Relationships between the effects of redox potential, α-Thenoyltrifluoroacetone and malonate on $O_2^{\cdot-}$ and H_2O_2 generation by submitochondrial particles in the presence of succinate and antimycin

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The rate of the antimycin-induced H_2O_2 and $O_2^{\cdot-}$ generation in beef heart submitochondrial particles is maximal at the [succinate]/[fumarate] ratio of ~1 : 5 and decays at both higher and lower redox potentials. Succinate dehydrogenase inhibitors, such as TTFA or malonate, stimulate active oxygen production in the presence of excess succinate but are inhibitory at E_h values more positive than the optimal. The modulation of $O_2^{\cdot-}$ and H_2O_2 generation by these inhibitors can be explained by their effects on the steady-state redox potential(s) of the component(s) of mitochondrial site 2.

Respiratory chain *Superoxide generation* *Redox titration* *α-thenoyltrifluoroacetone* *Ubisemiquinone* *Q-cycle*

1. INTRODUCTION

H_2O_2 and $O_2^{\cdot-}$ generation in site 2 of the mitochondrial respiratory chain has been amply studied in the last 10 yr [1]. It was proposed [2–4] that oxygen radicals are derived from autoxidation of the unstable ubisemiquinone formed as an intermediate in the QH_2-oxidizing centre of complex $b \cdot c_1$ (centre *o* of the Q-cycle [3,5]) and this model has received strong experimental support [6,7].

On the other hand, stimulation of the succinate-linked $O_2^{\cdot-}$ generation by TTFA has been considered in [8,9] as evidence for a specific role of the stable TTFA-sensitive ubisemiquinone [10–12] in the reaction.

This report shows that in the antimycin-blocked SMP, oxygen radical generation can be stimulated or inhibited by TTFA depending on the redox potential of the succinate/fumarate couple. The same effects are observed with malonate, although the latter does not exert any specific influence on the stable ubisemiquinone associated with succinate dehydrogenase [11]. We also confirm our preliminary observation [13] that redox dependence of the antimycin-induced oxygen radical production is bell-shaped rather than sigmoidal, as reported in [14,15]. On the basis of these findings, the modulation of $O_2^{\cdot-}$ and H_2O_2 generation by the succinate dehydrogenase inhibitors is suggested to be a mere consequence of the oxidizing effect of these inhibitors on ubiquinone.

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Abbreviations: TTFA, α-thenoyltrifluoroacetone; SMP, submitochondrial particles; $QH\cdot$, ubisemiquinone

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2. METHODS

Reagents and experimental procedures were generally the same as described earlier [2,7], O_2^- production was monitored by EPR spectroscopy with the use of the Tiron method [2,7,16], and H_2O_2 generation by the fluorescent scopoletin/horse radish peroxidase assay [17].

3. RESULTS

A typical effect of the [succinate]/[fumarate] ratio on the rate of H_2O_2 generation in the antimycin-inhibited SMP is shown in fig.1. In the presence of 20 mM fumarate, succinate additions first stimulate and then suppress H_2O_2 generation (trace a). Accordingly, in the presence of 1 mM succinate, fumarate markedly increases the reaction rate at 1–5 mM but is inhibitory at higher concentrations (trace b). Similar observations have been made with respect to O_2^- production measured by the Tiron method (data not included, cf. fig.3).

The dependences of H_2O_2 and O_2^- generation on the redox potential of the succinate/fumarate couple are given in fig.2A and can be seen to follow

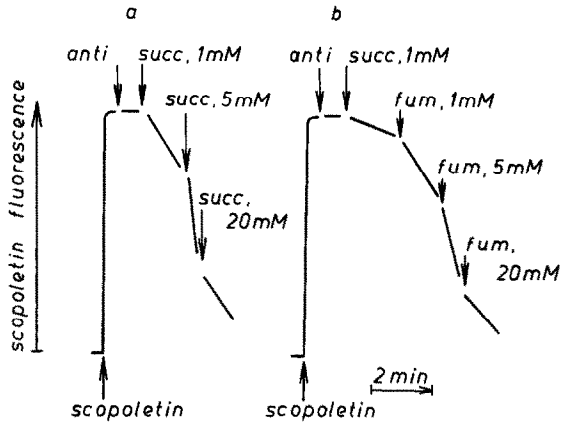

Fig.1. Effect of succinate and fumarate on the antimycin-induced H_2O_2 production. Beef heart SMP, 0.8 mg protein/ml. The basic incubation medium containing 20 mM HEPES–KOH buffer pH 7.5, 50 mM KCl, 1 μM the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 3 μM rotenone was supplemented with 0.3 μM horseradish peroxidase and (a) 0.15 M sucrose and 20 mM sodium fumarate or (b) 0.2 M sucrose. Additions: scopoletin, 2 μM; antimycin, 0.4 μg/ml, 25°C.

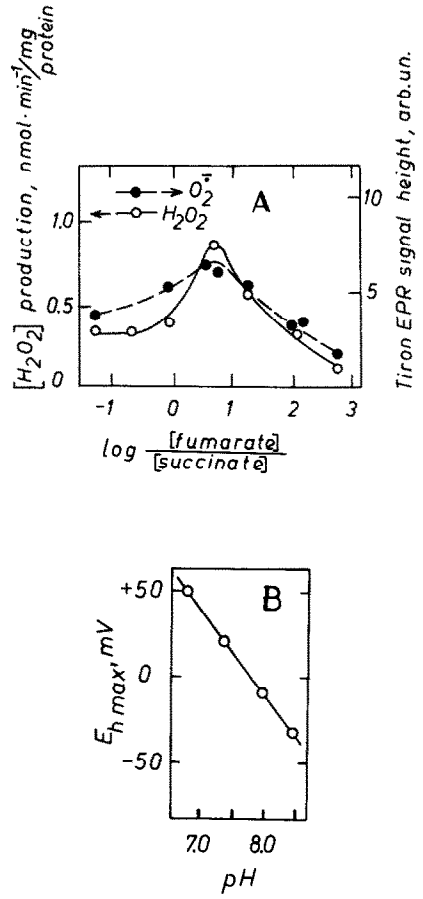

Fig.2. Redox-dependence of O_2^- (---●---) and H_2O_2 (—○—) generation in the antimycin-inhibited submitochondrial particles. (A) Beef heart SMP, 0.9 mg protein/ml. Experiments were carried out in the basic medium (see fig.1) supplemented with antimycin (0.5 μg/ml), various concentrations of fumarate (1–100 mM) and sucrose up to a total osmolarity of ~0.35 osM. 0.3 μM horseradish peroxidase + 2 μM scopoletin were added for H_2O_2 assay and 2 mM Tiron for O_2^- generation studies. The reactions were started by succinate addition and the steady-state rate of the scopoletin fluorescence decay or Tiron semiquinone EPR signal height were measured. Each point is the mean value of 3–5 measurements at various absolute concentrations of the succinate/fumarate redox buffer. (B) The bell-shaped redox dependence of H_2O_2 generation shown in (A) has been reproduced at different pH values and the E_h values at which the H_2O_2 production rate was maximal are plotted as a function of pH. A straight line with a slope −60 mV/pH unit is drawn through the points.

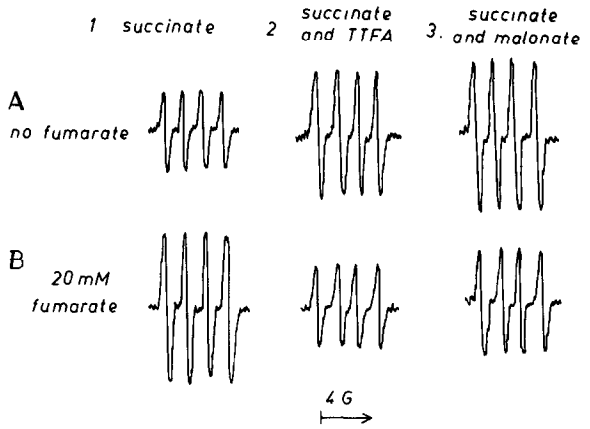

Fig.3. Effects of TTFA and malonate on the O_2^- generation in SMP. Beef heart SMP, 1 mg protein/ml in the basic incubation medium (see fig.1) supplemented with 0.5 μg/ml of antimycin, 2 mM Tiron and (A) 0.2 M sucrose or (B) 0.15 M sucrose and 20 mM sodium fumarate. EPR spectra have been recorded 5 min after addition of 5 mM succinate, 1, control; 2, in the presence of 0.1 mM TTFA; 3, in the presence of 0.5 mM malonate.

an asymmetrical extreme curve* rather than a sigmoidal Nernst plot as in [14,15]. At various absolute concentrations of succinate and fumarate, the maximal rate of both H_2O_2 and O_2^- generation was observed consistently at the [succinate]/[fumarate] ratio of about 1/5 corresponding to E_h ~40 mV at pH 7 [18], which is not too far from E_m of ubiquinone as measured by the succinate/fumarate titrations [19,20]. Very similar redox dependencies of O_2^- production have been obtained with several preparations of beef heart SMP as well as with SMP from several tumors (experiments in collaboration with Drs A. Peskin and E. Popova to be published elsewhere). Fig.2B shows that the redox potential characteristic of the maximal H_2O_2 production rate ($E_{h_{max}}$) displays pH-dependence of −60 mV/pH unit, which is typical of a hydrogen carrier like ubiquinone [19].

The bell-shaped redox dependence of oxygen radical production can provide a simple explanation for the stimulating effect of TTFA on this process [7–9]. It is conceivable that in the presence of succinate, oxygen and antimycin, TTFA addition should raise the steady-state redox potentials of the components localized between the antimycin and TTFA inhibition sites; notably oxidation of CoQ can be envisaged [20]. Thereafter, at low E_h of the succinate/fumarate couple, e.g., in the absence of added fumarate, which has been the case for most of the previous investigations, the oxidizing effect of TTFA on CoQ could easily result in enhancement of H_2O_2 and O_2^- generation. Were this true, it would follow that: (i) at [succinate]/[fumarate] ratios close to or below the optimal 1/5 TTFA would slow down rather than stimulate the succinate + antimycin-dependent oxygen radical formation; (ii) the effects of TTFA would be mimicked by other inhibitors of succinate dehydrogenase.

Both predictions are confirmed by the data given in fig.3. At the [succinate]/[fumarate] ratio of 1/4 TTFA and malonate bring about diminution of the O_2^--induced Tiron free radical signal (fig.3A), whereas in the absence of fumarate both inhibitors stimulate superoxide generation (fig.3B). Stimulation of the succinate-dependent H_2O_2 generation in the antimycin-inhibited SMP by malonate was mentioned in [20]. It should be noted that malonate, in contrast to TTFA, does not exert any specific inhibitory effect on the succinate dehydrogenase-associated stable ubisemiquinone [11].

Hence, the speculations on the specific role of

* As shown in our group by E. Popova (results to be published elsewhere), the steady-state height of the Tiron EPR signal is proportional to the square root of the O_2^--generation rate; that is why the redox profile of O_2^--production is apparently less steep than that of H_2O_2 generation.

the TTFA-sensitive ubisemiquinone species in the $O_2^{\cdot-}$-generation [8,9] are scarcely justified.

4. DISCUSSION

Relationships between the effects of redox potential, TTFA and malonate on the succinate-linked $O_2^{\cdot-}$ and H_2O_2 generation described here (see also [13]) seem to be rather clear and probably do not need further discussion. What is less clear, is why the rate of $O_2^{\cdot-}$ and H_2O_2 generation depends on the [succinate]/[fumarate] and, presumably, the $[QH_2]/[Q]$ ratio according to bell-shaped rather than sigmoidal curve. Conceivably, such a type of redox dependence is characteristic of an equilibrium yield of ubisemiquinone formed from Q and QH_2 via the reversible dismutation; hence, contribution of a stable **QH**· species to oxygen radical generation might be suggested.

However, the inhibitor analysis in [2,6,7] provides strong evidence that it is autoxidation of the unstable ubisemiquinone formed in centre *o* of the Q-cycle ($QH_o^{\cdot}$) that is the principal source of the $O_2^{\cdot-}$ radicals generated in the b–c_1 site of the respiratory chain in the presence of excess succinate. We have verified that throughout the range of redox potential used in this work the succinate-dependent $O_2^{\cdot-}$ (or H_2O_2) generation behaved exactly as in [2,6,7], i.e., the reaction did not occur unless an antimycin-type inhibitor (antimycin, HOQNO, funiculosin) was added and, when initiated by the latter, was suppressed by KCN, mucidin or myxothiazol (data not included). As discussed in [3,7], such a behaviour would be typical of $QH_o^{\cdot}$ but not of the stable ubisemiquinone species $QH_i^{\cdot}$ or $QH_{SDH}^{\cdot}$ associated with the CoQ-reducing centres of complex b–c_1 and succinate dehydrogenase, respectively. Therefore, it is likely that the bell-shaped redox profile of $O_2^{\cdot-}$ production, were it or not associated with the stable $QH^{\cdot}$ formation, is due to modulation of the same $QH_o^{\cdot}$-dependent reaction [2–4,7] rather than to a contribution of a second different process [8,9]. The mechanism of this modulation is at present under investigation.

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