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EFFECT OF SULFHYDRYL AND DISULFIDE AGENTS ON 3β AND 17β-HYDROXYSTEROID DEHYDROGENASE AND ON STEROID UPTAKE OF *PSEUDOMONAS TESTOSTERONI*

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SUMMARY

Uptake of testosterone and the activity of the 3β and 17β-hydroxysteroid dehydrogenase of membrane vesicles of *Pseudomonas testosteroni* were similarly inhibited by p-hydroxymercuribenzoate (PHMB), N-ethylmaleimide, $ZnSO_4$ and $CuSO_4$. The inhibition of 3β and 17β-hydroxysteroid dehydrogenase by PHMB could be reversed with dithiothreitol, whereas the inhibition of testosterone uptake could not be reversed. The transport process was inhibited by dithiothreitol, cysteine and glutathione. These results indicated that the 3β and 17β-hydroxysteroid dehydrogenase was involved in steroid uptake by membrane vesicles of *P. testosteroni* and, a disulfide bond present on a transport component, distinct from dehydrogenase activity, was also required.

INTRODUCTION

An active transport system for the uptake of steroids in membrane vesicles of *Pseudomonas testosteroni* has been described [1]. The uptake of testosterone* is dependent on the presence of NAD^+ and is optimal between pH 8 and pH 9. These conditions are similar to the requirements of the 3β and 17β-hydroxysteroid dehydrogenase activity of *P. testosteroni* [2, 3]. Characterization of steroids during the uptake process indicated that intravesicular steroids were products of the enzyme 3β and 17β-hydroxysteroid dehydrogenase [4]. Furthermore, only those steroids which were substrates of the enzyme were accumulated by membrane vesicles. The results presented here extend these studies on the relationship between uptake of steroids and the activity of 3β and 17β-hydroxysteroid dehydrogenase. The effects of various sulfhydryl and disulfide inhibitors on both processes have been investigated.

EXPERIMENTAL PROCEDURES

Materials. P. testosteroni 11996 was obtained from American Type Culture Collection, Rockville, Maryland; unlabelled testosterone from Steraloids, Inc., Pawling, New York; [7-^{3}H]-testosterone and [1,2-^{3}H]-testosterone from New England Nuclear Corporation, Boston, Massachusetts; NAD^+ from Sigma Chemical Company, Saint Louis, Missouri; p-chloromercuribenzoate (sodium salt) (PHMB), cysteine and glutathione from Schwarz–Mann, Orangeburg, New York; N-ethylmaleimide (N-EM) from Eastman Organic Chemicals, Rochester, New York; and, dithiothreitol from Calbiochem, San Diego, California.

Methods. Media used for growth, and conditions for induction of transport activity have been described [1, 5, 6]. Membrane vesicles were prepared after spheroplast formation using the lysozyme-EDTA method [7].

Testosterone transport assays. Steroid transport was assayed as previously described [7]. Reaction mixtures for transport studies contained in 0.2 ml: 50 μmol of Tris–HCl, pH 9.0, 200 nmol NAD^+, approx. 170 pmol of an aq. solution of labelled testosterone and 2 μg membrane protein. In inhibitor studies, the inhibitor and membrane protein were preincubated at 27°C for 10 min in a vol. of 0.1 ml before the addition of testosterone and NAD^+. The reaction mixture was incubated at 25°C for periods of time ranging from 1 to 5 min. At 5 min the uptake of testosterone was still proportional to the time of incubation. Controls for these experiments were membrane vesicles which has also been pre-incubated at 27°C for 10 min.

When reversal by dithiothreitol of the inhibition caused by PHMB was being investigated, dithiothreitol was added after the 10 min preincubation of membrane vesicle with PHMB and allowed to interact with the protein for 1 min at 27°C before the addition of NAD^+ and testosterone.

3β and 17β-Hydroxysteroid dehydrogenase assay. The assay for 3β and 17β-hydroxysteroid dehydrogenase was performed as described by Talalay[2] except that sodium pyrophosphate buffer was replaced with 0.75 ml of 1 *M* Tris–HCl, pH 9.0. In inhibitor studies, inhibitor and membrane protein were pre-incubated

* Trivial and systematic nomenclature of steroids. Androstenedione, 4-androstene-3,17-dione; 1,4-Androstadienedione, 1,4-androstadiene-3,17-dione; Testosterone, 17β-hydroxy-4-androsten-3-one.

Table 1. Effect of incubation on the activity of 3β- and 17β-hydroxysteroid dehydrogenase and on the uptake of testosterone

Experiment	3β- and 17β-hydroxysteroid dehydrogenase activity nmol NADH/min/mg protein No pre-incubation	Pre-incubation	% control	Uptake of testosterone nmol/min/mg protein No pre-incubation	Pre-incubation	% control
I	9.27	6.54	71	1.89 ± 0.19	1.10 ± 0.12	58
II	7.27	5.81	80	1.27 ± 0.09	0.62 ± 0.03	49

The assays were performed as described in the text. The values shown for uptake of testosterone are means derived from six experiments ± S.E. Controls represent those samples which had not been pre-incubated at 27°C for 10 min.

at 27°C for 10 min in a vol. of 1.5 ml before the addition of testosterone and NAD^+. Control samples were also preincubated at 27°C for 10 min.

RESULTS

Stability of the membrane-bound 3β and 17β-hydroxysteroid and the steroid uptake process. Talalay and Dobson[3] have reported that dilute suspensions of the purified 3β and 17β-hydroxysteroid dehydrogenase from *P. testosteroni* lose activity when incubated at alkaline pH in the absence of NAD^+. Incubation of the vesicle membrane at pH 9.0 for 10 min at 27°C resulted in a 20 to 30% loss in enzymatic activity (Table 1). When testosterone uptake was measured after a similar incubation of the vesicle membrane, 40–50% of the uptake disappeared.

The rate of NADH production appeared to be greater than the rate of uptake of testosterone, and inhibition of enzyme activity appeared to be less than that of steroid uptake. However, it should be noted that in uptake studies the net effect of entry and exit of the steroid is being measured. Testosterone enters the membrane vesicles as androstenedione which is rapidly converted to androstadienedione, for which there appears to be no permeability barrier, resulting in efflux of androstadienedione from the vesicle [4].

Effect of sulfhydryl reagents on the activity of 3β and 17β-hydroxysteroid dehydrogenase and on the uptake of testosterone. NAD^+ is known to exert a protective effect on sulhydryl groups [8]. Therefore, in studying the effects of sulfhydryl and disulfide reagents the inhibitior was pre-incubated with the vesicle membrane in the absence of NAD^+.

The effect of increasing concentrations of PHMB on the activity of 3β and 17β-hydroxysteroid dehydrogenase and on the uptake of testosterone was examined (Fig. 1). At a concentration of 10^{-4} M, PHMB caused a 73% inhibition of the dehydrogenase activity and a 64% inhibition of testosterone uptake. Clearly PHMB caused a marked inhibition of both processes although it is difficult to understand why this agent produced a greater inhibition of the enzyme.

When N-EM was used as the sulfhydryl reagent, a closer correlation between the inhibition of the two processes was obtained (Fig. 2). N-Ethylmaleimide completely inhibited both the 3β and 17β-hydroxysteroid dehydrogenase and the uptake of testosterone at a concentration of 30 mM.

Talalay and Dobson[3] reported that cysteine and glutathione did not protect purified *P. testosteroni* 3β

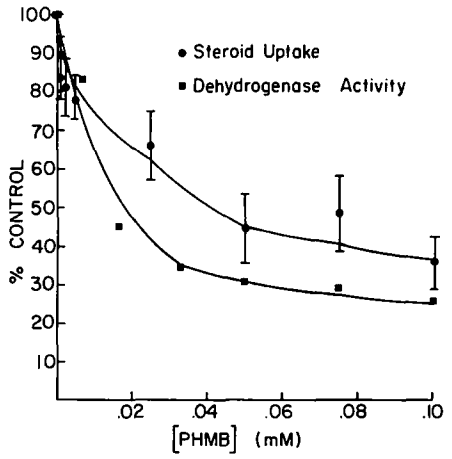

Fig. 1. Effect of PHMB on 3β- and 17β-hydroxysteroid dehydrogenase and on uptake of testosterone. The experiment was performed as described in Methods section. Each value shown for the uptake of testosterone represents the mean of six experiments ±S.E.

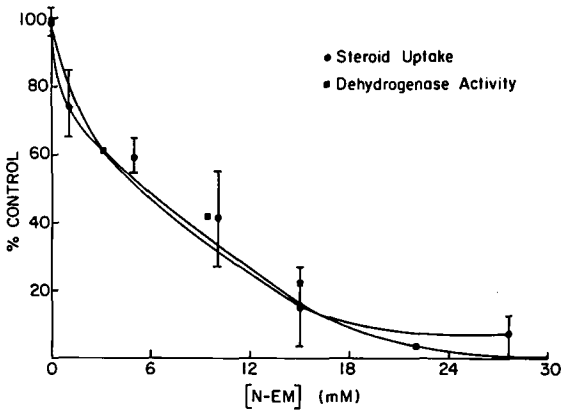

Fig. 2. Effect of N-EM on 3β- and 17β-hydroxysteroid dehydrogenase and on uptake of testosterone. The experiment was performed as described in Methods section. Each value shown for the uptake of testosterone represents the mean of six experiments ±S.E.

Table 2. Effect of sulfhydryl and disulfide agents on 3β- and 17β-hydroxysteroid dehydrogenase and on uptake of testosterone

	3β- and 17β-hydroxysteroid dehydrogenase activity (% control)	Uptake of testosterone (% control)
PHMB (1.0 m*M*)	0	0
PHMB (1.0 m*M*) + dithiothreitol (1.0 m*M*)	37	0
PHMB (1.0 m*M*) + dithiothreitol (5.0 m*M*)	85	0
Dithiothreitol (10.0 m*M*)	96	4
Cysteine (10.0 m*M*)	100	59
Glutathione (10.0 m*M*)	96	39

The assays were performed as described in the text. The sequence of addition of the various agents is indicated in the text.

and 17β-hydroxysteroid dehydrogenase against inactivation by PHMB. However, when the membrane-bound enzyme, pre-incubated in 1.0 mM PHMB, was exposed to dithiothreitol prior to the addition of NAD^+ and testosterone, the inhibition of the dehydrogenase due to PHMB could be reversed (Table 2). On the other hand, the inhibition of the uptake process by PHMB could not be reversed with dithiothreitol. Incubation of the sulfhydryl protecting reagents, dithiothreitol, cysteine or glutathione at concentrations of 10 mM, with the vesicle membrane inhibited the transport of testosterone but had no effect upon the activity of the 3β and 17β-hydroxysteroid dehydrogenase.

Effect of heavy metals on the 3β and 17β-hydroxysteroid dehydrogenase and on the uptake of testosterone. $CuSO_4$ and $ZnSO_4$ exerted inhibitory effects on both the activity of the 3β and 17β-hydroxysteroid dehydrogenase and on the uptake of testosterone (Fig. 3 and Fig. 4). The inhibition by $CuSO_4$ of the 3β and 17β-hydroxysteroid dehydrogenase activity closely paralleled the inhibition of the uptake process, whereas inhibition of the uptake process by $ZnSO_4$ was more marked than inhibition of the dehydrogenase activity. This may reflect a further inhibition by $ZnSO_4$ of some other component required for the transport of testosterone.

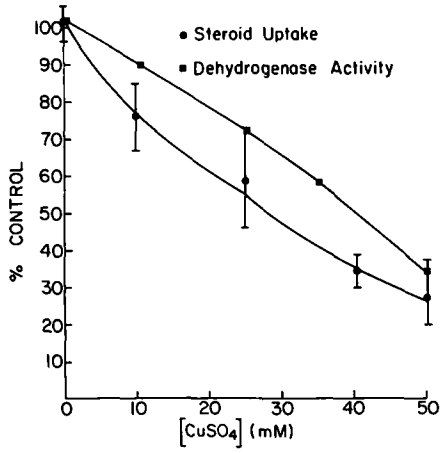

Fig. 3. Effect of heavy metals on 3β- and 17β-hydroxysteroid dehydrogenase and on uptake of testosterone. The experiment was performed as described in Methods section. Each value shown for the uptake of testosterone represents the mean of six experiments ±S.E.

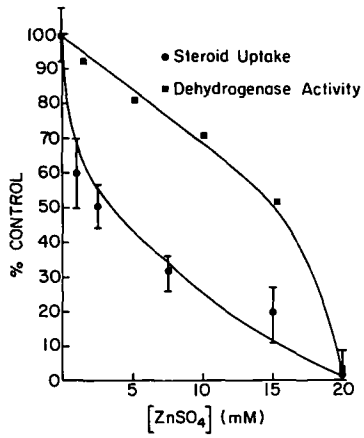

Fig. 4. Effect of $ZnSO_4$ on 3β- and 17β-hydroxysteroid dehydrogenase and on uptake of testosterone. The experiment was performed as in Fig. 3 except that $ZnSO_4$ was substituted for $CuSO_4$.

DISCUSSION

Several lines of evidence indicate that 3β and 17β-hydroxysteroid dehydrogenase plays an important role in the uptake of steroids by membrane vesicles of *P. testosteroni*. The uptake of testosterone is dependent on the presence of NAD^+ and is optimal between pH 8 and 9, similar to the requirements of 3β and 17β-hydroxysteroid dehydrogenase. Only those steroids which serve as substrates for the enzyme are accumulated by membrane vesicles and the intravesicular steroids are products of the enzyme reaction. Furthermore, inhibition of 3β and 17β-hydroxysteroid dehydrogenase activity by pre-incubation at 27°C or addition of PHMB, N-EM, $CuSO_4$ or $ZnSO_4$ during pre-incubation, resulted in a similar or greater inhibition of steroid uptake.

In an earlier report [1], we indicated that no consistently significant inhibition of steroid uptake could be detected using dithiothreitol (5 mM), $ZnSO_4$ (5 mM), PHMB (10^{-4} M) or N-EM (10^{-2} M). In the previous experiments, these agents were added to the

reaction mixture at time zero, whereas in the experiments reported here, inhibitors were pre-incubated with the membrane for 10 min before the addition of testosterone and NAD^+.

Dithiothreitol was able to reverse the inhibition of the membrane-bound 3β and 17β-hydroxysteroid dehydrogenase due to PHMB. Talalay and Dobson were unable to effect reversal of the inhibition due to PHMB using purified *P. testosteroni* 3β and 17β-hydroxysteroid dehydrogenase indicating that membrane-bound dehydrogenase may be protected from irreversible inhibition.

Although dithiothreitol was able to reverse the inhibition of 3β and 17β-hydroxysteroid dehydrogenase due to PHMB, steroid uptake could not be restored. The lack of reversibility of the inhibition of the steroid transport system caused by PHMB could not be accounted for by the presence of excess PHMB on the vesicle membranes. Washing of the membranes after exposure to the sulfhydryl reagent and prior to the addition of dithiothreitol did not effect a reversal of the inhibition. Indeed, when the effect of three disulfide reagents, including dithiothreitol, on both processes was investigated, it was found that each of these inhibited the transport process without inhibiting the 3β and 17β-hydroxysteroid dehydrogenase. It appears therefore that a disulfide bond present on a component of the transport process but not present on the dehydrogenase is necessary for transport.

In summary, uptake of steroids by membrane vesicles requires the functional integrity of the 3β and 17β-hydroxysteroid dehydrogenase. During transport, testosterone is converted to androstenedione and NAD^+ is reduced to NADH. In addition, transport is dependent upon the electron transport chain [1]. The requirements for the enzyme activity and electron transport may be linked *via* the production of NADH. A disulfide bridge not present on the 3β and 17β-hydroxysteroid dehydrogenase is also required for steroid transport.

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