MITOCHONDRIAL H_2O_2 FORMATION: RELATIONSHIP WITH ENERGY CONSERVATION*

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1. Introduction

In previous communications it was shown that the mitochondrial respiratory chain has to be regarded as a novel source of cellular H_2O_2 production [1–3]. As detected either by the peroxidase linked oxidation of the fluorescent dye scopoletin [1], or by the formation of complex ES of the cytochrome *c* peroxidase [2, 3], H_2O_2 appeared to be generated in state 4 mitochondria [4] with succinate as the substrate. Either uncouplers or ADP + P_i abolished it, while antimycin or state 3 → 4 transitions [4] restored again mitochondrial H_2O_2 formation.

In the present paper the scopoletin method [1, 5, 6] has been utilized to clarify the site of H_2O_2 formation. Though it was suggested in previous communications [1–3] that the respiratory chain linked H_2O_2 formation reflected the degree of reduction of an autoxidizable electron carrier on the substrate side of the antimycin block, the present study will show that the phenomenon can not be explained exclusively on this basis. Our experiments indicate that the rate of mitochondrial H_2O_2 formation is also under the control of energy conservation.

2. Materials and methods

Scopoletin (purum) was purchased from Fluka A.G., Switzerland, peroxidase from horse radish, type VI, from Sigma Chemical Company, St. Louis, Mo., USA. The uncoupler S13 (5-Cl, 3-*t*-butyl, 2′-Cl, 4′-NO_2-salicylanilide) was a gift of Dr. Metcalf, Monsanto Co., St. Louis, Mo., USA. Rat heart mitochondria were prepared in sucrose-EDTA buffer (0.25 M sucrose, 2 mM EDTA-Tris, pH 7.4) according to the method described by Chance and Hagihara for pigeon heart mitochondria [7]. Protein was determined by the biuret method [8]. Mitochondrial H_2O_2 formation was determined by the scopoletin method [5, 6] as described earlier [1]. Redox titrations with the succinate/fumarate couple have been carried out in a dual wavelength spectrophotometer which was designed and constructed in the Johnson Research Foundation, University of Pennsylvania.

3. Results and discussion

The production of H_2O_2 in mitochondria has been studied by the scopoletin method [1].

Interferences with this technique, arising from the presence of peroxisomes or endogenous electron donors, are not significant when mitochondria from heart (rat, beef, pigeon) are employed [9]. Moreover in rat heart mitochondria no significant H_2O_2 destruction could be detected [9]. In fact its accumulation was proportional to the time of incubation with substrate [9] and did not decrease with time when substrate oxidation was stopped. Thus the scopoletin method has been found to be a simple and sensitive technique for investigating the mechanism of H_2O_2 formation in heart mitochondria.

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3.1. *The effect of antimycin*

Having been shown that mitochondrial H_2O_2 formation is linked to the respiratory chain [1–3] the question to be investigated first was whether the rate of mitochondrial H_2O_2 production can be correlated only to the degree of reduction of a respiratory chain component or whether the energy coupling mechanism is also involved.

In order to distinguish whether the redox state or the energy state controls mitochondrial H_2O_2 formation the electron flux was blocked by antimycin in tightly coupled and uncoupled mitochondria. As shown in fig. 1A and B, tightly coupled rat heart mitochondria were supplemented with succinate, horse radish peroxidase (HRP) and scopoletin in oxygen saturated sucrose-Tris buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4).

The rate of scopoletin fluorescence decrease observed immediately after the addition of horse radish peroxidase in fig. 1A and B indicated the rate of mitochondrial H_2O_2 production under these conditions (suceinate, state 4). In fig. 1A, after uncoupling by 25 nM S13, H_2O_2 formation was abolished.

Addition of antimycin, at a concentration sufficient to inhibit electron transport, stimulated the rate of H_2O_2 formation only in uncoupled mitochondria (fig. 1A) but was inhibitory in tightly coupled mitochondria (fig. 1B). The stimulation by antimycin observed in the uncoupled mitochondria brought the rate of H_2O_2 formation to a value close to that in state 4, before the addition of S13. The inhibition induced by antimycin in coupled mitochondria decreased the rate of H_2O_2 formation to one sixth of its initial value. Though all electron carriers on the substrate side of the antimycin block should be reduced to the same extent in coupled and uncoupled mitochondria the rate of H_2O_2 formation in the presence of antimycin instead was very different. This experiment clearly indicates that mitochondrial H_2O_2 formation can not simply be controlled by the degree of reduction of a respiratory chain component on the substrate side of the antimycin block. A component, whose degree of reduction is controlled by the energy coupling mechanism, must then be involved.

3.2. *The sensitivity to uncoupling*

In previous communications as well as in fig. 1A it was shown that mitochondrial H_2O_2 formation can

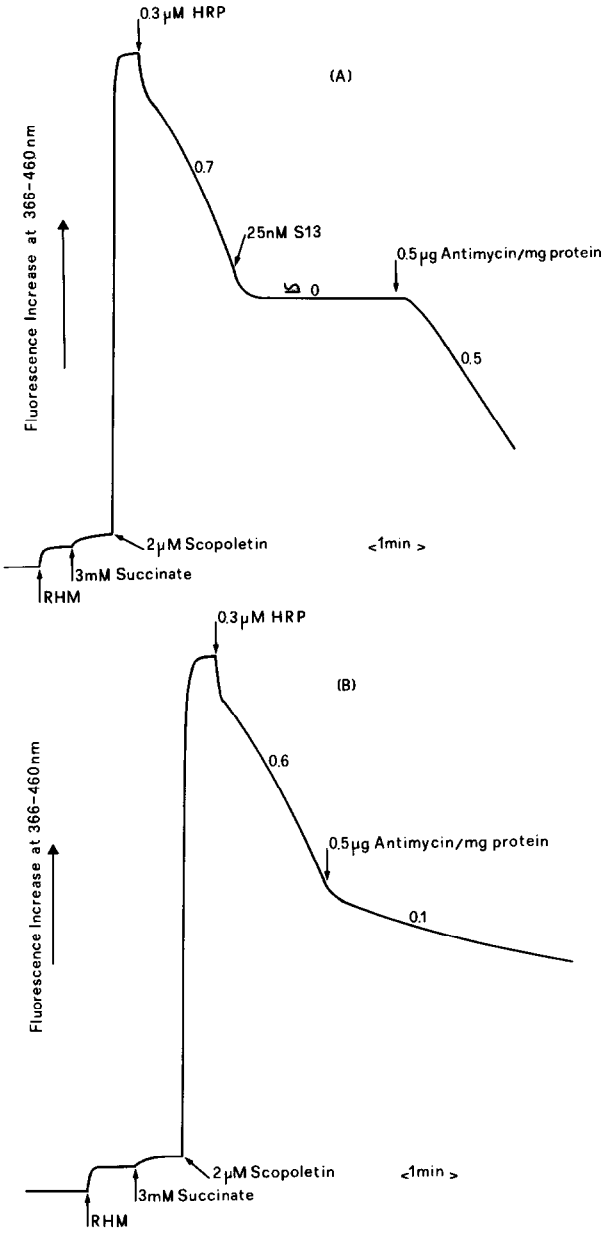

Fig. 1. Effect of antimycin on the rate of H_2O_2 formation in uncoupled and tightly coupled rat heart mitochondria. Experimental conditions: 0.25 M sucrose, 10 mM Tris-HCl pH 7.4, 0.6 mg protein/ml with a respiratory control index of 3.5. The medium was saturated with oxygen. The experiments of (A) and (B) were carried out under identical conditions except that in (A) 25 nM of the uncoupler S 13 was added prior to the antimycin addition. RHM = rat heart mitochondria, HRP = horse radish peroxidase.

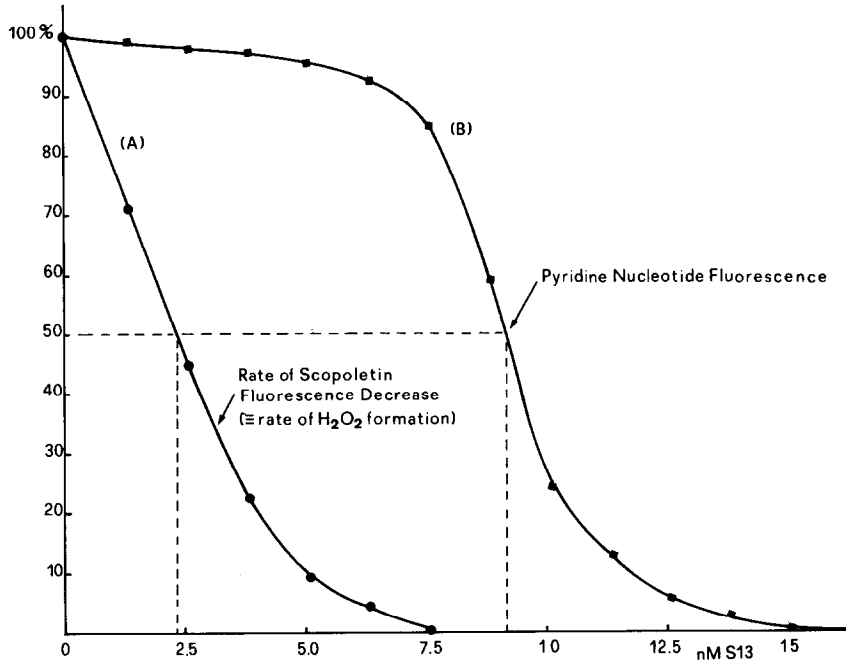

Fig. 2. Sensitivity of mitochondrial H_2O_2 formation and energy-linked reduction of endogenous pyridine nucleotides to the uncoupler S13. Both titrations were carried out with tightly coupled rat heart mitochondria (0.6 mg protein/ml with a respiratory control index of 3.5) supplemented with 3 mM succinate in oxygen saturated sucrose-Tris buffer (see fig. 1) under identical conditions except that in (A) 2 μM scopoletin and 0.3 μM HRP were present. The excitation and emission wavelengths in both experiments were 366 and 460 nm, respectively. The 100% value of each curve corresponds to the maximum rate of H_2O_2 formation (A) and the maximum decrease of the uncoupler sensitive pyridine nucleotide fluorescence (B).

be abolished by the addition of uncouplers. In fig. 2 the sensitivity of H_2O_2 formation to the uncoupler S13 was compared with the energy-linked, succinate induced reduction of endogenous pyridine nucleotides [10]. This plot shows a marked difference in the sensitivity of the two reactions to the uncoupler. In the presence of 2.4 nM S13 50% of the H_2O_2 formation has been inhibited. In contrast 9 nM S13 were necessary to uncouple 50% of the succinate induced reduction of pyridine nucleotides. It is well known that different energy-linked reactions are uncoupled at different uncoupler concentrations. Kraayenhof reported a 22-fold difference in S13 sensitivity for different energy-linked reactions in mitochondria [11]. The nearly 4-fold higher sensitivity to the uncoupler S13 of mitochondrial H_2O_2 formation over the energy-linked succinate induced reduction of endogenous pyridine nucleotides provides further evidence that this reaction is controlled by the energy coupling mechanism.

3.3. *The dependence on the redox potential*

The experiment of fig. 1 indicates a close correlation between antimycin binding and H_2O_2 formation. For the identification of the unknown autoxidizable component of the mitochondrial respiratory chain the dependence of the rate of H_2O_2 formation as a function of the redox potential has been investigated. Fumarate and succinate (Em = 24 mV at pH 7.0) [12] in the presence of antimycin were utilized in different concentrations to adjust the potential of the mitochondrial electron carriers to known values. Since fumarate penetrates poorly the membrane of intact mitochondria these experiments have been carried out with membrane fragments prepared from beef heart mitochondria by sonic disruption [13]. These membrane fragments, when supplemented with succinate and antimycin, produce H_2O_2 as well [9]. When the redox potential of the respiratory chain components of membrane fragments in the presence of antimycin

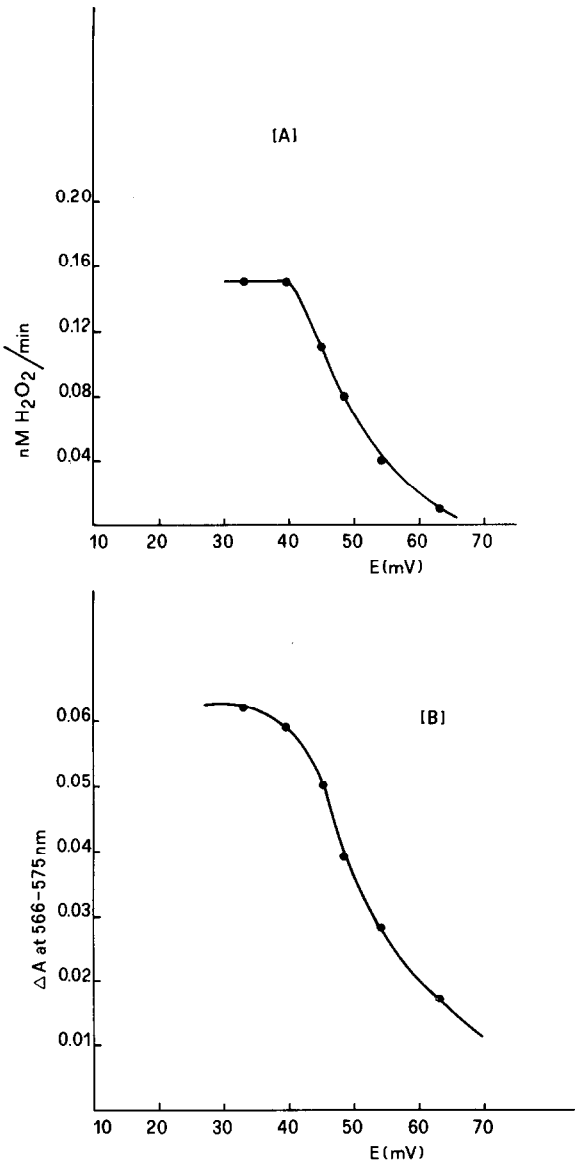

Fig. 3. The dependence of the rate of H_2O_2 formation and the degree of reduction of *b* cytochromes on the redox potential. Sonicated mitochondrial fragments (1.6 mg protein/ml) were suspended in sucrose-Tris buffer (see fig. 1) in the presence of 0.5 μg antimycin/mg protein. For further explanation see text.

was changed by the succinate/fumarate couple the rate of H_2O_2 formation was varied. Fig. 3A shows the dependence of the rate of H_2O_2 formation on the fumarate/succinate couple (expressed in mV) and in fig. 3B under identical conditions the dependence of the degree of reduction of the *b* cytochromes measured at 566–575 nm. Due to the impossibility of measuring H_2O_2 formation in the presence of dithionite, only the succinate reducible cytochromes in the presence of antimycin were considered. Fig. 3 indicates that the rate of H_2O_2 formation and the degree of cytochrome *b* reduction respond very similarly to the change of redox potential between 65 and 30 mV. The plots of the two reactions are sigmoidal with a maximum inflection of about 45 mV. At higher potentials however ($\gg$ 65 mV) a reduction of *b* cytochromes was observed which would not be correlated to the rate of H_2O_2 formation (compare fig. 3 A, B). Wikstrom and Berden (cf. fig. 10 A of [14]), under the conditions we have employed in the experiment of fig. 3 (except that their maximum reduction point was obtained by dithionite addition) have shown that a *b*-type cytochrome with a mid-point potential of 40 mV in the presence of antimycin has the spectroscopic properties of cytochromes b_{566} + b_{558}.

4. Conclusions

The inhibition of H_2O_2 formation by antimycin in tightly coupled mitochondria (fig. 1B), the stimulation in uncoupled (fig. 1A) and the high sensitivity to the uncoupler S13 (fig. 2) strongly suggest that the energy coupling mechanism is directly involved in mitochondrial H_2O_2 formation. Furthermore, the similar response to redox potential changes of both H_2O_2 production and cytochromes b_{558} + b_{566} reduction (fig. 3 A and B) indicate more precisely the involvement of phosphorylation site II in this process.

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