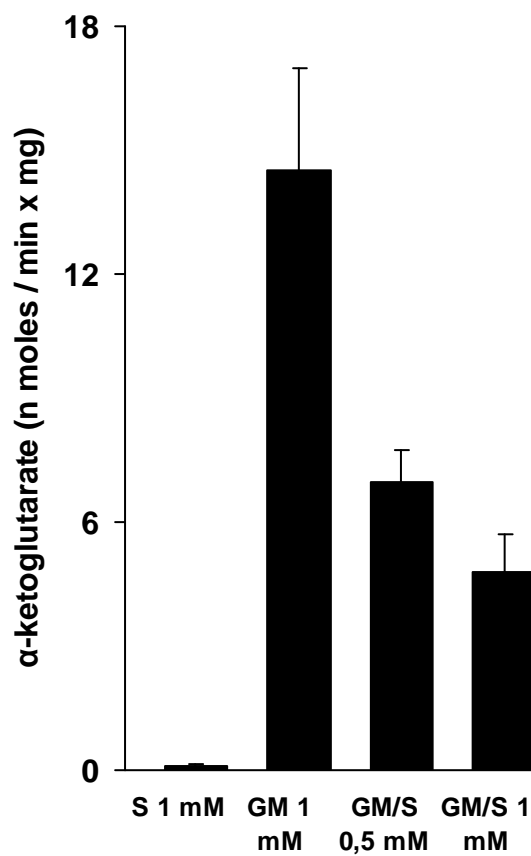


Fig.3

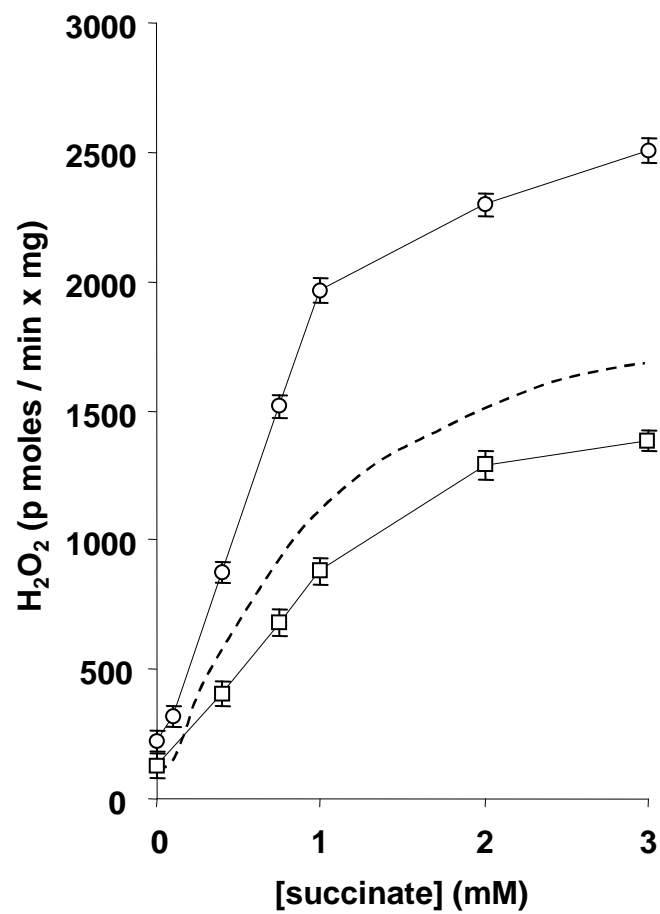


Fig.4