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TESTOSTERONE-DEPENDENT OXYGEN CONSUMPTION IN MEMBRANE VESICLES OF *PSEUDOMONAS TESTOSTERONI*

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SUMMARY

Oxygen consumption was measured in membrane vesicles of *Pseudomonas testosteroni* using conditions similar to those identified for testosterone transport in these vesicles. Testosterone and NAD^+, which are primary requirements for testosterone transport, were both required for maximum oxygen consumption suggesting that testosterone transport and oxygen consumption were linked. Testosterone-dependent oxygen consumption was inhibited by 95% by 1 mM KCN indicating that the electron-transport chain could be involved in this process. Respiration appears to play an important role in the transport of steroids by membrane vesicles of *P. testosteroni*.

INTRODUCTION

Pseudomonas testosteroni is a gram-negative bacterium capable of growing on certain C_{19} and C_{21} steroids as its sole carbon source [1]. A specific and efficient steroid transport system is induced during adaptive growth on these steroids and retained in membrane vesicles prepared from these bacteria [2]. Membrane vesicles from uninduced cultures on the other hand revealed no significant transport activity for steroids [2].

Testosterone transport is an active transport process [2]. Transport is linked to 3β and 17β-hydroxysteroid dehydrogenase activity [3–6] which catalyzes the reversible reaction: Testosterone + $NAD^+ \rightleftharpoons$ Androstenedione + NADH + H^+ [7]. Testosterone transport is also dependent on energy generated by the transfer of electrons as judged by inhibition of transport by cyanide, amytal, and antimycin A [9], the requirement of the electron acceptor NAD^+ [2], and the inhibition of transport in the absence of oxygen [8]. The steroid itself may function as the electron donor *via* generation of NADH + H^+ [3].

Oxygen consumption itself has not been measured in membrane vesicles of *P. testosteroni*. The measurement of respiration under conditions suitable for testosterone transport would be direct evidence for the involvement of the electron transport chain in steroid transport.

EXPERIMENTAL PROCEDURES

Materials

P. testosteroni 11996 was obtained from American Type Culture Collection, Rockville, Maryland; yeast extract from Difco Laboratories, Detroit, Michigan; testosterone, B-nicotinamide adenine dinucleotide (NAD^+), lysozyme, ribonuclease -A, deoxyribonuclease 1, all from Sigma, St. Louis, MO; ethylenediaminotetraacetic acid disodium salt (EDTA), from Matheson, Coleman and Bell, Norwood, OH.

Methods

Bacterial cells were grown and membrane vesicles prepared as described [6] except that the final centrifugation is carried out at 45,000 *g* for 30 min.

Oxygen consumption assay. A typical reaction mixture for oxygen consumption studies contained in 3.0 ml: 0.30 M Tris–HCl buffer, pH 9.0, 39 μM testosterone, 1.24 mg of membrane protein, and 37 mM NAD^+. The concentration of reagents is approximately 18 times greater than that necessary for optimum transport. The reaction mixture was placed in a glass sample chamber and equilibrated in a model 5301 standard bath assembly of a Y.S.I. model 53 Biological Oxygen Monitor. Membrane protein was added to start the reaction and a Y.S.I. 5331 oxygen probe was inserted within 15 s. The decrease in oxygen in the medium was recorded on a Cole Parmer Recorder for 9–20 min. The initial slope over 6 min was used to calculate the percent oxygen consumed per minute. A value of 90% is represented by air saturated reaction mixture minus membrane protein.

Preparation of steroid solutions. For oxygen consumption studies the stock solution of testosterone (1 mg/ml in absolute ethanol) was diluted in distilled water to a concentration of 10 and 12 μg/ml. The small amount of ethanol (<0.1%) transferred to the reaction mixture did not influence the results of the assays.

Protein determination. All protein determinations were carried out using the method of Lowry *et al.*[7].

* Trivial and systematic nomenclature of steroids: Testosterone—17β-hydroxy-4-androsten-3-one; Androstenedione—4-androstene-3,17-dione; 5α-Dihydrotestosterone—17β-hydroxy-5α-androstan-3-one; Dehydroepiandrosterone—3β-hydroxy-5-androsten-17-one.

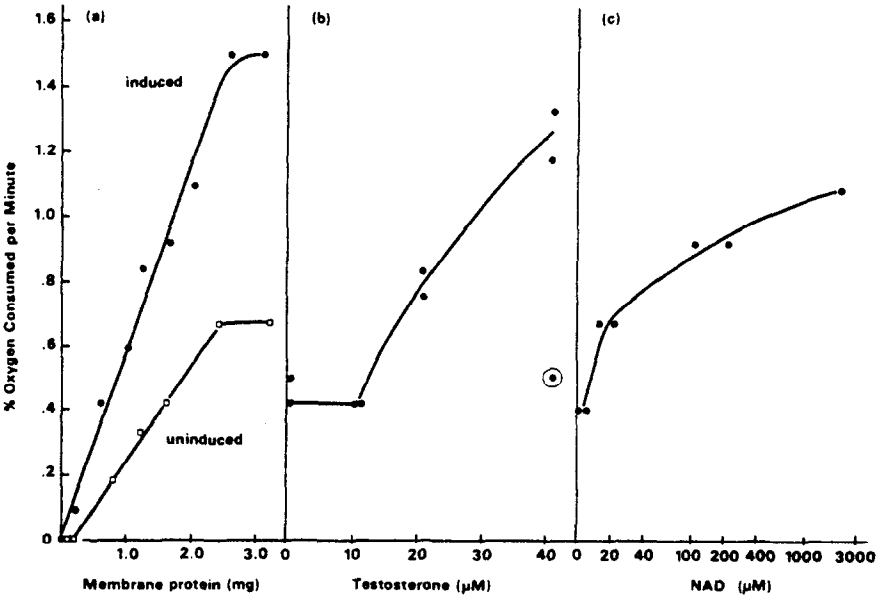

Fig. 1. Oxygen consumption during testosterone transport with increasing (a) membrane protein (b) testosterone concentrations (c) NAD^+ concentrations. The assays were performed as described in Methods. The reaction mixtures contained in 3.0 ml: 0.29 M or 0.30 M Tris–HCl buffer, pH 9.0 and (a) induced vesicles—41 μM testosterone, 35 mM NAD^+ and 0–3.2 mg of membrane protein, uninduced vesicles—39 μM testosterone 37 mM NAD^+ and 0–3.1 mg of membrane protein (b) 0–41 μM testosterone, 37 mM NAD^+ and 1.24 mg of membrane protein (c) 49 μM testosterone, 0–3 mM NAD^+ and 1.56 mg of membrane protein. Addition of membrane protein was used to start the reaction. A control tube without (a and b) membrane protein (c) membrane protein and NAD^+ served as the blank. ●——● represents oxygen consumption using induced vesicles and □——□ represents oxygen consumption using uninduced vesicles. In (b), the two points plotted for each testosterone concentration represent duplicate experiments. In one experiment KCN was added to a final concentration of 1 mM ⊙.

RESULTS AND DISCUSSION

Oxygen consumption was measured in membrane vesicles of *P. testosteroni* using conditions identified for steroid transport [2]. The percentage of oxygen consumed per min in the presence of testosterone increased linearly up to approximately 2.5 mg of membrane protein for both induced and uninduced vesicles (Fig. 1a). Larger amounts of vesicles resulted in no further increase in oxygen uptake. Membrane vesicles prepared from induced bacteria had a greater rate of oxygen consumption and more than double the maximum oxygen consumption assayed compared to vesicles prepared from uninduced bacteria.

Addition of testosterone caused an increase in oxygen consumption above a basal level (Fig. 1b). The amount of oxygen consumed per minute increased from 0.46% at 10 μM testosterone to 1.25% at 41 μM, almost a 3-fold increase. Higher concentration of testosterone could not be tested due to solubility limitations of the steroid in aqueous solution.

Consumption of oxygen in the presence of membrane vesicles and testosterone was stimulated by NAD^+ (Fig. 1c). Oxygen consumption was increased from 0.42 to 1.08% per min at the highest concentrations of NAD^+ tested. This was a 2.6-fold stimulation at 2.5 mM NAD^+.

Maximum oxygen consumption therefore required the presence of induced vesicles, testosterone, and NAD^+ as does steroid transport in membrane vesicles of *P. testosteroni* [2]. No further requirements have been determined for either system. Maximum oxygen consumption is therefore linked to testosterone transport and is referred to here as testosterone-dependent oxygen consumption.

In the absence of testosterone or NAD^+, induced vesicles showed an oxygen consumption of approximately 0.42–0.50% per min. Since this basal level of oxygen did not require NAD^+ or testosterone, it is not linked to testosterone transport.

In the transport of testosterone across the vesicle membrane, testosterone is converted to androstenedione by the 3β and 17β-hydroxysteroid dehydrogenase [3, 8]. The NADH + H^+ that is produced in the reaction can then transfer electrons to the electron-transport chain *via* an NADH dehydrogenase as reported in *Escherichia coli* [10]. NADH dehydrogenase activity has been identified in *P. testosteroni* membrane vesicles [11]. Testosterone can therefore serve as the physiological donor to the electron-transport chain [3]. The terminal cytochrome oxidase of the electron-transport chain transfers electrons to oxygen which in turn is reduced to water [12]. An increase in testosterone transport would therefore increase oxygen consumption.

The inhibition of transport in the absence of oxygen [9] indicated the necessity of oxygen as an electron acceptor in testosterone transport. Potassium cyanide a known inhibitor of the terminal cytochrome

oxidase inhibits testosterone-dependent oxygen consumption (Fig. 1) and testosterone transport [9]. Both transport and testosterone-dependent oxygen consumption therefore require the activity of the electron-transport chain.

The studies reported here indicate that oxygen is consumed during testosterone transport and provides further evidence for the involvement of the electron-transport chain in steroid transport.

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