3β,17β-Hydroxysteroid dehydrogenase of *Pseudomonas testosteroni*

Kinetic evidence for the bifunctional activity at a common catalytic site

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3β,17β-Hydroxysteroid dehydrogenase (3β17βHSDH) is an NAD-dependent dehydrogenase which has a double specificity for the 3- and 17-positions on the steroid skeleton. When dehydroepiandrosterone (DHEA) is used as steroid substrate, and the assay coupled with ketosteroid-isomerase, the two reactions occur alternately and each reaction on the 3-position produces a chromophoric molecule. These two reactions can follow one another without dissociation of the coenzyme from the enzyme binding site. This is confirmed by competition experiments with another dehydrogenase.

3β17β-Hydroxysteroid dehydrogenase *Δ5-3-Ketosteroid isomerase* *Bifunctional enzyme*
Dehydroepiandrosterone Pseudomonas testosteroni *NAD regeneration*

1. INTRODUCTION

3β,17β-Hydroxysteroid dehydrogenase (3β17β HSDH, EC 1.1.1.51) is an inducible enzyme produced by *Pseudomonas testosteroni*. This enzyme catalyses the reversible oxidation of both 3- and 17-hydroxy groups of some hydroxysteroids. Since the original report [1], it has been confirmed by several authors that the 3 and 17 activities are associated with the same protein [2,3].

It is not known, however, if 3 and 17 dehydrogenations occur at the same active site, whether they share a common catalytic site but have different steroid binding regions or if separate active sites are present [4]. This report deals with this open question.

When dehydroepiandrosterone (DHEA) is used as steroid substrate, the enzyme is able to catalyse both reduction of the 17-oxo group and oxidation of the 3-hydroxy group. In this way, the cyclic transformation of coenzyme in catalytic quantity was applied to an interesting analytical method of NAD amplification [5].

We show here that in such a reaction pathway, the reaction in the 17-position can be followed by the reaction in the 3-position without dissociation of the coenzyme from the enzyme binding site.

2. MATERIALS AND METHODS

3β17βHSDH and ketosteroid isomerase were gifts from SEMPA-Chimie (Palaiseau, France). Purification procedures are described in [6,7] for the two enzymes. 3β17βHSDH purchased from Sigma was also used with the same results. 3β17βHSDH activity was measured by the rate of NADH formation monitored at 340 nm. The assay mixture contained 1.5 μmol NAD^+, 0.3 μmol testosterone, 0.3 mmol sodium pyrophosphate

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Abbreviations: isomerase, Δ5-3-ketosteroid isomerase; DHEA, dehydroepiandrosterone (3β-hydroxy-17-oxo-androst-5-ene); YADH, yeast alcohol dehydrogenase

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(pH 9.5), 0.1 ml methanol in a total volume of 3 ml. The reaction was initiated by addition of enzyme. One unit is defined as the formation of 1 μmol NADH/min.

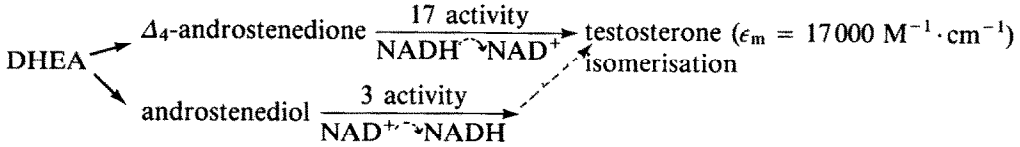
The velocity of the reaction catalysed by 3β17βHSDH coupled with isomerase was measured by the rate of Δ4-androstenedione (and testosterone) production monitored at 248 nm (ϵ_m = 17000 $M^{-1} \cdot cm^{-1}$). The assay mixture contained 0.6 μmol DHEA, 0.15 mmol sodium phospohate (pH 7.4), 0.3 ml methanol, in a total volume of 3 ml. The reaction was initiated by addition of both isomerase (5 units) and 3β17βHSDH (0.125 units). In competition experiments by the YADH reaction, the velocity of the cyclic reaction was measured in the presence of YADH with ethanol as substrate (30 μl). In control experiments, we did not observe inhibition or activation due to YADH or ethanol added separately.

3. RESULTS

3.1. *Reaction pathway*

The reaction pathway of the 3β17βHSDH-isomerase reaction with DHEA as substrate is shown in fig.1. The irreversible isomerisation of Δ5-androstenedione to Δ4-androstenedione acts as a driving force and produces a chromophoric molecule. Δ5-Androstenedione is in a very low steady-state concentration since isomerase activity is in large excess.

As described in [5,8], further reactions on the two precedent products can occur giving rise to testosterone:



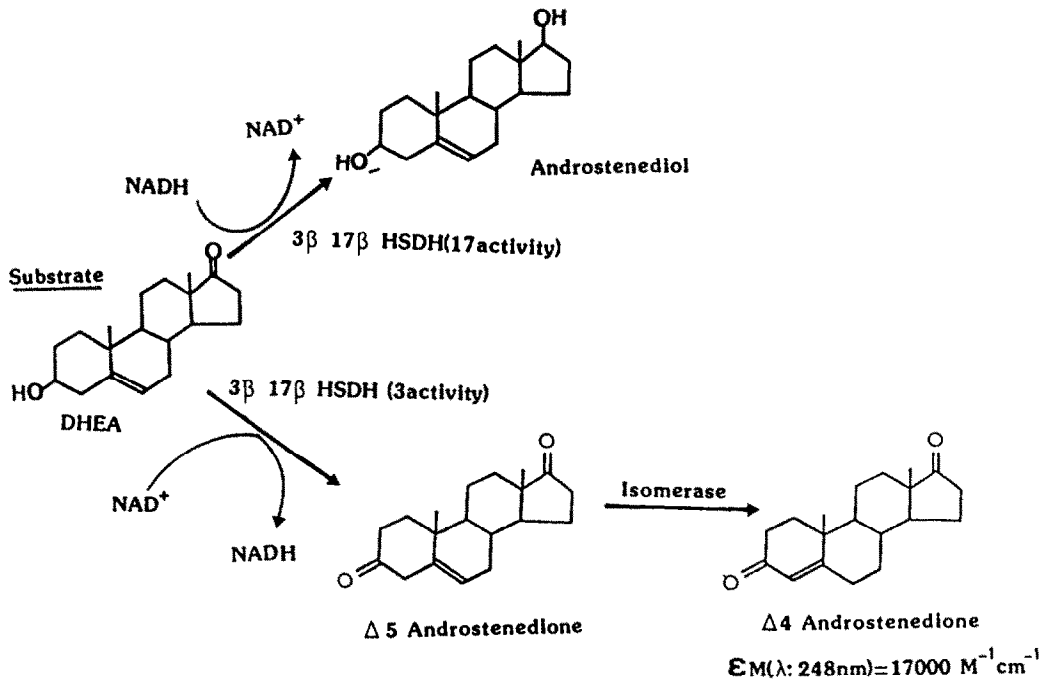


Fig.1. Reaction pathway of the 3β17βHSDH-isomerase reaction with DHEA as substrate.

In these experimental conditions, only 10% of the absorbance variation is due to testosterone produced by these further reactions.

Simplifying the following discussion, only the first two reactions giving rise to androstenediol and Δ4-androstenedione (fig.1) will be considered. For our purpose, the secondary reactions in the 3- and 17-positions which produce testosterone are equivalent to the first ones.

A new chromophoric molecule results from oxidation of the 3β-hydroxy group on androstenediol followed by isomerisation. Conversely the reduction of the 17-oxo group of Δ4-androstenedione does not produce a new one.

3.2. *Reaction with coenzyme in catalytic concentration*

The characteristic features of the kinetic records performed with catalytic concentrations of coenzyme are shown in fig.2. In the first step, the initial form of the coenzyme is consumed, and the initial reaction rate (V_o) is then lower when the reaction starts with NAD^+ in place of NADH. When a 'coenzymic steady state' is reached, the reaction rates (V_s) are the same whatever the initial form of the coenzyme: reduced or oxidized. The difference between these two steps (V_o, V_s) becomes progressively less distinct as coenzyme concentration increases. When the initial NAD^+ or NADH concentration is about 6 μM, the reaction rate remains constant and identical with the two forms of the coenzyme. From this point, the reaction rate has its maximum value. The increase of the initial NAD^+ (or NADH) concentration above 6 μM does not involve an increase in reaction rate.

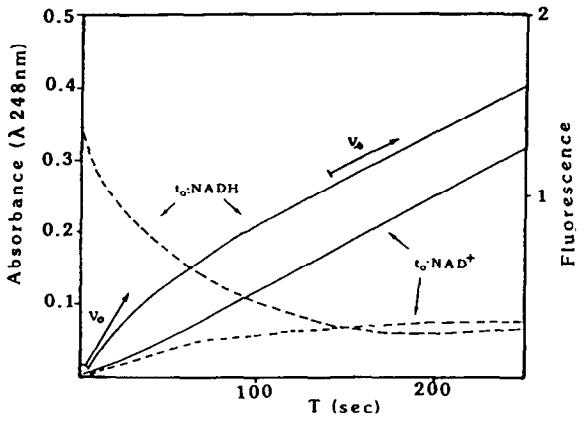


Fig.2. Kinetic records with low coenzyme concentrations. (——) Absorbance (λ = 248 nm), (– – –) fluorescence (λ_{ex} = 340 nm, λ_{em} = 455 nm). As indicated by arrows reaction starts with NAD^+ = 1 μM or NADH = 1 μM. V_s is the reaction velocity measured when the coenzymic steady state is reached.

Table 1

Maximal activities of the 3β17βHSDH-isomerase reaction

Symbols	Initial coenzyme concentrations	d[Androstenedione]/dt (μmol/min)	d[NADH]/dt (μmol/min)
V_{smax}	NAD^+ or NADH > 10 μM	0.22	–
V_3	NAD^+, 1 mM	–	+0.22
V_{17}	NADH, 0.1 mM	–	−0.0215

Values are for one unit of 3β17βHSDH

3.3. *Reaction with coenzyme in stoichiometric concentration*

When NAD is in a high initial concentration (millimolar range), the formation or consumption of NADH can be monitored at 340 nm. The values of these activities measured at saturating coenzyme concentrations are reported in table 1 (the concentrations required for half maximal velocities are approx. 80 μM for NAD^+ and 10 μM for NADH).

3.4. *Competition by YADH reaction*

The 3β17βHSDH-isomerase reaction was also performed in the presence of YADH and ethanol. There is competition between the reaction catalyzed by YADH and the 17 activity of

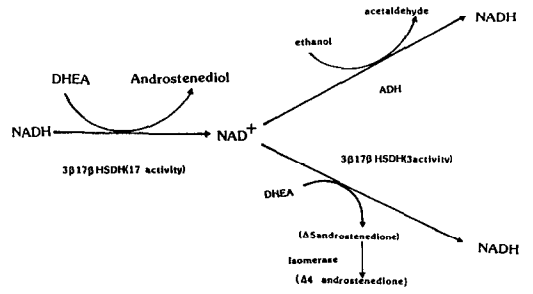


Fig.3. Competition for NAD^+ consumption by the YADH reaction.

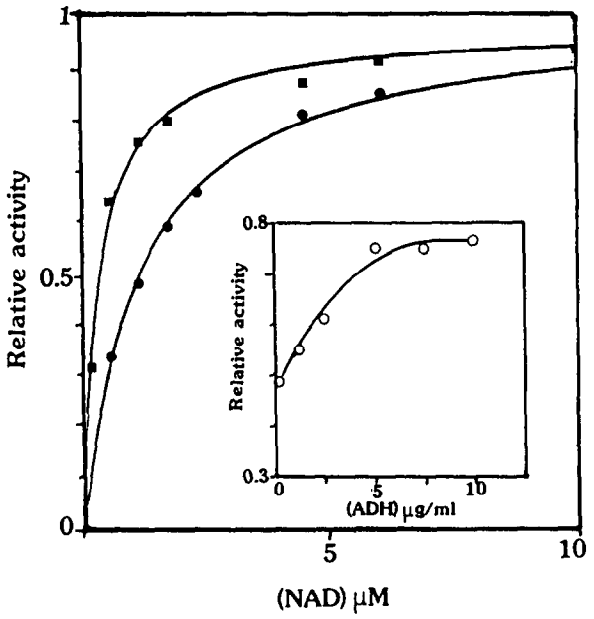


Fig.4. Activity vs NAD^+ concentration. (●) 3β17βHSDH-isomerase; the curve is calculated with $V_m = 1$, $K_m = 1.2\,\mu M$. (■) 3β17βHSDH-isomerase + YADH (10 μg/ml) and ethanol; the curve is calculated with $V_m = 0.97$, $K_m = 0.36\,\mu M$. (Inset) Effect of YADH concentration, $[NAD^+] = 1.2\,\mu M$.

3β17βHSDH for the consumption of NAD^+ (fig.3). The reaction rate has been measured in the presence of YADH and ethanol at various NAD^+ concentrations (fig.4). The 3β17βHSDH-isomerase reaction is not inhibited but activated by the YADH reaction, and only at low NAD^+ concentrations. This implies a decrease of the apparent K_m. The maximal activity is not modified. The concentration of YADH was chosen to give a maximal effect as shown in the control experiment (fig.4, inset).

4. DISCUSSION

Examination of the foregoing results reveals that the hypothesis of two separate catalytic sites is not satisfactory. When the reaction starts with NADH, the NAD^+ concentration is initially negligible. Thus only the 17 activity should be initially observed without formation of androstenedione. In fact, during the early step of the reaction course, the reaction rate (V_o, fig.2) is not negligible and even higher with NADH than with NAD^+.

At high initial NADH concentration (table 1), the Δ4-androstenedione production rate (V_{smax}) is 10-fold higher than the NADH consumption velocity (V_{17}). The main fraction of the NADH consumed by the 17 activity is regenerated despite a high NADH/NAD^+ ratio. This suggests that the NAD^+ molecules produced by the 17 reaction can react in the 3 reaction, without dissociation from a single binding site. This is not true for the reverse reaction: when the reaction starts with a high initial NAD^+ concentration, the NADH production is stoichiometric with the Δ4-androstenedione production ($V_3 = V_{smax}$). In this case, all the NADH molecules produced by the 3 reaction are released in the reaction medium. The existence of a common site for the two activities is clearly confirmed by the inefficiency of the competition of an additional NAD^+ consuming reaction.

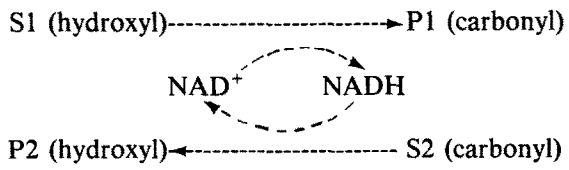
In the presence of the competing reaction, with high YADH/3β17βHSDH ratio, most of the NAD^+ molecules released from the 3β17βHSDH binding site are shunted from the 3 reaction to the YADH reaction. If NAD^+ does dissociate from a 17 specific site and reassociate with a 3-specific site, the YADH reaction should cause a complete depletion of the velocity of androstenedione production. The activation effect which is actually observed reflects a shift in the coenzymic steady state. The activity is measured in the linear phase of the reaction course (V_s, fig.1), when NAD is only 20% in its reduced form. In the presence of the reaction catalyzed by YADH, this steady state is shifted toward a completely reduced state for NAD. This appears as an apparent enhanced affinity because, like other NAD-dependent dehydrogenases, 3β17βHSDH has a higher affinity for the reduced form of the coenzyme [4]. The symmetrical effect was also observed (not shown): in the presence of an additional NADH-consuming reaction (LDH + pyruvate) the apparent K_m for NAD is then increased to 5.8 μM, without significant alteration of V_{smax}. The published data on 3β17βHSDH were interpreted by their authors in various ways. Detailed considerations of the binding of various steroids have led to the conclusion that two different active sites, with a high degree of specificity are present on the enzyme surface [1]. The opposite interpretation was also proposed [9,10]: studies on a group of 17β-hydroxysteroids argue in favor of a large steroid

binding site. The two activities could result from two different fitting possibilities for the steroid. Inhibition by substrate excess, classically observed with most of the substrates, is then interpreted by the formation of the dead-end ternary complex which results from one of the two binding possibilities. The results reported above demonstrate that the two activities proceed with the same coenzyme binding site. This eliminates the possibility of two distinct catalytic sites.

Other published results are consistent with this view. Binding studies indicate that there is statistically one NAD binding site per subunit (3β17βHSDH is tetrameric), these sites being equivalent and independent [4]. The inhibition by a synthetic cyano-ketosteroid of both 3β and 17β activities has been reported [11] indicating a structural homology between the regions involved in each reaction.

Unsuccessful attempts at affinity labelling by substrate and coenzyme analogs have been reported [4,5]. However, as far as we know, no more direct evidence that could be obtained by these methods has been published to demonstrate the common origin of the two activities.

The idea that one enzyme active site may catalyse different stereospecific reactions is not completely surprising, especially for a steroid dehydrogenase. It was clearly demonstrated that the two activities of 3α,20β-HSDH (EC 1.1.1.53) from *Streptomyces hydrogenans* and of 17β-oestradiol dehydrogenase-20αHSDH are relevant for a single catalytic site [12,13]. The 4 dehydrogenases, whose 3-dimensional structures have been determined [14], have subunits made of 2 domains, one of which is similar in all 4. This domain binds the NAD, the other binding the substrate. Our results suggest that a single NAD binding domain is implicated in the 2 specificities. It seems likely that the two specificities also involve a single steroid binding domain, with possibly a broad steroid binding site as has been suggested [9]. The existence of two steroid binding domains, each able to interact with a single NAD binding domain does not seem consistent with the known structural features of other dehydrogenases. The 3β17βHSDH-isomerase reaction used in here is quite similar to the reaction catalysed by horse liver alcohol dehydrogenase (HLADH) when two substrates are consumed in a cyclic reaction [15]:



In our case the two substrates are located on the same molecule: DHEA. These spontaneous NAD-regenerating reactions are essential for the development of studies dealing with artificial and catalytically competent NAD-dehydrogenase complexes. The grafting of a functional NAD molecule at a molecular distance from the active site of HLADH has been described [16,17]. A prerequisite for such studies is a method that allows the alternative transformation of bound NAD molecules. Since the two activities have the same site on 3β17βHSDH, the reaction described in this work appears to be a convenient model for this purpose.

4. CONCLUSION

Despite numerous and precise data published on 3β17βHSDH, the fundamental question concerning the origin of the double specificity on a single site or on 2 different ones has not received clear answers. The kinetic results reported here demonstrate, in a simple way, the existence of a single catalytic site.

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