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REGULATION OF 3-KETOSTEROID-1-EN-DEHYDROGENASE ACTIVITY OF *ARTHROBACTER GLOBIFORMIS* CELLS BY A RESPIRATORY CHAIN

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Summary—It has been shown that 3-ketosteroid-1-en-dehydrogenase localized in a cytoplasmic membrane donates reducing equivalents to a respiratory chain directly which passes them over to oxygen. Microbial hydrocortisone oxidation is coupled with energy generation in the form of the H^+ transmembrane potential. Electron transfer via a respiratory chain is the limiting stage in the process of hydrocortisone 1-en-dehydrogenation

INTRODUCTION

Microbial steroid 1-en-dehydrogenation is known to involve an inducible enzyme-3-ketosteroid-1-en-dehydrogenase (EC 1.3.99.4). In many bacteria this enzyme is associated with cell membrane structures [1. 2]. However, it is thought that 3-ketosteroid-1-en-dehydrogenase may be also a soluble enzyme [3, 4]. Sih and Bennet[1] and Levy and Talalay[2] supposed that 3-ketosteroid-1-en-dehydrogenase contains flavin, as a prosthetic group, which is directly involved in steroid dehydrogenation.

Dehydrogenation of steroid compounds is possible only in the presence of an electron acceptor. The natural acceptor has not yet been identified, but it is supposed to be vitamin K_2 [5]. As was shown Abul-Hajj, vitamin K_2 [35] extracted from *Nocardia restrictus* by iso-octane stimulated the 1-en-dehydrogenase activity of cell-free bacterial preparations. Artificial electron acceptors, which are currently used in the studies of 3-ketosteroid-1-en-dehydrogenase, e.g. menadione or phentazinemethasulfate (PMS) according to Sih and Bennett[1], accept the reducing equivalents directly from flavin of steroid dehydrogenase. However, the question of the terminal physiological acceptor of electrons from 3-keto-steroid-1-en-dehydrogenase and the enzymic systems involved in this process still need further investigation.

The present paper deals with the localization of 3-keto steroid-1-en-dehydrogenase in the cell, its connection with a respiratory chain and the factors which regulate the dehydrogenase activity.

EXPERIMENTAL

The bacterial culture *Arthrobacter globiformis* was used. The culture was grown on glucose–corn medium [6]. The cells were washed twice with the medium containing (mM): Tris–phosphate buffer, pH 7.0, 50; $MgCl_2$-5; and EDTA, 0.5; and centrifuged for 10 min at 10,000 *g*. The sediment was suspended in the same buffer and used in the experiment.

Cytoplasmic membranes were obtained by differential centrifugation of cell homogenate. Cells were ultrasonically disrupted using an ultrasonic disintegrator MSE-150 (Great Britain) as follows. Cell suspension (40 mg/ml) was sonicated 5 × for 60 s at an interval of 90 s at the maximal level of cavitation noise at 0–4°C. Homogenate was centrifuged twice at 10,000 *g* for 10 min. The sediment containing native cells was discarded, and the supernatant was centrifuged at 24,000 *g* for 120 min. Sedimented cytoplasmic membranes were suspended in buffer and used in the experiment.

Oxygen uptake by cells and cytoplasmic membrane preparation was measured with an LP-7 polarograph (CSSR) with a closed Clark-type platinum electrode covered with a Teflon film. Respiration medium was of the same composition as the medium used for cell washing. Sample volume was 2 ml, temp. 22–25°C.

An electric component of H^+ transmembrane potential was assessed by the distribution of a hydrophobic penetrating cation tetraphenyl phosphonium bromide (TPP^+) between incubation medium and cells. The cation concentration was determined by a selective electrode manufactured at the Vilnyus State University (Faculty of Natural Sciences, Department of Biochemistry and Biophysics). The medium contained 25 mM Tris–phosphate buffer (pH 7.4), 1 μm TPP^+ and 10 mM $MgCl_2$; sample vol. 5 ml, at 25°C.

The effect of cyanide and PMS on the rate of hydrocortisone transformation to prednisolone was examined in the following conditions: 2 mg cells (dry wt) and 12.5 mg prednisolone were placed into 150 ml flasks containing 25 ml of 10 mM phosphate buffer (pH 7.2) and incubated with shaking at 28°C

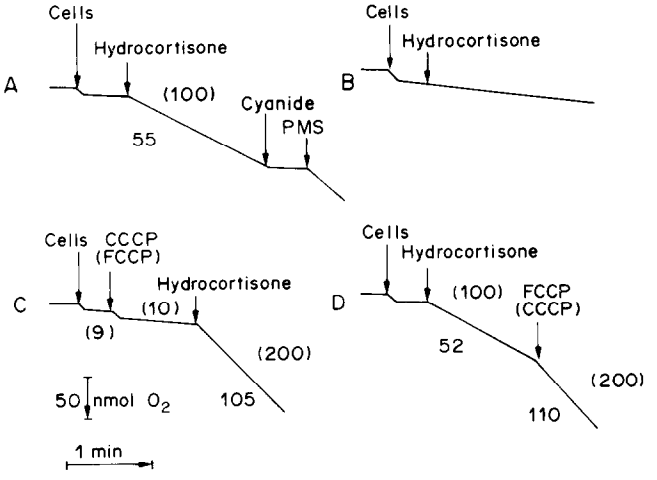

Fig. 1. The effect of cyanide and hydrocortisone on oxygen consumption by *A. globiformis* cells. Concentration of cells is 0.65 mg/ml (dry wt); hydrocortisone, 250 μg/ml; cyanide, 1 mM; phenazine methasulfate (PMS) 0.1 mM; FCCP and CCCP 5 μM. Curves A, C and D correspond to the cells grown in the presence of an inducer; curve B, to the cells grown without an inducer of 3-ketosteroid-1-en-dehydrogenase. Numbers in brackets indicate the hydrocortisone 1-en-dehydrogenation rate, nmoles·min^{-1} mg^{-1} cells (dry wt). Numbers without brackets indicate the respiration rate, nmoles O_2·min^{-1} mg^{-1} cells (dry wt).

(180–200 rpm). Cyanide and PMS were added at concentrations of 1 and 0.02 mM, respectively.

The degree of hydrocortisone transformation was determined by semiquantitative TLC on Silufol plates (CSSR) [6]. Protein was assayed by the Biuret method [7].

RESULTS AND DISCUSSION

Previously [8] it was supposed that oxygen dissolved in the medium is the terminal acceptor of electrons released as the result of steroid 1-en-dehydrogenation. However, the mechanism of this process remained obscure. To elucidate this question, we studied the influence of hydrocortisone on oxygen uptake by *A. globiformis* cells.

Figure 1 presents the typical polarographic curves of oxygen consumption by *A. globiformis* cells grown in the presence of an inducer of 3-ketosteroid-1-en-dehydrogenase–cortisone acetate (curves A, C, D) and without it (curve B). As can be seen, the addition of hydrocortisone to the cells grown in the presence of the inducer accelerated oxygen consumption (curve A), whereas the respiration rate of the cells grown without the inducer did not increase (curve B).

The above results indicate that the reducing equivalents are transported from 3-ketosteroid-1-en-dehydrogenase to oxygen. It is known that 3-ketosteroid-1-en-dehydrogenase does not interact with oxygen directly [1, 2]. Then what enzymic systems are involved in electron transfer from dehydrogenase to oxygen? Most probably this transfer occurs via a respiratory chain, as shown by the experiments with potassium cyanide. Cyanide is known to be the inhibitor of the terminal oxidase of the respiratory chain of bacteria [9], which does not influence the 3-ketosteroid-1-en-dehydrogenase activity [1]. As seen in Fig. 1 (curve A), cyanide added to the cells after hydrocortisone almost completely inhibited the respiration (by 95%). The inhibition of oxygen consumption was eliminated by the addition of PMS. PMS is an autooxidable compound capable of direct interaction with 3-ketosteroid-1-en-dehydrogenase and transfer the electrons to oxygen, by-passing the respiratory chain.

Figure 2 illustrates the effect of potassium cyanide and PMS on the rate of hydrocortisone dehydrogenation by *A. globiformis* under aerobic conditions. In the absence of cyanide, hydrocortisone, was almost completely (95%) converted to prednisolone (curve B), whereas the addition of cyanide markedly inhibited dehydrogenation (curve A), and the transformation was as little as 10% as compared with the control. Figure 2 also demonstrates the elimination of

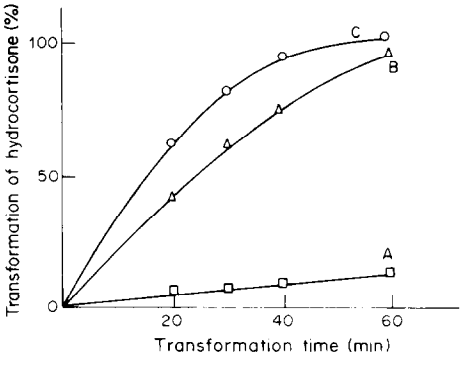

Fig. 2. The effect of cyanide and phenazinemethasulfate (PMS) on hydrocortisone transformation. A—cells + cyanide (1 mM); B—without cyanide; C—cells + cyanide (1 mM) + PMS (0.02 mM).

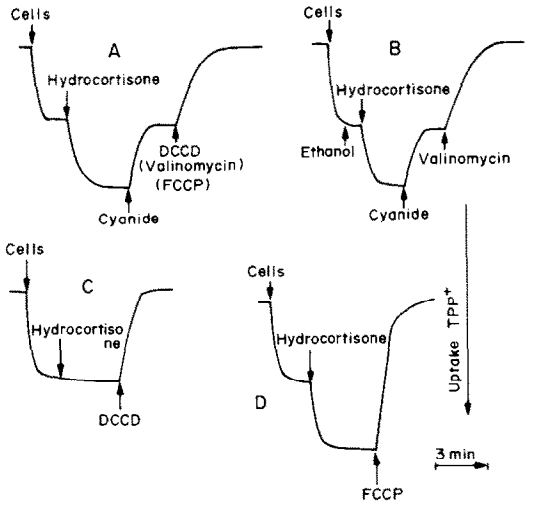

Fig. 3. The effect of hydrocortisone on energy-dependent uptake of tetraphenyl phosphonium by *A. globiformis* cells. Concentration of cells is 0.4 mg/ml; hydrocortisone, 200 μg/ml; dicyclohexyl carbodiimide (DCCD), 40 μM trichloromethoxycarbonyl cyanidephenylhydrasone (FCCP), 5 μM; valinomycin, 0.2 μM; and ethanol, 100 mM. A, B, D—cells grown in the presence of an inducer (acetate cortisone); C—without an inducer.

the inhibitory effect of cyanide on dehydrogenation by the addition of PMS to the reaction medium (curve C).

The above results indicate that during hydrocortisone hydrogenation electrons are transferred from 3-ketosteroid-1-en dehydrogenase to oxygen via the respiratory chain.

This conclusion is confirmed by the results of measuring the transmembrane potential of *A. globiformis* cells during hydrocortisone dehydrogenation (Fig. 3). Figure 3 illustrates the active uptake of TPP^+ cations by cells due to the energy of endogenous substrate oxidation (curve A). The addition of hydrocortisone stimulated the further uptake of the TPP^+ from the incubation medium (transmembrane potential generation). The addition of cyanide, which inhibits cell respiration resulted in the release of TPP^+ from cells (discharge of the potential). It is noteworthy, that in response to cyanide addition the cells released only that part of the cation which was accumulated during hydrocortisone oxidation. A complete deenergization of cells was observed only in response to the following addition of valinomycin, an ionophore of potassium ions (curve B), dicyclohexyl carbodiimide (DCCD) (curve C), an inhibitor of ATPase and protonophore of the respiratory chain activity [10, 11], or carbonyl-cyanide-*m*-chlorophenylhydrazone (CCCP) or carbonyl-cyanide trifluoromethoxyphenylhydrazone (FCCP) (curve D) an uncoupler of oxidative phosphorylation [9].

Curve B is a control for curve A because hydrocortisone was added to the medium as an ethanol solution. As can be seen, the addition of ethanol did not lead to the generation of the potential.

Curve C shows that the cells grown in the absence of the inducer of 3-ketosteroid-1-en-dehydrogenase are incapable of TPP^+ uptake in response to hydrocortisone addition.

These results show that hydrocortisone dehydrogenation by *A. globiformis* is coupled to the generation of the transmembrane potential, which is evidence for the participation of the respiration chain in this process.

However, these findings do not provide the answer to the question of how the reducing equivalents are transferred from 3-ketosteroid-1-en-dehydrogenase to the respiratory chain. Since the 3-ketosteroid-1-en-dehydrogenase activity does not depend on NAD^+ or $NADP^+$ [1, 2], we supposed that 3-ketosteroid-1-en-dehydrogenase is directly associated with the respiratory chain and transmits electrons to one of its components. To check this assumption, we isolated and studied *A. globiformis* cytoplasmic membranes capable of hydrocortisone dehydrogenation. Figure 4 illustrates the effect of hydrocortisone on oxygen consumption by the membrane preparation. As can be seen (curve A), the addition of hydrocortisone to the aerobic suspension of membranes resulted in oxygen consumption. In the presence of ethanol (hydrocortisone was added in the form of ethanol solution), no membrane respiration was observed. Oxygen consumption by the membrane fraction in the presence of hydrocortisone was

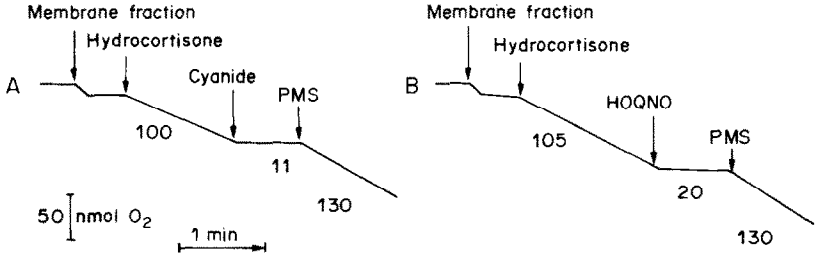

Fig. 4. Effect of hydrocortisone on the rate of oxygen uptake by *A. globiformis* cytoplasmic membranes. Additions: membranes 1 mg protein/ml; hydrocortisone—250 mg/l; cyanide 1 mM; 2-*n*-nonyl-4-hydroxy quinoline-*N*-oxide (HOQNO) μM; PMS 50 μM. Numbers indicate the respiration rate, nmoles O_2 min^{-1} mg^{-1} protein.

markedly inhibited by cyanide (curve A) or 2*N*-nonyl-4-hydroxy-quinoline-*N*-oxide [HOQNO] (curve B), whereas PMS eliminated the inhibitory effect.

The results presented in Fig. 4 indicate that 3-ketosteroid-1-en-dehydrogenase is localized in a cytoplasmic membrane and transfers the reducing equivalents directly to the respiratory chain, obviously, to menaquinone, before the site of inhibition by HOQNO. These results are in line with the data reported by Abul-Hajj[5].

The study of kinetic regulation of hydrocortisone-1-en-dehydrogenation by *A. globiformis* [12] has revealed that the transport of reagents across a cell envelope does not limit the transformation process.

The fact that the respiratory chain is involved in hydrocortisone oxidation suggests that the rate of the steroid transformation is determined by the factors which regulate the respiratory chain activity. As is known, the rate of electron transfer via the respiratory chain is determined by the membrane potential. Since hydrocortisone dehydrogenation by *A. globiformis* cells is coupled with the generation of energy in the form of transmembrane potential (Fig. 3), one should expect that the discharge of the potential would be followed by the acceleration of hydrocortisone transformation to prednisolone. In fact, as can be seen in Fig. 1 (curves C and D), in the presence of the oxidation phosphorylation uncoupler FCCP, CCCP or valinomycin an ionophore of K^+ (not shown in the figure), the rates of both hydrocortisone transformation to prednisolone and oxygen consumption increased. Curve A corresponds to the cell activity in the absence of the uncoupler. The results of measuring the rate of hydrocortisone transformation (Fig. 1) indicate that the oxygen consumption and hydrocortisone transformation occur stoichiometrically (2 moles prednisolone are produced per 1 mole consumed oxygen). Such a relationship is observed only with cells whose endogenous respiration is almost zero. If otherwise, the stoichiometry is disturbed. Probably it is caused by the competition between the dehydrogenases of endogenous substrates and 3-ketosteroid-1-en-dehydrogenase for the transfer of electrons to the respiratory chain.

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