J. steroid Biochem. Vol. 23, No. 3, pp. 307–312, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

REDOX REACTIONS IN HYDROCORTISONE TRANSFORMATION BY *ARTHROBACTER GLOBIFORMIS* CELLS

A. Y. ARINBASAROVA, A. G. MEDENTSEV, V. K. AKIMENKO, K. A. KOSHCHEYENKO and G. K. SKRYABIN

Institute of Biochemistry and Physiology of Microorganisms, U.S.S.R. Academy of Sciences, Pushchino, Moscow region, 142292, U.S.S.R.

(*Received* 5 *November* 1984)

Summary—Hydrocortisone and prednisolone transformation by *Arthrobacter globiformis* cells in aerobic and anaerobic conditions was studied. 3-Ketosteroid-1-en-dehydrogenase activity was shown to be the major factor regulating the direction of transformation. When it is high (aerobic conditions), the end products of hydrocortisone transformation are prednisolone or its 20β-hydroxy derivative. The latter is produced via 1-en-dehydrogenation, which is not a limiting stage of the process. Low 3-ketosteroid-1-en-dehydrogenase activity (in the presence of cyanide) or its complete inhibition (strictly anaerobic conditions) result in the direct reduction of 20-keto group of hydrocortisone.

INTRODUCTION

Many bacteria, including *A. globiformis*, are known to be able to transform steroid compounds with the help of inducible enzymes, e.g. 3-ketosteroid-1-en-dehydrogenase, which dehydrogenates the A ring of the steroid in the 1,2-position; 3-ketosteroid-1,2-reductase, which catalyzes the reduction of the 1,2-double bond of the A ring; and 20β-hydroxysteroid dehydrogenase, which is responsible for the reduction of 20-keto group, etc. [1].

The analysis of the data available on microbial transformation indicates that the intensity and direction of transformation may be regulated differently:

(i) by selective induction of certain enzymes with various steroid compounds [2–5];

(ii) by changing the transformation conditions, e.g. using the inhibitors of respiration and oxidative phosphorylation [5, 6], and artificial electron acceptors and donors [5–7], or adding certain metabolites (TCA cycle substrates, fatty and amino acids, or nitrous bases) [5, 6, 9];

(iii) by selective inhibition or activation of certain reactions [5–8].

Previously it has been shown that hydrocortisone is transformed to prednisolone and 20β-hydroxy derivatives of hydrocortisone and prednisolone [10–11]. It has been supposed that hydrocortisone 20β-hydroxy derivative is produced as the result of the successive action of 3-ketosteroid-1-en-dehydrogenase, 20β-hydroxysteroid dehydrogenase and 3-ketosteroid-1,2-reductase. No direct 20β-reduction of hydrocortisone has been shown.

The aim of the present work was to study the relationships between redox reactions which occur during hydrocortisone transformation to prednisolone and its 20β-hydroxy derivatives.

EXPERIMENTAL

Materials

The bacterium *Arthrobacter globiformis* 193 was obtained from the culture collection of the Institute of Biochemistry and Physiology of Microorganisms, U.S.S.R Academy of Sciences; hydrocortisone (11β, 17α, 21-trihydroxy-4 pregnene-3,20-dione) and prednisolone (11β, 17α, 21-trihydroxy-1,4-pregnadiene-3,20-dione) were from "Serva"; cortisone acetate (17α-hydroxy-21-acetoxy-4-pregnen-3,11,20-trione), 20β-hydroxy derivatives of hydrocortisone and prednisolone (11β, 17α, 20,21-tetrahydroxy-4-pregnene-3-one and 11β, 17α, 20,21-tetrahydroxy-1,4 pregnadiene-3-one) were from plant "Akrihin" (U.S.S.R.).

Methods

Growth conditions and the cell biomass assay were as previously described [11]. Cortisone acetate was used as an inducer of steroid-transforming enzymes [12]. The inducer was added in the form of an ethanol solution simultaneously with the inoculum (the final steroid concentration was 200 μg/ml).

Oxygen consumption assay. Oxygen consumption by cells was determined with a polarograph LP-7 (Czechoslovakia), using a Clark-type platinum electrode covered with a "Teflon" film, in 10 mM Tris–phosphate buffer (pH 7.0) at 22–25°C. Oxygen concentration in the medium was assumed to be 250 μM. The final sample volume was 2 ml.

Amount of pyridinenucleotide assay. The degree of pyridine-nucleotide reduction in bacterial cells was determined fluorimetrically at 460 nm.

Steroid transformation. Anaerobic transformation was carried out in an anaerobic box "Hirasawa" (TE-Her, AZ-series, Japan). Cells (50 mg dry wt)

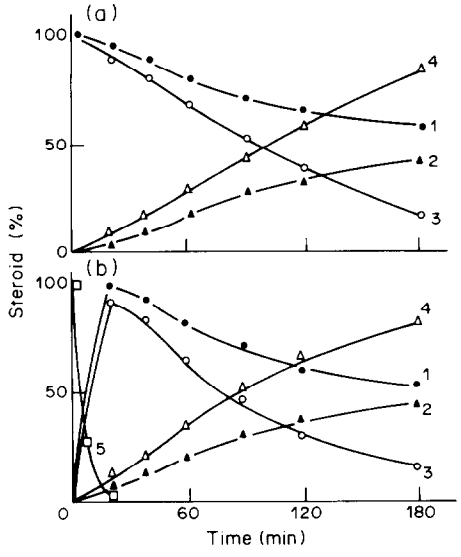


Fig. 1. The effect of glucose on prednisolone (A) and hydrocortisone (B) transformation by *A. globiformis* cells. A and B: (1) prednisolone in the absence of glucose, (2) 20β-hydroxy derivative of prednisolone in the absence of glucose, (3) prednisolone in the presence of glucose, (4) 20β-hydroxy derivative in the presence of glucose, (5) hydrocortisone in the absence and in the presence of glucose.

[1.25 mg/ml] were added to 300 ml flasks containing 40 ml buffer solution. To remove oxygen, the cell suspension and the initial substrate solutions were anaerobically preincubated for 10–15 min.

Aerobic steroid transformation was carried out as follows: 25 mg (dry weight) cells (1 mg/ml) and hydrocortisone of prednisolone at a final concentration of 0.2 mg/ml (0.55 mM) were placed in 250 ml flasks containing 25 ml of Tris–phosphate buffer and aerobically incubated on a shaker (180–200 rpm).

The steroid substrates were introduced into the medium as ethanol solutions (the final ethanol concentration did not exceed 1%).

The rate of hydrocortisone and prednisolone transformation was determined by TLC on Silufol plates [12].

Glucose and cyanide, if necessary, were added at final concentrations of 1% (wt) and 1 mM, respectively.

RESULTS AND DISCUSSION

Figure 1 (A and B) illustrates the aerobic transformation of prednisolone and hydrocortisone by *A. globiformis* cells in the presence of glucose (curves 3) and without it (curves 1). It can be seen (Fig. 1A) that on incubation of bacterial cells with prednisolone as an initial transformation substrate, the consumption of prednisolone (curve 1) was accompanied by the accumulation of its 20β-hydroxy derivative (curve 2). This process was intensified by the addition of glucose to the incubation medium. After 3 h transformation in the presence of glucose, the content of 20β-hydroxy derivative of prednisolone (curve 4) was 85–90% of the initial amount of prednisolone, i.e. 2.0–2.5 times as high as compared with prednisolone production in the absence of glucose. The rate of accumulation of 20β-hydroxy derivative of prednisolone in the presence of glucose correlates with the rate of prednisolone decrease in the incubation medium (curve 3).

On incubation of bacterial cells with hydrocortisone (Fig. 1B), complete hydrocortisone consumption (curve 5) and stoichiometric production of prednisolone (curve 1) were observed as early as 20 min. Further incubation was followed by the decrease of prednisolone (curve 1) and accumulation of its 20β-hydroxy derivative (curve 2). After addition of glucose, the rate of hydrocortisone conversion to prednisolone (curve 5) did not change, but the rate of prednisolone transformation (curve 3) to its 20β-hydroxy derivative (curve 4) was increased 2.0–2.5-fold.

A stimulating effect of glucose and other substrates on the reduction of 20-keto group of various steroids has been reported elsewhere [5, 6]. The mechanism of such an effect is believed to reside in the activation of metabolic processes resulting in the increase of the amount of reduced pyridine-nucleotides-NAD(P)H, which are the coenzymes of 20β-hydroxysteroid dehydrogenase [1].

On incubation of cells with hydrocortisone or with prednisolone no 20β-hydroxy derivative of hydrocortisone was detected. Glucose added to the incubation medium did not provoke its production.

The data available [10, 13] and the results presented in Fig. 1 suggest that hydrocortisone aerobic transformation by *A. globiformis* cells proceeds by the following scheme:

$$\text{hydrocortisone} \xrightarrow{\text{1-en-dehydrogenation}} \text{prednisolone} \xrightarrow{\text{20β-reduction}} \text{20β-hydroxy derivative of prednisolone}$$

The results shown in Fig. 1 indicate that the rate of production of prednisolone 20β-hydroxy derivative in the same when both hydrocortisone and prednisolone are metabolized. This means that the intermediate reaction of hydrocortisone 1-en-dehydrogenation is not a limiting stage under aerobic conditions.

Previously [13] it has been shown the oxidation of 20β-hydroxy group of prednisolone by *Mycobacterium globiforme* (*A. globiformis*) but there was no evidence for two (or one) enzymes for 20β-reduction and oxidation of 20β-hydroxy group. The absence of hydrocortisone 20β-hydroxy deriva-

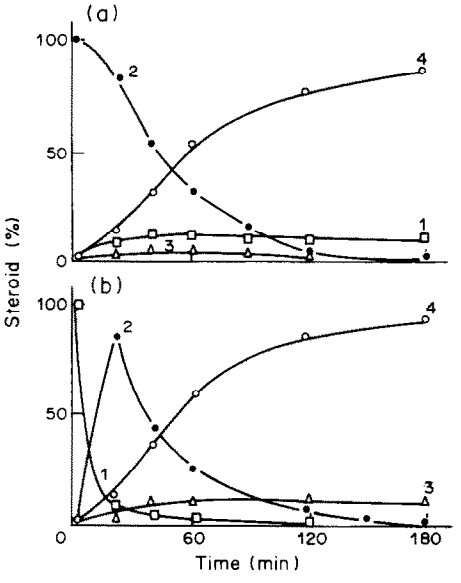


Fig. 2. The effect of cyanide on hydrocortisone (A) and prednisolone (B) transformation by bacterial cells under aerobic conditions: (1) prednisolone, (2) hydrocortisone, (3) 20β-hydroxy derivative of prednisolone, (4) 20β-hydroxy derivative of hydrocortisone.

tive in the reaction medium is obviously because hydrocortisone is a substrate for two enzymes: 3-ketosteroid-1-en-dehydrogenase and 20β-hydroxysteroid dehydrogenase. Due to a high 1-en-dehydrogenase activity, all hydrocortisone is rapidly oxidized to prednisolone, and the direct 20β-reduction of hydrocortisone becomes improbable. Also it may well be that the 20β-hydroxy derivative of hydrocortisone is produced, but is immediately oxidized to the 20β-hydroxy derivative of prednisolone under the influence 3-ketosteroid-1-en-dehydrogenase. So, the absence of hydrocortisone 20β-hydroxy derivative in the reaction medium under aerobic conditions is due to the high 3-ketosteroid-1-en-dehydrogenase activity.

If this is so, it would be expected that when there is low or no 3-ketosteroid-1-en-dehydrogenase activity, the hydrocortisone 20β-hydroxy derivative should accumulate in the incubation medium.

Previously [14] we have shown that in *A. globiformis* cells, 3-ketosteroid-1-en-dehydrogenase bound to cytoplasmic membranes released the reduction equivalents directly to the respiratory chain, which transfers them to oxygen. The dehydrogenase activity of the enzyme was inhibited by cyanide and was absent under strictly anaerobic conditions. Therefore the ability of *A. globiformis* to directly reduce the 20-keto group of hydrocortisone was studied in the presence of cyanide or under strictly anaerobic conditions.

In fact, the incubation of cells with hydrocortisone in the presence of cyanide (Fig. 2A) was associated with a drastic decrease of prednisolone accumulation (curve 1). Hydrocortisone decrease (curve 2) was accompanied by the production of its 20β-hydroxy derivative (curve 4). After 3 h incubation, the content of hydrocortisone 20β-hydroxy derivative was 85–90% of the initial amount of the steroid. So, on inhibition of 3-ketosteroid-1-en-dehydrogenase activity by cyanide, the cells acquired the ability to reduce the hydrocortisone 20-keto group.

Prednisolone transformation in the presence of cyanide [Fig. 2B] (curve 1) was associated with the accumulation of hydrocortisone (curve 2) and its further transformation to 20β-hydroxy derivative (curve 4).

The above results indicate that the major route of production of hydrocortisone 20β-hydroxy derivative from prednisolone involves its reduction to hydrocortisone catalyzed by 3-ketosteroid-1,2-reductase, and further direct reduction of 20-keto groups by 20β-hydroxysteroid dehydrogenase. Such a route of prednisolone transformation is obviously conditioned by the fact that the 3-ketosteroid-1,2-reductase activity is far higher than the 20β-hydroxysteroid dehydrogenase activity.

Since in the presence of cyanide the respiratory chain is incompletely inhibited (by 95%) [Fig. 3] (curve 1), 3-ketosteroid-1-en-dehydrogenase activity is partly retained. Therefore, some of hydrocortisone 20β-hydroxy derivative may be produced by the mechanism involving the formation of prednisolone 20β-hydroxy derivative [Fig. 2B] (curve 3), which is then converted to hydrocortisone 20β-hydroxy derivative by 3-ketosteroid-1,2-reductase.

In the presence of cyanide, the rate of

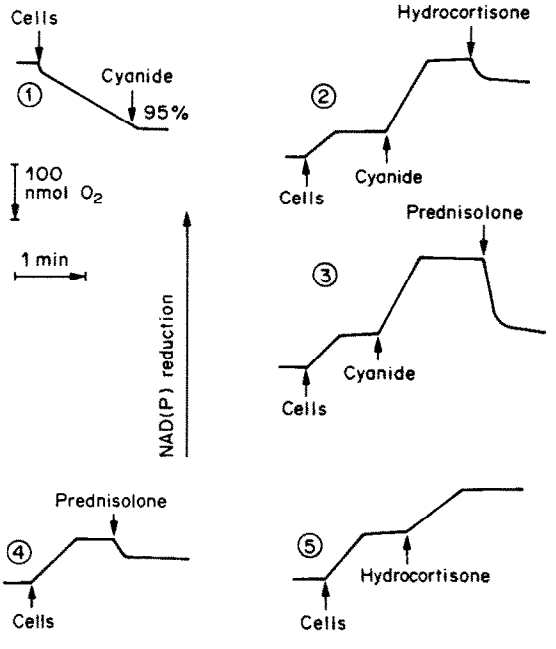


Fig. 3. The effect of cyanide, prednisolone and hydrocortisone on respiration (1) and reduction of pyridinenucleotides (PN) (2, 3, 4, 5) in *A. globiformis* cells. Additions: cells, 1.5 mg (dry wt)/ml; hydrocortisone and prednisolone, 0.2 g/l (0.55 mM); cyanide, 1 mM.

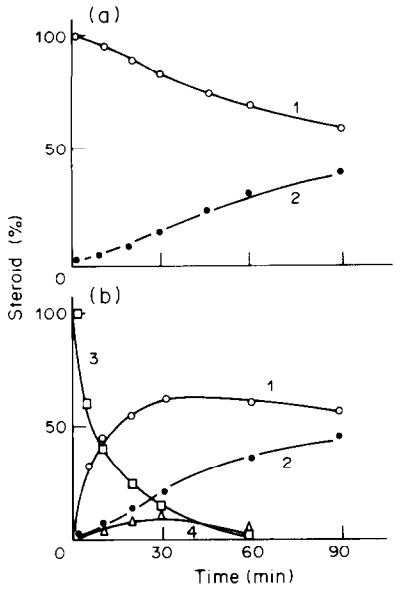


Fig. 4. Hydrocortisone (A) and prednisolone (B) transformation by *A. globiformis* cells under anaerobic conditions. (1) hydrocortisone, (2) 20β-hydroxy derivative of hydrocortisone, (3) prednisolone, (4) 20β-hydroxy derivative of prednisolone.

20β-reduction markedly increases (Fig. 2): after 3 h incubation without cyanide the content of 20β-hydroxy derivatives was 40% of the initial amount of the steroid [Fig. 1A] (curve 2), whereas in the presence of cyanide it makes up 85–90% [Figs 2A and 2B] (curves 3 and 4). The ability of cyanide to activate 20β-reduction of steroids was reported earlier [5, 6]. It was supposed that the stimulating effect of this inhibitor was associated with the increase in the content of reduced pyridinenucleotides in the cell. This assumption is supported by the results presented in Fig. 3. As is seen (curve 1), cyanide (1 mM) inhibits (by 95%) the endogenous respiration of *A. globiformis* cells and appreciably increases the amount of reduced pyridinenucleotides (curve 2). Hydrocortisone added after cyanide decreases the content of NAD(P)H [Fig. 3] (curve 2). In the presence of prednisolone, the degree of reduction of pyridinenucleotides decreases much more quickly and to a much lower level (curve 3) than in the presence of hydrocortisone. This is probably due to a higher rate of the reaction catalyzed by 3-ketosteroid-1,2-reductase, as compared with the reaction conditioned by 20β-hydroxysteroid dehydrogenase. This is confirmed by a relatively rapid accumulation of hydrocortisone on incubation of cells in the presence of cyanide [Fig. 2B] (curve 2). A lower intracellular content of reduced pyridinenucleotides in the presence of prednisolone, as compared with that in the presence of hydrocortisone, is probably because the reduction of 1 mole prednisolone to 20β-hydroxy derivative of hydrocortisone requires 2 moles of NADH (20β- and 1,2-reduction of the double bond), whereas on reduction of 1 mole hydrocortisone 1 mole of NADH is consumed (only 20β-reduction).

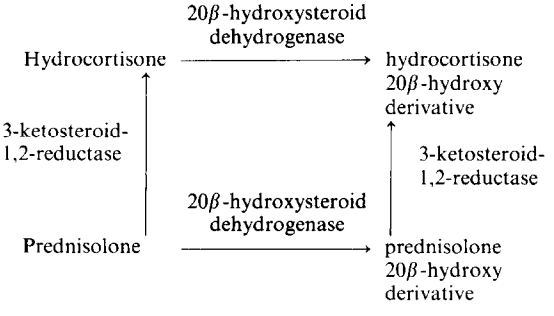
As shown in Fig. 3 (curve 4), in the absence of cyanide prednisolone also decreases the amount of reduced pyridinenucleotides in cells. Hydrocortisone added to cells in the absence of cyanide in contrast, results in an increase in the NAD(P)H content (curve 5). The latter seems to be caused by the competition between 3-ketosteroid-1-en-dehydrogenase and NADH-dehydrogenase for the transfer of reduced equivalents to the respiratory chain, which results in the decrease in the NADH oxidation rate and hence in the increase in the content of reduced pyridinenucleotides in the cell. The latter may also be due to the reverse electron transfer from menaquinone to NADH-dehydrogenase.

The direct reduction of hydrocortisone 20-keto group was also demonstrated on incubation of hydrocortisone with *A. globiformis* cells under strictly anaerobic conditions. As seen in Fig. 4, during the incubation no prednisolone was produced as an intermediate product, and the consumption of hydrocortisone [Fig. 4A] (curve 1) was accompanied by the accumulation of its 20β-hydroxy derivative [Fig. 4A] (curve 2). So, when 3-ketosteroid-1-en-dehydrogenase does not function [14], hydrocortisone 20β-hydroxy derivative is produced because of the direct reduction of its 20-keto group.

During prednisolone transformation (Fig. 4B), 60% of hydrocortisone (curve 1), 10% of prednisolone 20β-hydroxy derivative (curve 4) and 15% of hydrocortisone 20β-hydroxy derivative (curve 2), along with prednisolone (curve 3) were observed in the medium after 30 min of incubation. After 60 min incubation only hydrocortisone and its 20β-hydroxy derivative were detected.

Both under anaerobic conditions, and in the presence of cyanide, not only 20β-, but also 1,2-reduction processes were stimulated due to retardation of the reverse reaction of 1-endehydrogenation. The increase in the amount of reduced pyridinenucleotides, obviously, is not of much importance, as is evidenced by the absence of the products of 1,2-reduction (of hydrocortisone and its 20β-hydroxy derivative) under aerobic conditions even after the addition of glucose to the reaction medium (Figs 1A and 1B).

Thus, the transformation of hydrocortisone and prednisolone by *A. globiformis* cells under anaerobic conditions or in the presence of cyanide seems to occur as follows:



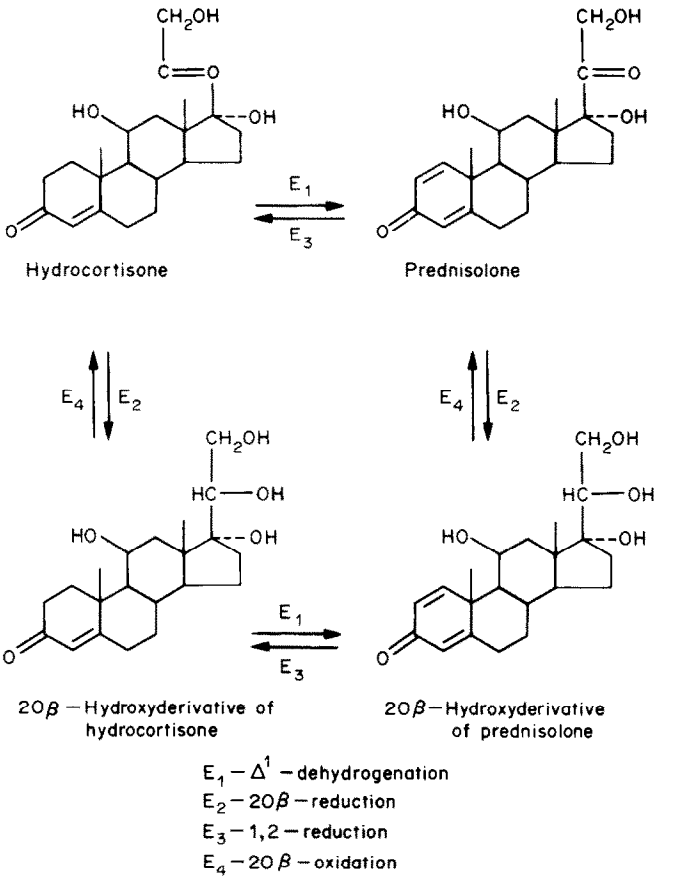


Fig. 5. Scheme of hydrocortisone transformation.

Under these conditions, hydrocortisone is directly transformed to its 20β-hydroxy derivative, whereas prednisolone transformation may proceed by two pathways. Since the 3-ketosteroid-1,2-reductase activity is markedly higher than the 20β-hydroxysteroid dehydrogenase activity, as is evidenced by the rapid accumulation of hydrocortisone (Figs 2B and 4B), the major pathways of prednisolone transformation involves its reduction to hydrocortisone and its 20β-hydroxy derivative.

The data available in the literature [10, 11, 13] and our results indicate that hydrocortisone and prednisolone transformation is carried out by a complex polyenzymic system, as shown in Fig. 5. Under aerobic conditions, prednisolone is directly transformed to its 20β-hydroxy derivative. Anaerobic transformation of prednisolone occurs via 1,2- and 20β-reduction, i.e. prednisolone→hydrocortisone→ hydrocortisone 20β-hydroxy derivative. Hydrocortisone transformation under aerobic conditions proceeds as follows: hydrocortisone→prednisolone→ prednisolone 20β-hydroxy derivative.

On inhibition of the respiratory chain activity by cyanide or under anaerobic conditions, when the 3-ketosteroid-1-en-dehydrogenase activity is wholly inhibited, the direction of transformation is changed. Under these conditions, the direct 20β-reduction of hydrocortisone takes place. Thus, the 3-ketosteroid-1-en-dehydrogenase activity is the main factor which regulates the direction of hydrocortisone transformation.

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