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INACTIVATION OF LYOPHILIZED DPNH DEHYDROGENASE FROM *ESCHERICHIA COLI* BY OXYGEN

M. GUTMAN*, A. SCHEJTER AND Y. AVI-DOR

Department of Biochemistry, Tel-Aviv University, Tel-Aviv and Department of Biochemistry, Israel Institute for Technology, Technion, Haifa (Israel)

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SUMMARY

Lyophilized *Escherichia coli* membranes lose 50% of their DPNH oxidase activity when exposed to O_2. The oxidation of succinate or lactate is not affected by this treatment. The enzyme damaged by O_2 is the membrane DPNH dehydrogenase (DPNH-ferricyanide oxidoreductase, EC 1.6.99.3). The K_m values for DPNH and the electron acceptor $K_3Fe(CN)_6$ are the same in the native and the inactivated enzyme. The v_{max} of the latter is decreased by 50% for both substrates. The accessibility of non-heme iron to *o*-phenanthroline is partly increased. It is suggested that the non-heme iron of DPNH dehydrogenase participates in intramolecular electron conductance between the oxidizing and the reducing sites of the enzyme.

INTRODUCTION

LION AND BERGMANN[1,2] found that lyophilized *Escherichia coli* lose 99.9% of their viability after 4 h of exposure to O_2. This effect does not occur when aqueous suspensions of the bacteria are submitted to the same treatment, nor when the lyophilization is performed in the presence of iodide, nitrite or thiourea[3]. It was further suggested[4] that the inactivation of DPNH oxidase system of the bacteria may be the cause of the loss of viability.

In this study it is shown that, in fact, the DPNH oxidase system of the dried bacteria is partially inactivated by the O_2 exposure, but the extent of the effect does not explain the loss of viability. The effect was also studied on isolated dried membranes, and it is shown that the only enzyme affected is DPNH dehydrogenase (DPNH–ferricyanide oxidoreductase, EC 1.6.99.3). The isolated enzyme[5] undergoes similar inactivation by O_2, without showing significant changes in the amounts of its active components.

MATERIALS AND METHODS

Growth of the bacteria, preparation of membranes, isolation of DPNH dehydrogenase and lyophilization were performed as described elsewhere[5].

* Present address: Molecular Biology Division, Veterans Administration Hospital, San Francisco, Calif., U.S.A.

Exposure to O_2

Air was used as a source of O_2, as described by LION AND BERGMANN[1]. The material to be exposed was lyophilized. After lyophilization was completed, the ampules were sealed under vacuum. To ensure that no moisture could penetrate the ampules, the air was bled into them through a cooling coil immersed in an acetone–solid CO_2 bath, and then sealed. In other experiments, the open ampules were kept in a desiccator containing $Mg(ClO_4)_2$ and were exposed to air at 32°. For control, vacuum-sealed ampules were kept under the same conditions. At desired times the contents of the ampules were suspended in water, and their enzymic activities were determined.

Labile sulfide, non-heme iron and protein content of the samples were determined as described elsewhere[5]. Flavin content was estimated spectrophotometrically at 470 mμ. Succinate oxidase and lactate oxidase activities were measured with a Clark oxygen electrode; malate oxidase was measured manometrically. DPNH oxidation was measured spectrophotometrically at 340 mμ. Succinate dehydrogenase and lactate dehydrogenase were measured by recording the disappearance of O_2 after inhibition of respiration by 3 mM KCN, in the presence of excess of phenazine methosulfate[6]. The reduction of cytochrome b_1 and of flavin was followed spectrophotometrically at 428 and 470 mμ, respectively.

The determination of the activities of DPNH oxidase system and DPNH dehydrogenase was already described[5].

RESULTS

The effect of O_2 on the enzymic activities of dried bacteria and bacterial membranes

In order to show that the structural integrity of the bacteria was not a necessary condition for the occurrence of the O_2 effect, the inactivation of DPNH oxidase system by exposure to O_2 was measured using whole dried bacteria and bacterial membranes. The results are as follows: in both cases the inactivation takes place during the first 2–4 h, and levels off at about 50% of the initial activity. This value is not exceeded even after 48 h of exposure.

With the purpose of establishing the site of the O_2 effect, various oxidase and dehydrogenase activities located in the bacterial membrane were measured prior to and after exposure to O_2. No change was detected in respiration rate with succinate, D- or L-lactate. On the other hand oxidation of malate decreases similarly to that of DPNH oxidase activity. The oxidation of malate in *E. coli* is DPN^+ dependent, therefore, the decreased malate oxidase activity is probably due to the inactivation of DPNH oxidase. The similar extent of inactivation of both activities favours this conclusion.

The fact that only DPNH-coupled respiration is damaged indicated that the DPNH-specific component of the respiratory chain is affected. This is further supported by a comparison of the rates of reduction of flavin and cytochrome *b*, in frozen and O_2-exposed membranes. The amount of DPNH-reducible flavin and the rate of its reduction were diminished by exposure to O_2. The amount of cytochrome *b* reduced by DPNH was not changed, but due to lower activity of the flavoprotein, the rate of cytochrome *b* reduction was slower in O_2-exposed membranes. Thus, it can be concluded that O_2 acts on the flavoprotein–cytochrome *b* region of the bacterial membrane respiratory chain.

The effect of O_2 on isolated DPNH dehydrogenase

The DPNH-coupled flavoprotein is the O_2-sensitive component of bacterial DPNH oxidase system. This can be further established by measuring the inactivation by O_2 of membrane-bound DPNH dehydrogenase activity. To measure this, we followed the O_2 effect on the DPNH–ferricyanide reductase activity of the bacterial membranes, and the isolated DPNH dehydrogenase[5]. The results shown in Fig. 1 indicate that both the membrane-bound and the solubilized enzyme are inactivated at the same rate, which is also the rate of inactivation of DPNH-dependent respiration of the whole membranes.

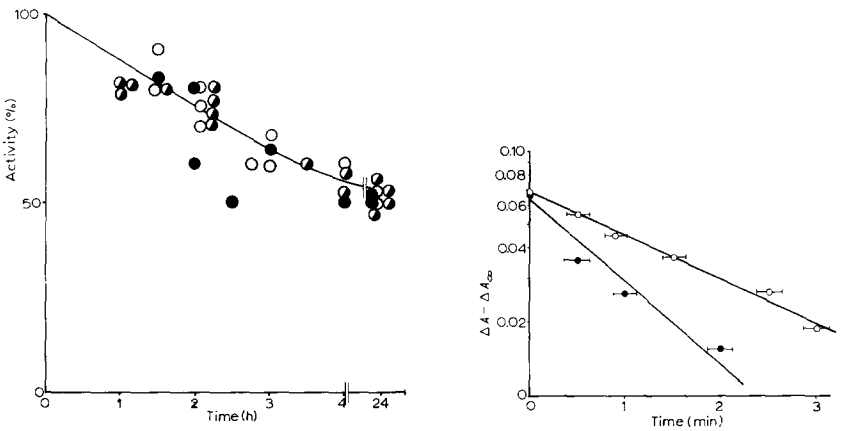

Fig. 1. The inactivation of enzymic activities during exposure to O_2. ○, DPNH oxidase; ●, membrane-bound DPNH dehydrogenase; ◑, solubilized DPNH dehydrogenase.

Fig. 2. The kinetics of the reaction of non-heme iron with *o*-phenanthroline. The enzyme, vacuum stored or exposed to O_2 (0.1 mg/ml), was reduced with DPNH 0.5 mM in the presence of 250 μM *o*-phenanthroline. At desired times the spectra from 600 to 450 mμ were recorded (in 13 sec) and changes of $A_{540\ m\mu}$ were measured. ○, vacuum-stored enzyme; ●, O_2-exposed enzyme.

The effect of O_2 on the substrate affinities of DPNH dehydrogenase

The electron transfer catalysed by DPNH dehydrogenase requires the binding to the enzyme of two different molecules, DPNH and an electron acceptor. The effect of O_2 could be due to the change in the affinity of the enzyme for one or both compounds. This possibility was tested by measuring K_m values for DPNH and for

TABLE I

THE EFFECT OF O_2 EXPOSURE ON THE K_m AND v_{max} OF *E. coli* DPNH DEHYDROGENASE

The measurements were carried as described elsewhere[5].

	$K_3Fe(CN)_6$		DPNH	
	K_m (μM)	v_{max} ($\mu equiv \cdot min^{-1} \cdot mg^{-1}$)	K_m (μM)	v_{max} ($\mu equiv \cdot min^{-1} \cdot mg^{-1}$)
Control	175	3.3	7	4.1
O_2 exposed	175	1.7	7	2.4

$K_3Fe(CN)_6$. As is shown in Table I, the K_m values of the partially inactivated enzyme were identical to those of the untreated DPNH dehydrogenase; however, the v_{max} values of the inactivated enzyme were lower in both cases by 40–50%.

DPNH dehydrogenase from *E. coli* contains flavin, labile sulfide and non-heme iron. Some of the non-heme iron atoms of the reduced enzyme can form a red, enzyme-bound ferrous *o*-phenanthroline complex[5]. The kinetics of this reaction are first order. It was found that after exposure to O_2, the rate of the reaction with *o*-phenanthroline was doubled (Fig. 2). No changes were detected in the fluorescense of the flavin nor in the amount of labile sulfide.

It was reported that reduction of the enzyme, either by DPNH or by ascorbate in the presence of *o*-phenanthroline, causes a decrease in the enzymic activity[5]. The same property, to the same extent, is detected in O_2-exposed enzyme.

DISCUSSION

O_2 at high pressure exerts well-known effects on the activities of a number of mitochondrial enzymes located at the oxidizing end of the respiratory chain[7]. In this study, a different type of O_2 effect is described. It operates on bacterial membranes, at atmospheric O_2 pressure and occurs only when the membranes are in the dry state. The extent of the effect, though not sufficient to explain the loss of viability of the bacteria[1], can be used to characterize the bacterial DPNH dehydrogenase.

That this is the enzyme affected by the exposure to O_2, can be concluded from the fact that the respiration which depends on the substrates that do not require DPN^+ for their oxidation is not affected by the O_2 treatment. The inactivation of respiration is shown when the donors used are DPNH or malate. It is well known that malate requires DPN^+ in order to be oxidized. Thus, the similar rates of inactivation of DPNH oxidase and malate oxidase activities indicate that the damage occurs at the level of electron transfer from DPNH to the respiratory chain. The enzyme involved at this level is DPNH dehydrogenase, a non-heme iron flavoprotein that was previously isolated[5]. The experiment of Fig. 1 clearly shows that O_2 acts directly upon DPNH dehydrogenase, without requirement of attachment to the membrane. The inactivation by O_2 is irreversible, and therefore different from the effect of hyperbaric O_2 on mitochondrial respiration[8], although in both cases O_2 acts at the same enzyme level. The most important question raised by these observations is that of the mechanism of the O_2 effect.

The mitochondrial enzymes DPNH–cytochrome *c* reductase and yeast lactate dehydrogenase, also flavoproteins, are both inactivated by O_2, but in these cases the effect occurs in solution[9,10]. It has been shown that the inactivation of these enzymes is accompanied by a release of their flavin prosthetic groups into the solution[9,10]. In the case of bacterial DPNH dehydrogenase, the amount of protein-bound flavin is not affected by the inactivation, and the same applies to the other active site components, labile sulfide and non-heme iron. Furthermore, the K_m values measured for DPNH and $K_3Fe(CN)_6$ are not affected by the exposure to O_2. The inactivation observed is a direct consequence of the decrease in the v_{max} values, which are the same for both substrates (Table I).

The peculiar behaviour of DPNH dehydrogenase can be explained if it is assumed that the donor and the acceptor molecules bind the enzyme at different sites,

which in themselves are not affected by O_2 exposure, with the result that both K_m's remain unchanged. As a consequence, the rate-limiting step of the overall electron transfer process that is affected by the O_2 exposure is that of the electron transfer from the oxidizing to the reducing site of the enzyme. An enzyme mechanism for a non-heme iron-flavoprotein based on two different sites bridged by non-heme iron has already been proposed[11]. In this mechanism the role of non-heme iron is to conduct the electrons between the sites. A change in the properties of non-heme iron upon O_2 exposure might be excepted if the above mechanism is assumed. In fact, in the O_2-exposed enzyme the accessibility of reduced non-heme iron to *o*-phenanthroline increased, the rate of the reaction being doubled (Fig. 2). The reasons for this change are not clear, but the increased accessibility of the non-heme iron to a bulky ligand such as *o*-phenanthroline, together with the very small change in the total amount of accessible non-heme iron, suggest a conformational change affecting a small region of the molecule.

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