J. Steroid Biochem. Molec. Biol. Vol. 38, No. 2, pp. 257–263, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

CHARACTERISTICS OF 16-DEHYDROPROGESTERONE REDUCTASE IN CELL EXTRACTS OF THE INTESTINAL ANAEROBE, *EUBACTERIUM* SP. STRAIN 144

WILLIAM E. WATKINS* and THOMAS L. GLASS†
Department of Microbiology, North Dakota State University, Fargo, ND 58105, U.S.A.

(Received 24 July 1990)

Summary—16-Dehydroprogesterone reductase (16-DHPR) activity was present in cell extracts of *Eubacterium* sp. strain 144 only when the organism was grown in the presence of steroids containing a Δ^{16-17} double bond and C-20-ketone. Cells grown with 16-dehydropregnenolone contained 16-DHPR activity but lacked Δ^{4-5}-3-keto steroid reductase activity. Pyruvate or sodium dithionite served as electron donors for 16-DHPR and both reactions required methyl viologen as an electron carrier. Neither NADH nor NADPH, with or without flavin nucleotides, were used by 16-DHPR. Enzyme activity was detected in the cytoplasmic fraction (40%) and membrane fraction (20%) of crude cell extracts, but 40% of the activity was unaccounted for following ultracentrifugation. 16-DHPR activity was unaffected by pH in potassium phosphate buffer over the range 5.0 to 8.5, but was inhibited by Tris–HCl above pH 7.0. 16-DHPR activity was inhibited by sulfhydryl reagents, but inhibitors of electron transport reactions or metal chelators did not affect the enzyme.

INTRODUCTION

The intestinal flora of man and rats catalyzes the 16α-dehydroxylation-reduction of 16α-hydroxysteroids producing 17-iso-steroids [1–3]. In 1980, two strains of *Eubacterium* sp. (144 and 146) were isolated from rat feces and shown to perform this reaction [4]. Studies with strain 144 indicated that the overall steroid transformation was catalyzed by two enzymes: 16α-hydroxyprogesterone dehydroxylase (16α-dehydroxylase) which formed 16-dehydroprogesterone from 16α-hydroxyprogesterone and 16-dehydroprogesterone reductase (16-DHPR) which reduced 16-dehydroprogesterone to 17-isoprogesterone [5]. Properties of 16α-dehydroxylase have been described and the enzyme has been purified [6, 7].

Initial attempts to detect 16-DHPR activity in cell extracts of strain 144 were unsuccessful [5] necessitating studies with resting cells [8]. Although grown in the presence of 16-dehydroprogesterone, the 16-DHPR activity of these cells was poor unless hemin was also added to the culture medium and pyruvate or H_2 was provided during assay to serve as an electron donor for 16-DHPR activity. Moreover, once these requirements were met, strain 144 exhibited two other steroid-transforming activities not previously known. These were reduction of the Δ^{4-5} double bond (designated progesterone reductase; PR) and 3α-hydroxysteroid dehydrogenase. It was proposed that strain 144 required hemin to synthesize a cytochrome-containing electron transport system that supplies electrons to 16-DHPR and PR coupled to the oxidation of pyruvate and H_2 [8]. Cells grown in hemin-supplemented medium, lacking 16-dehydroprogesterone, did not show 16-DHPR or PR activities suggesting that the synthesis of both enzymes was steroid-induced.

16-DHPR activity was detected in the fungus, *Rhizopus nigricans*, but properties of the enzyme were not studied [9]. 16-DHPR is not known to occur in any bacteria other than *Eubacterium* spp. 144 and 146. Based on analysis of deuterated 17-isopregnenolone formed by mixed rat cecal bacteria from D_2O and 16-dehydropregnenolone, Bjorkhem *et al.* [10] concluded that 16-DHPR catalyzed a *trans* addition of hydrogen to the 16α and 17β positions of the steroid. Other than this and the results described above, nothing else is known concerning 16-DHPR. The present study was done to extend our earlier results by developing a cell extract assay for 16-DHPR and determining some of its features.

*Present address: Department of Nutrition, University of California, Davis, CA 95616, U.S.A.
†To whom correspondence should be addressed.

MATERIALS AND METHODS

Culture conditions

Eubacterium sp. strain 144 was grown in the medium described by Feighner *et al.* [11] as previously modified [8, 12]. Hemin was added to the medium to give a final concentration of 1 μg/ml instead of 0.5 μg/ml [8]. The medium was prepared and the organism grown using the Hungate anaerobic technique [13], as modified by Bryant [14], or by using the serum bottle modification [15] of Hungate's procedures. Medium (1.5 l) was adjusted to pH 7.2 with 10 N KOH, boiled under CO_2 gassing, and transferred to 2 l serum flasks. CO_2 was bubbled through the medium until cooled to room temperature, Na_2CO_3 was added, and gassing continued until the Na_2CO_3 was dissolved and neutralized. The flasks were sealed and autoclaved (25 min, 121°C). Na_2S, prepared as a separately sterilized anaerobic solution, was added to give a final concentration of 0.1 g/l. Prior to inoculation, a solution of 16-dehydropregnenolone (1 mg/ml methanol) was made anaerobic by gassing with argon for 10 min. The steroid was then added to the medium to give 10 μg/ml and 1% (v/v) methanol. Inocula for the serum flasks were grown in 20 ml serum bottles containing 10 ml medium. Generally, two 1.5 l batches of medium were each inoculated with 4 ml of an 18 h culture. The flasks were incubated at 37°C until the absorbance was 0.3–0.35 (660 nm) as measured with a Beckman model 24 spectrophotometer and 1 cm lightpath cuvettes.

Cell extracts

During harvesting of the cells and extract preparation, precautions were taken to minimize exposure to oxygen by flushing the cells and extracts with argon whenever they were exposed to air. Buffers were made anaerobic by boiling and cooling under argon followed by the addition of dithiothreitol (DTT) and stored in serum bottles. Cells were harvested by centrifugation (5000 *g*, 4°C, 15 min) and each cell pellet was resuspended in 2 ml of 50 mM potassium phosphate buffer containing 1 mM DTT. In initial experiments with pyruvate as the electron donor for 16-DHPR, the pH of this buffer was 5.5; the pH was increased to 7.5 when sodium dithionite replaced pyruvate in the assays. The cell pellets were combined and the cells were broken by two passes through a chilled French pressure cell at 16,000 psi. The broken cells (10–20 ml) were treated with a small amount of DNAase and $MgCl_2$ and centrifuged at 10,000 *g*, 0°C, 20 min under argon using stainless steel tubes closed with O-ring seal covers. The supernatant (crude cell extract) was either stored in serum bottles under H_2 at 4°C or centrifuged at 105,000 *g*, 4°C, 2 h to give the cytoplasmic and membrane fractions. The membrane pellet was resuspended in a minimal volume of buffer (above) with a tissue homogenizer. The cytoplasmic and membrane fractions were stored in serum bottles under H_2 at 4°C.

16-DHPR assay

Two assays were developed to measure 16-DHPR activity. When pyruvate was the electron donor, the assay (2.0 ml final vol) contained 25 mM potassium phosphate buffer, pH 5.5; 0.05 mM DTT; 0.5 mM methyl viologen (MV); 0.1 mM coenzyme A; 0.26 mM 16-dehydroprogesterone; 10% (v/v) methanol (added with steroid); 20 mM sodium pyruvate and crude cell extract or cytoplasmic fraction (2.0–2.5 mg protein/ml). When sodium dithionite (2.0 mM) was the electron donor, coenzyme A and pyruvate were omitted, the buffer pH was increased to 7.5, the MV concentration increased to 2 mM, and the amount of crude extract or soluble fraction added was reduced to 0.5–1.0 mg/ml. All assays were done in anaerobe culture tubes under continuous argon gassing. With both procedures, reaction components, except electron donor and steroid, were incubated with the enzyme for 5 min at 37°C and the reactions initiated by adding the electron donor followed by 16-dehydroprogesterone. Unless otherwise indicated, the reactions were incubated for 2 min and 0.2 ml removed, added to 0.5 ml of 0.5 N H_3PO_4 to stop the reaction, and the samples frozen at −20°C until steroid extraction. Under this condition, 1 unit (U) of activity is equal to 1 nmol 17-isoprogesterone formed per 2 min and specific activity is equal to U/mg protein.

Steroid analysis

Steroids were extracted with diethylether, separated by HPLC, detected by u.v. absorbance at 254 nm, and quantitated by external standardization as previously described [12] except for the following changes: samples were filtered with Nylon Acrodiscs 13 (0.45 μm pore size, Gelman Sciences, Ann Arbor, Mich.), a 4.6 × 150 mm Ultrasphere C18 (5 μ particle size) reverse-phase column (Beckman Instruments Inc., San

Ramon, Calif.) was used, and the solvent system was methanol/H_2O (78:22, v/v). For external standardization, 17-isoprogesterone was prepared from 16-dehydroprogesterone using resting cells with pyruvate as the electron donor. A 400-ml culture of strain 144 was grown as previously described [8] except that 16-dehydropregnenolone, instead of 16-dehydroprogesterone, was added to the medium. Preliminary experiments indicated such cells possessed 16-DHPR activity, but lacked PR activity. The cells were harvested and suspended in 50 mM potassium phosphate buffer, pH 5.5 plus 1 mM DTT under argon. The reaction contained (in a final vol of 20 ml) cell suspension (13 ml), 25 mM potassium phosphate buffer, pH 5.5, 0.05 mM DTT, 20 mM sodium pyruvate, 16-dehydroprogesterone (1 mg), and 20% (v/v) methanol (added with the steroid). The reaction was carried out in a serum bottle under Ar at 37°C and was initiated by the addition of steroid. After 4 h, 0.4 ml of 0.5 N H_3PO_4 was added to stop the reaction and the mixture was centrifuged (10,000 *g*, 22°C, 10 min). The supernatant was extracted twice with 90 ml diethylether, the extract evaporated to dryness, and the residue dissolved in 1 ml methanol. 17-Isoprogesterone was separated from residual 16-dehydroprogesterone by HPLC (above) using a 4.6 × 250 mm Ultrasphere C18 column and flow rate of 0.8 ml/min. Fractions (0.4 ml) were collected and those containing 17-isoprogesterone were pooled, evaporated to dryness, and the steroid dissolved in methanol. HPLC analysis showed that 98% of the 16-dehydroprogesterone was converted to 17-isoprogesterone.

Protein

Protein was measured using the bicinchoninic acid technique of Smith *et al.* [16] and commercially prepared reagents (Pierce Chemical Co., Rockford, Ill.). Bovine serum albumin was the standard.

Chemicals

16α-Hydroxy-4-pregnene-3,20-dione (16α-hydroxyprogesterone); 4-pregnene-3,20-dione (progesterone); 3β-hydroxy-5,16-pregnadien-20-one (16-dehydropregnenolone); 5,16-pregnadien-3β-ol; 5,16-pregnadiene-3β-20α-diol; and 3β-hydroxy-16α-methyl-5,16-pregnadien-20-one were obtained from Steraloids Inc., Wilton, N.H. 4,16-Pregnadiene-3,17-dione (16-dehydroprogesterone), bovine serum albumin, p-chloromercuribenzoate (p-CMB), *o*-phenanthroline, α,α-dipyridyl, hemin, methyl viologen, and *Clostridium pasteurianum* ferredoxin were from Sigma Chemical Co., St Louis, Mo. HPLC grade methanol was from Burudick and Jackson, Muskegon, Mich.

RESULTS

Electron donors for 16-DHPR

16-DHPR activity was detectable in crude cell extracts of strain 144. With pyruvate as the electron donor, the reaction required CoA and MV but not thiamin pyrophosphate (Table 1). The pyruvate-dependent reduction of MV was observed visually and this reaction required CoA. These results indicate that a pyruvate: MV oxidoreductase catalyzes the oxidation of pyruvate, generating reduced MV that supplies electrons to 16-DHPR. Since ferredoxin is the physiological electron carrier for pyruvate oxidation in many anaerobes, the ability of ferredoxin from *C. pasteurianum* to substitute for MV was tested, but no 16-DHPR activity was found. Likewise, extracts prepared from cells grown in iron-rich medium (50 μM $FeSO_4$) showed pyruvate-dependent 16-DHPR activity only when MV was present.

Flavin nucleotides did not substitute for MV in the pyruvate-dependent reaction, nor could NADH or NADPH, with or without flavin nucleotides, replace pyruvate as an electron donor for 16-DHPR. Dithionite, which can reduce MV chemically, could substitute for pyruvate when MV was present, but dithionite alone did not donate electrons directly to 16-DHPR (Table 1). At the dithionite concentration (2 mM) used in these assays, chemical reduction of 16-dehydroprogesterone was not observed in controls lacking cell extract. However, at higher dithionite concentrations (10–20 mM), this did occur and part of the

Table 1. Electron donors for 16-dehydroprogesterone reductase activity

Electron donor	17-Isoprogesterone (nmol/mg protein)
Pyruvate	0
Pyruvate + MV	65
Dithionite	0
Dithionite + MV	68

Reactions contained crude cell extract (2.5 mg/ml) pyruvate (1 mM), dithionite (2 mM), MV (0.5 mM) and the buffer pH as 5.5. CoA (0.1 mM) was added to the pyruvate-containing reactions. The reactions were initiated by adding 16-dehydroprogesterone and incubated for 10 min. No 16-DHPR activity was detected with the following electron donors: pyruvate and FAD or FMN (0.2 mM each), NADH or NADPH (1 mM each) with or without FAD or FMN (0.2 mM each).

16-dehydroprogesterone was converted to progesterone (or 17-isoprogesterone). For this reason, concentrations of dithionite higher than 2 mM were not used in the assays.

Subcellular distribution of 16-DHPR

16-DHPR activity was found in both the cytoplasmic and membrane fractions of crude cell extracts (Table 2). The specific activity of 16-DHPR in the membrane material was twice that of the cytoplasm. About 40% of the total 16-DHPR activity was recovered in the cytoplasm and about 20% was present in the membranes. However 40% of the total activity was unaccounted for following ultracentrifugation. Whether this represents a loss or underestimation of 16-DHPR activity is not clear. The enzyme's activity in crude cell extract and the cytoplasmic fraction was stable for at least a week when stored anaerobically at 0°C. The estimate of 16-DHPR activity in the membrane fraction is based on material resuspended in buffer with a tissue homogenizer. Inactivation of the enzyme, heterogeneity of the preparation or restricted access of the steroid to all of the enzyme in this material may have led to lower estimates of the actual 16-DHPR activity in the membranes. Subsequent attempts to solubilize 16-DHPR from the membrane fraction in potassium phosphate buffer, pH 7.5 containing 1% (v/v) Triton X-100 resulted in the loss of all activity. All further experiments were done using the cytoplasmic fraction as the source of 16-DHPR.

It should be noted that when the subcellular distribution of 16-DHPR activity was measured using pyruvate as the electron donor, activity was found in the crude extract and cytoplasm, but not the membranes. This result indicates that the pyruvate: MV oxidoreductase was present in the cytoplasm but not the membranes.

Properties

16-DHPR activity was proportional to protein from 0.5 to 2.0 mg/ml with a 2 min incubation. At 1 mg protein/ml, the reaction was linear for 4 min (data not shown). Typical results showing the effect of pH on 16-DHPR activity are illustrated in Fig. 1A. The enzyme was relatively unaffected by pH in potassium phosphate buffer over the range 5.0–8.0. At pH values above 7.0, 16-DHPR was progressively inhibited by Tris–HCl and activity was lost at pH 8.5.

Typically, the optimal methanol concentration for 16-DHPR activity was 10% (v/v).

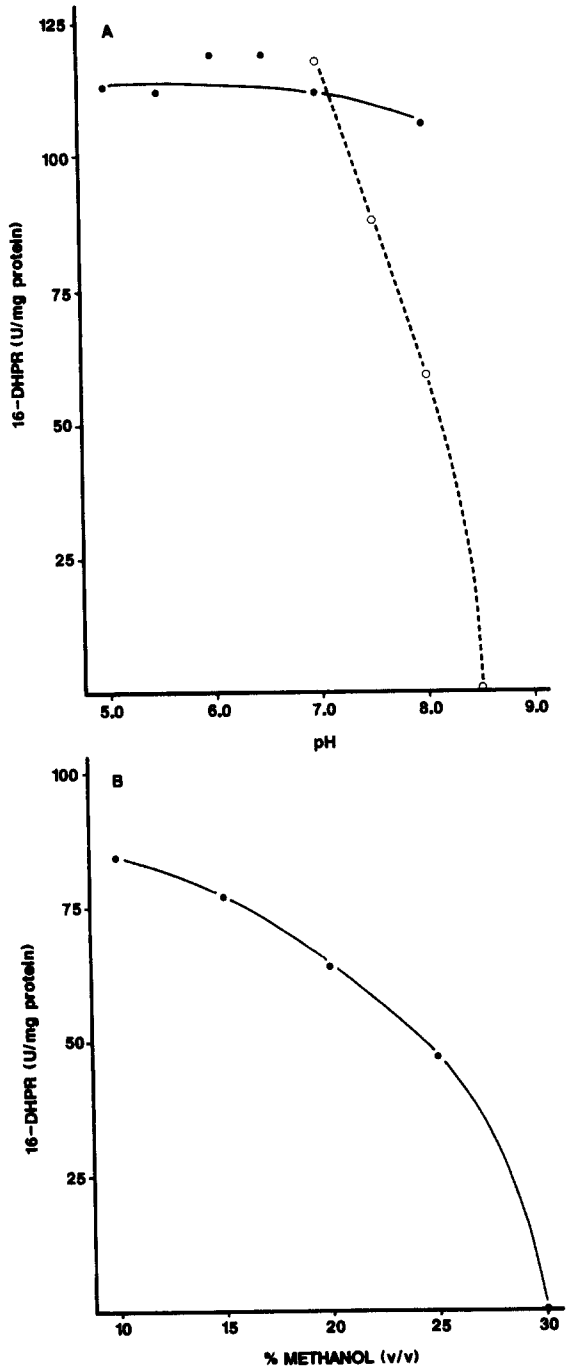

Fig. 1. Effect of pH (A) and methanol (B) on dithionite-dependent 16-dehydroprogesterone reductase activity. (A) Assays contained 25 mM potassium phosphate buffer (●) or 50 mM Tris–HCl buffer (○).

Table 2. Subcellular distribution of dithionite-dependent 16-dehydroprogesterone reductase activity

Fraction	Total protein (mg)	Sp. act. (U/mg)	Total act. (U)	% Total act.
Crude extract	107	127	13,589	100
Cytoplasm	77	65	5005	37
Membrane	18	156	2808	21

Higher methanol concentrations were inhibitory, but 50% of the activity was still present at 25% (v/v) methanol and activity was not lost until the methanol concentration was increased to 30% (v/v) (Fig. 1B). 16-DHPR typically showed hyperbolic substrate saturation kinetics with both MV and 16-dehydroprogesterone (Fig. 2). The enzyme was saturated by MV above 0.5 mM and by steroid above 0.15 mM. No attempts were made to estimate kinetic parameters for these substrates because of the presence of competing enzymes. Hydrogenase was present in the cytoplasmic fraction (as indicated by the H_2-dependent reduction of MV) and could compete with 16-DHPR for reduced MV. Similarly, 16α-dehydroxylase activity was also present and catalyzed the rapid hydroxylation of 16-dehydroprogesterone maintaining an equilibrium mixture of this steroid and 16α-hydroxyprogesterone. Although 16-DHPR pulled the overall reaction in the direction of 17-isoprogesterone formation, the actual amount of 16-dehydroprogesterone present at any given time was less than added to the assays. Despite this, the apparent K_m of 16-DHPR for 16-dehydroprogesterone is clearly less than 0.15 mM.

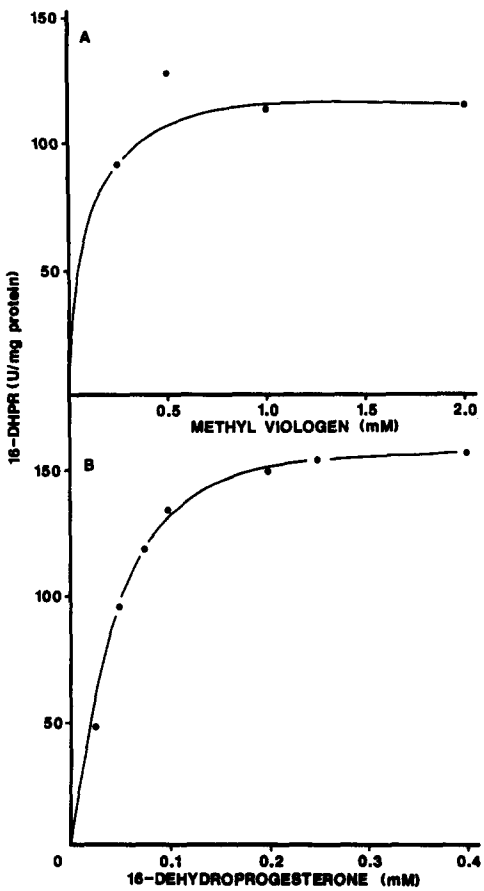

Fig. 2. Effect of methyl viologen (A) and 16-dehydroprogesterone (B) concentrations on dithionite-dependent 16-dehydroprogesterone reductase activity.

Table 3. Effects of metabolic inhibitors on dithionite-dependent 16-dehydroprogesterone reductase activity

Inhibitor	Sp. act. (U/mg protein)
Control (+DTT)	93
EDTA	97
NaN_3	106
NaCN	112
CO	117
o-Phenanthroline	88
α,α-Dipyridyl	92
Control (−DTT)	112
$HgCl_2$	3
p-CMB	8

Cell extracts were prepared with (+) or without (−) DTT. Sulfhydryl inhibitors (0.5 mM) were tested against extracts without DTT in assays from which DTT was omitted. All other inhibitors [1 mM, except EDTA (4 mM) and CO (100% v/v)] were tested against DTT-containing extracts and assays. Inhibitors were incubated with enzyme for 10 min at 37°C before initiating the reactions.

Inhibitors

Several compounds that can inhibit electron transport-coupled reactions were tested for their effects on 16-DHPR activity (Table 3). However, with the exception of sulfhydryl reagents, no inhibitions of 16-DHPR were detected. Subsequent experiments have also shown that acriflavin does not inhibit 16-DHPR activity.

Steroids

Several steroids, structurally similar to 16-dehydroprogesterone, were tested for their ability to inhibit 16-DHPR activity. However, none of the following steroids was inhibitory: 5,16-pregnadien-3β-ol, 5,16-pregnadiene-3β,20α-diol, 3β-hydroxy-16α-methyl-5,16-pregnadien-20-one and 16-ketoprogesterone. Only 16-dehydropregnenolone "inhibited" 16-DHPR activity when measured with 16-dehydroprogesterone as the substrate. Since both steroids contain the Δ^{16-17} double bond 20-ketone, we regard this "inhibition" as competition between two substrates, rather than between a substrate and non-reactive substrate analog, for the active site of 16-DHPR.

Induction specificity

Steroids tested as inhibitors of enzyme activity were also tested for their ability to induce the synthesis of 16-DHPR. As above, only those steroids with an unmodified Δ^{16-17} double bond and C20 ketone were capable of eliciting 16-DHPR synthesis (Table 4). No result was obtained when 5,16-pregnadien-3β-ol was ex-

Table 4. Induction specificity of dithionite-dependent 16-dehydroprogesterone reductase

Steroid	Sp. act. (U/mg protein)
16-Dehydroprogesterone	146
16-Dehydropregnenolone	130
Progesterone	0
5,16-Pregnadien-3β-ol	No growth
5,16-Pregnadien-3β,20α-diol	0
3β-hydroxy-16α-methyl-5,16-pregnadien-20-one	0

Each steroid was added to a separate flask at 10 μg/ml and 1% (v/v) methanol. Cells were grown to an absorbance of 0.3–0.35, harvested, broken, and centrifuged to give the cytoplasmic fraction. Reactions contained cytoplasmic fraction (1 mg/ml).

amined as an inducer, because strain 144 failed to initiate growth in the presence of this steroid. Subsequent experiments showed that when 5,16-pregnadien-3β-ol was added to growing cultures of strain 144 (0.1 absorbance), growth of the bacterium was partially inhibited by the steroid.

DISCUSSION

Earlier studies using antibiotic-treated cultures [12] and resting cell assays [8] suggested that the synthesis of 16-DHPR and PR was induced by 16-dehydroprogesterone. With cultures grown in the presence of progesterone, resting cell assays showed PR, but not 16-DHPR, activity (T. L. Glass, unpublished data). This suggested that the synthesis of these enzymes was differentially regulated. Steroids containing the Δ^{4-5}-3-ketone structure were felt to induce the synthesis of PR and steroids containing the Δ^{16-17}-20-ketone to induce the synthesis of 16-DHPR. 16-Dehydroprogesterone induces the synthesis of both enzyme since it contains both functional groups. This was confirmed in the present study by specific activity measurements which showed that 16-dehydroprogesterone and 16-dehydropregnenolone, but not progesterone, induced 16-DHPR synthesis. Moreover, extracts from 16-dehydropregnenolone-induced cells lacked PR activity. The induction specificity for 16-DHPR synthesis is very high. Steroids lacking the Δ^{16-17} double bond (progesterone) or the C20-ketone (5,16-pregnadiene-3β,20α-diol) did not induce 16-DHPR synthesis.

During this study, we noted that the specific activity of 16-DHPR varied among extract preparations by more than 2-fold (60–140 U/mg protein). The basis for this is not known but may be related to the inducible nature of 16-DHPR. We did not follow the transformation of the inducer, 16-dehydropregnenolone, during the growth of strain 144 but, based on previous experience with 16-dehydroprogesterone [8], it is likely the inducer was converted to 17-isopregnenolone prior to cell harvest. The variation in 16-DHPR specific activity then may reflect different amounts of enzyme remaining in the cells following exhaustion of the inducer.

16-DHPR is unique in terms of reductive steroid transformations carried out by intestinal anaerobes because of it's requirement for low redox potential electrons for activity. In other steroid double bond reductions, electrons are provided by reduced pyridine nucleotides with or without flavin nucleotides as stimulatory cofactors [17–19]. Resting cells of *Eubacterium lentum* VPI 11122 can use H_2 as a reductant for the 21-dehydroxylation of corticosteroids, but the enzyme, in cell extracts, shows maximum activity when reduced flavin nucleotides are the electron donors [11, 20]. In strain 144, the oxidations of pyruvate, H_2, or dithionite are all coupled to the reduction of MV which, in turn, donates electrons to 16-DHPR. Concurrent studies on the PR activity of strain 144 (identified as a Δ^{4-5}-5α-steroid reductase in cell extracts) have demonstrated electron donor and MV requirements identical to those of 16-DHPR (T. L. Glass, unpublished results). These data support the proposal that a common electron transport pathway supplies reducing equivalents to both steroid double bond reductases in strain 144 [8].

The nature of the physiological electron carrier that MV substitutes for is unknown. The inability to detect MV-independent 16-DHPR activity suggests the carrier was either inactivated or diluted out by extract preparation. Growth of strain 144 in the presence of hemin is required to obtain cells with maximum 16-DHPR and PR activities. This was proposed to reflect the involvement of a cytochrome linking the oxidation of pyruvate and H_2 to the steroid double bond reductases [8]. Given the low amounts of cytochromes present in bacteria that normally contain them, loss of MV-independent 16-DHPR activity through dilution of a cytochrome would not be surprising. Direct evidence for the presence and role of a cytochrome in 16-DHPR activity is still required. Moreover, this does not exclude the involvement of additional electron carriers in coupling pyruvate and H_2 oxidation to 16-DHPR activity.

16-DHPR exhibits several properties that clearly distinguish it from 16α-dehydroxylase [6, 7]. Whereas 16α-dehydroxylase activity is detectable in extracts prepared from cells grown in the absence of steroids, 16-DHPR activity is

not present under this condition. 16α-Dehydroxylase shows optimum activity at 25% (v/v) methanol, while 16-DHPR activity is inhibited above 10% (v/v) methanol. Both enzymes are highly specific for their substrates and intolerant of modifications at or near the substrate functions acted upon. However, 16-ketoprogesterone is a competitive inhibitor of 16α-dehydroxylase [7] but is not recognized by 16-DHPR. Although an apparent K_m of 16-DHPR for 16-dehydroprogesterone could not be determined, we estimate it's value is less than 0.15 mM. 16α-Dehydroxylase has apparent K_ms of 0.52 and 0.25 mM for 16α-hydroxyprogesterone and 16α-hydroxypregnenolone respectively [7].

Certain similarities between 16-DHPR and 16α-dehydroxylase do exist. Both activities are detectable in the cytoplasmic and membrane fractions of strain 144 cell extracts [5]. Both enzymes show broad pH optima in potassium phosphate buffer and both are inhibited by sulfhydryl reagents but not other metabolic inhibitors [6, 7].

Several years ago, a preliminary examination of strain 144 cell extract fractions showed 16α-dehydroxylase activity but failed to detect 16-DHPR activity [5]. In retrospect the absence of 16-DHPR activity may have resulted from insufficient exposure (5 h) to the inducing steroid. However, even if 16-DHPR synthesis were fully induced, the use of reduced pyridine nucleotides as electron donors in the assays would not have detected 16-DHPR activity.

Acknowledgements—This work was supported by grant DK 37636 from the National Institute of Diabetes and Digestive and Kidney Diseases. The assistance of Yuming Xu with some of the experiments is gratefully appreciated. This paper was published with the approval of the Director of the North Dakota Agricultural Experiment Station as journal article No. 1880.

REFERENCES

1. Erikson H., Gustafsson J.-Å. and Sjövall J.: Steroids in germfree and conventional rats. 4. Identification and bacterial formation of 17α-pregnane derivatives. *Eur. J. Biochem.* **6** (1968) 219–226.
2. Erikson H. and Gustafsson J.-Å.: Excretion of steroid hormones in adults: steroids in faeces from adults. *Eur. J. Biochem.* **18** (1971) 146–150.
3. Bokkenheuser V. D., Winter J., Hylemon P. B., Ayengar N. K. N. and Mosbach E. H.: Dehydroxylation of 16α-hydroxyprogesterone by fecal flora of man and rat. *J. Lipid Res.* **22** (1981) 95–102.
4. Bokkenheuser V. D., Winter J., O'Rourke S. and Ritchie A. E.: Isolation and characterization of fecal bacteria capable of 16α-dehydroxylating corticoids. *Appl. Environ. Microbiol.* **40** (1980) 803–808.
5. Winter J., O'Rourke S., Bokkenheuser V. D., Hylemon P. B. and Glass T. L.: 16α-Dehydration of corticoids by bacteria isolated from rat fecal flora. *J. Steroid Biochem.* **16** (1982) 231–237.
6. Glass T. L., Lamppa R. S. and Hylemon P. B.: Characterization of 16α-hydroxyprogesterone dehydroxylase in cell extracts of the intestinal anaerobic bacterium, *Eubacterium* sp. 144. *Biochim. Biophys. Acta* **792** (1984) 59–64.
7. Glass T. L. and Lamppa R. S.: Purification and properties of 16α-hydroxyprogesterone dehydroxylase from *Eubacterium* sp. strain 144. *Biochim. Biophys. Acta* **837** (1985) 103–110.
8. Glass T. L. and Burley C. Z.: Stimulation of 16-dehydroprogesterone and progesterone reductases of *Eubacterium* sp. 144 by hemin and hydrogen or pyruvate. *Appl. Environ. Microbiol.* **49** (1985) 1146–1153.
9. Meister P. D., Peterson D. H., Murray H. C., Eppstein S. H., Reineke L. M., Weintraub A. and Leigh H. M.: Microbiological transformations of steroids. II. The preparation of 11α-hydroxy-17α-progesterone. *J. Am. Chem. Soc.* **75** (1953) 55–57.
10. Bjorkhem I. H., Erickson H. and Gustafsson J.-Å.: Microbial formation of 17α-C_{21} steroids: stereochemistry of saturation of the Δ^{16}-double bond. *Eur. J. Biochem.* **20** (1971) 340–343.
11. Feighner S. D., Bokkenheuser V. D., Winter J. and Hylemon P. B.: Characterization of a C_{21} neutral steroid hormone transforming enzyme, 21-dehydroxylase, in crude cell extracts of *Eubacterium lentum*. *Biochim. Biophys. Acta* **574** (1979) 154–163.
12. Glass T. L. and Burley C. Z.: Biotransformation of 16-dehydroprogesterone by the intestinal anaerobic bacterium, *Eubacterium* sp. 144. *J. Steroid Biochem.* **21** (1984) 65–72.
13. Hungate R. E.: A roll tube method for cultivation of strict anaerobes. In *Methods in Microbiology* (Edited by J. R. Norris and D. W. Ribbons). Academic Press, New York, Vol. 3B (1969) pp. 117–132.
14. Bryant M. P.: Commentary on the Hungate technique for culture of anaerobic bacteria. *Am. J. Clin. Nutr.* **25** (1972) 1324–1328.
15. Miller T. L. and Wolin M. J.: A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. *Appl. Microbiol.* **27** (1974) 985–987.
16. Smith P. K., Krohn R. I., Hermanson G. T., Mallia A. K., Gartner F. H., Provenzano M. D., Fujimoto E. K., Goeke N. M., Olson B. J. and Klenk D. C.: Measurement of protein using bicinchoninic acid. *Analyt. Biochem.* **150** (1988) 76–85.
17. Glass T. L., Wheeler L. A., Sutter V. L. and Finegold S. M.: Transformation of 4-androsten-3,17-dione by growing cultures and cell extracts of *Clostridium paraputrificum*. *Biochim. Biophys. Acta* **573** (1979) 332–342.
18. Stokes N. A. and Hylemon P. B.: Characterization of Δ^4-3-ketosteroid-5β-reductase and 3β-hydroxysteroid dehydrogenase in cell extracts of *Clostridium innocuum*. *Biochim. Biophys. Acta* **836** (1985) 255–261.
19. White B. A., Cacciapuotti A. F., Fricke R. J., Whitehead T. R., Mosbach E. H. and Hylemon P. B.: Cofactor requirements for 7α-dehydroxylation of cholic and chenodeoxycholic acid in cell extracts of the intestinal anaerobic bacterium, *Eubacterium* species V.P.I. 12708. *J. Lipid Res.* **22** (1981) 891–898.
20. Feighner S. D. and Hylemon P. B.: Characterization of a corticosteroid 21-dehydroxylase from the intestinal anaerobic bacterium, *Eubacterium lentum*. *J. Lipid Res.* **21** (1980) 585–593.