NON-OLIGOMERIC NATURE OF PORCINE TESTICULAR 20α-HYDROXYSTEROID DEHYDROGENASE

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1. Introduction

It is known that the majority of pyridine nucleotide-linked dehydrogenases have oligomeric quaternary structures. However, 20α-hydroxysteroid dehydrogenase which was purified from porcine testes [1] is peculiar in this respect. It behaved as the 35 000 dalton molecule not only in gel-filtration and ultracentrifugation analyses but also in SDS-acrylamide gel electrophoresis [1] thus it is apparently non-oligomeric. In the study reported below, we have demonstrated that the testicular 20α-hydroxysteroid dehydrogenase is not assembled into a polymeric form even while performing its enzymic function.

2. Materials and methods

2.1. *20α-Hydroxysteroid dehydrogenase*

Porcine testicular 20α-hydroxysteroid dehydrogenase was purified to homogeneity as reported previously [1]. Routinely in the purification procedure, the enzyme activity was assayed by measuring 340 nm light absorption of $NADPH_2$ after incubation of the enzyme with 17α-hydroxyprogesterone (60 μM) and $NADPH_2$ (176 μM) in 100 mM sodium phosphate buffer, pH 7.0. For the inhibition and centrifugation studies reported below, [4-^{14}C]17α-hydroxy-progesterone (Radiochemical Centre, Amersham, UK) was used as the substrate after dilution to a specific radioactivity of 264 cpm/nmole. The product of the enzyme reaction, 17α,20α-dihydroxypregn-4-en-3-one, was isolated by thin-layer chromatography and the amount was determined by the radioactivity [1].

2.2. *Centrifugation*

A linear concentration gradient of sucrose or glycerol was prepared between 5 and 20% (w/v). The gradient was 5 ml in volume and uniformly contained sodium phosphate (100 mM), cysteine (10 mM), [4-^{14}C]17α-hydroxyprogesterone (60 μM), $NADPH_2$ (176 μM) and ethanol (139 μM) at pH 7.0. After overlaying with 0.1 ml of a solution containing 20α-hydroxysteroid dehydrogenase (25 μg protein) and all the above-mentioned compounds except sucrose or glycerol, the gradient was centrifuged on an ultracentrifuge (Hitachi Model 65-P, rotor RPS 40T-2). Immediately after centrifugation, 0.22 ml fractions were collected from the bottom of the centrifuge tubes. Some of the fractions were incubated at 37°C before the enzyme reaction was stopped with methylene dichloride.

Yeast alcohol dehydrogenase (Miles Lab.) and horseradish peroxidase (Sigma) were used as reference proteins. Solutions of these enzymes were centrifuged on separate gradients in the same fashion as above and the location of the enzyme was determined by assaying the enzyme activity by conventional methods.

3. Results

3.1. *Effect of ionic strength on 20α-hydroxysteroid dehydrogenase activity*

Fig.1A shows that the increase of ionic strength of the solution only slightly affected the 20α-hydroxysteroid dehydrogenase activity. The effect of cysteine was observable regardless of the ionic strength of the solution. Similarly, sucrose, a typical non-electrolyte,

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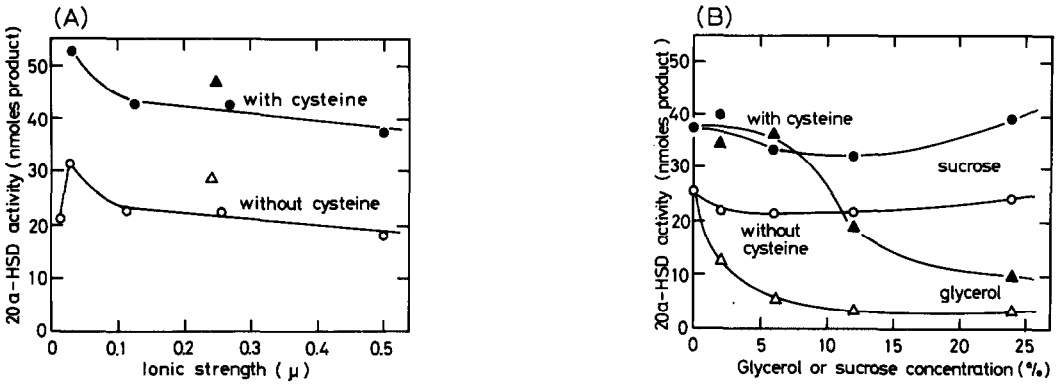

Fig.1. Effects of salt, glycerol and sucrose concentrations on the activity of testicular 20α-hydroxysteroid dehydrogenase (20α-HSD). (A) The ionic strength of the solution was changed by the addition of NaCl (○, ●) to 1 mM sodium phosphate buffer, pH 7.0, or by the increase of the concentration of the phosphate buffer (△, ▲). (B) The concentrations of glycerol and sucrose are expressed in % (w/v). In both (A) and (B), the enzyme (25 μg) was incubated at 37°C for 30 min in the solution (2.5 ml), with 10 mM cysteine (●, ▲), or without cysteine (○, △). Other incubation conditions are described in the Materials and methods.

had no significant effect on the enzyme activity (fig.1B). On the other hand, glycerol was inhibitory to the enzyme, while cysteine partially reversed the inhibition.

3.2. *Active enzyme centrifugation*

The rate of sedimentation of 20α-hydroxysteroid dehydrogenase was determined on the sucrose gradient while the enzyme was functioning in the presence of the substrate and cofactor. Fig.2A shows the distribution of 17α,20α-dihydroxypregn-4-en-3-one which was produced on the gradient. When the enzyme reaction was stopped immediately after centrifugation, it was revealed that the dehydrogenase had traveled down about one third of the gradient as it was performing reduction of the steroid at a constant rate. The location of the enzyme on the gradient was confirmed by the increment of the amount of the reduced steroid when the solutions were incubated after fractionation of the gradient. It was concluded that the active form of 20α-hydroxysteroid dehydrogenase sedimented slightly slower than horseradish peroxidase which was used as a reference protein. Similar results were obtained when the enzyme was centrifuged on the sucrose gradient at 26° C, as well as when glycerol was used in place of sucrose at 15°C (fig.2B). Incubation of the collected fractions after dilution and addition of new substrate and cofactor resulted in most evident localization of the dehydrogenase on the gradient.

In the experiment above-mentioned, it was noted that the substrate steroid was more soluble in the glycerol solutions than in the sucrose solutions. Based on the radioactivity detected after centrifugation, it was shown that each fraction contained 14.0 ± 0.4 nmol of steroids in the case of glycerol, whereas the sucrose gradient contained only 3.0 ± 0.2 nmol of steroid per fraction. In the latter case, 75–80% of the radioactivity which was added initially, was found at the bottom of the tube after centrifugation. Micelle formation can account for the result. Nevertheless, on both glycerol and sucrose gradients, no spurious radioactive peak was observed in control runs without the enzyme.

4. Discussion

The observation that the activity of 20α-hydroxysteroid dehydrogenase was not influenced by salt concentrations suggested that the association–dissociation phenomena such as that observed in the case of adrenocortical cholesterol side-chain cleavage enzyme [2,3] is not involved in the case of this dehydrogenase. It was then observed that the dehydrogenase, while it was acting, sedimented slightly slower than horseradish peroxidase (mol. wt. 40 000) [4]. Under the same conditions of centrifugation,

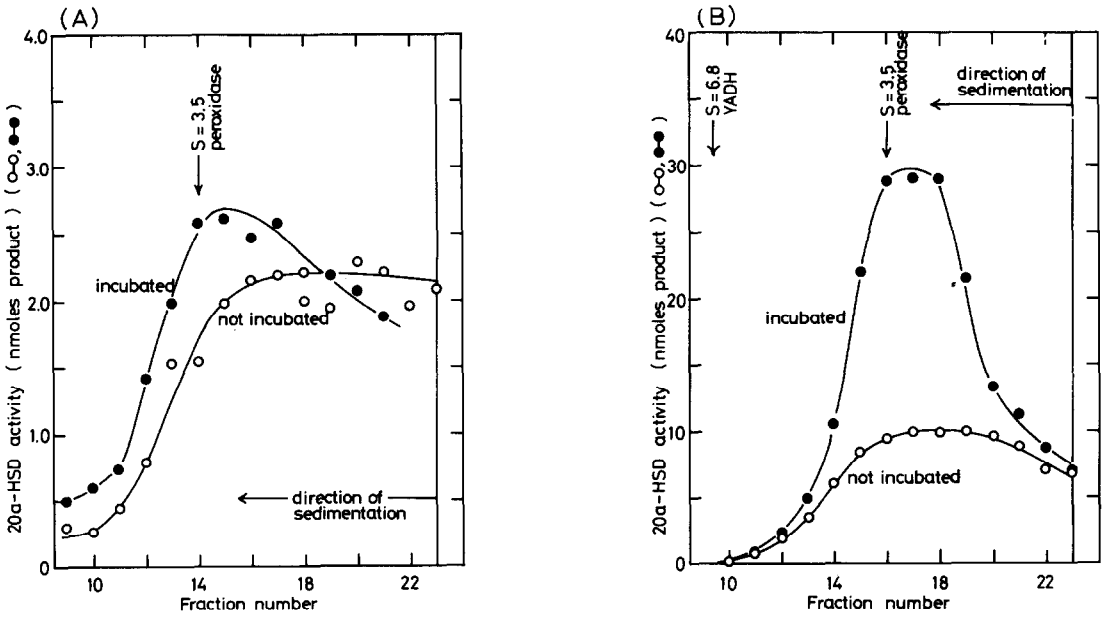

Fig.2. Sedimentation of testicular 20α-hydroxysteroid dehydrogenase as it is performing its enzymic function. (A) Sucrose gradients. Centrifugation was carried out at 2°C for 28 h at 37 000 rev/min (max 180 000 *g*). (B) Glycerol gradients. Centrifugation at 15°C for 14 h at 35 000 rev/min (max 157 000 *g*). In both (A) and (B), the enzyme reaction was stopped immediately after centrifugation (○) or after the fractions were incubated at 37°C for 30 min (●). In the case of (B), 2.5 ml buffer containing cysteine, [4-^{14}C]17α-hydroxyprogesterone and $NADPH_2$ as described above was newly added to each fraction before incubation. The activity of 20α-hydroxysteroid dehydrogenase (20α-HSD) is expressed as nmoles of the reduced steroid which was found in each fraction. The locations of the reference proteins, horseradish peroxidase and yeast alcohol dehydrogenase (YADH), are shown by the arrows.

yeast alcohol dehydrogenase which is a tetrameric enzyme of mol. wt. 150 000 [5] did not dissociate into its subunits. The result implies that the testicular 20α-hydroxysteroid dehydrogenase performs its enzymic action as a monomer protein of mol. wt. 35 000. This is analogous to the action of 3α-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni* which is active in the monomer form, but much less active when it is dimerized [6].

It is interesting to note that three mammalian hydroxysteroid dehydrogenases, placental estradiol 17β-dehydrogenase [7], testicular 20α-hydroxysteroid dehydrogenase [1] and testicular testosterone 17β-dehydrogenase [8], are similar to each other in size (mol. wt. around 35 000). It is known, however, that the placental 17β-dehydrogenase exists in a dimeric form of mol. wt. 72 000 [9]. It was also reported that the testicular 17β-dehydrogenase had a sedimentation coefficient of 5.3 S when it was centrifuged on a sucrose gradient [8], suggesting that dimerization of the enzyme easily occurs. On the other hand, no evidence of polymerization of the testicular 20α-hydroxysteroid dehydrogenase has ever been obtained by the present workers in active enzyme centrifugation at various temperatures (2–26°C).

The mechanism of the inhibitory action of glycerol on 20α-hydroxysteroid dehydrogenase is not clear at present. It seems that the inhibition is not due to the solubility problem of the substrate steroid. We supposed that the enzyme may be polymerized by glycerol, but the result of the above-mentioned experiment suggested this is not the case. Although it is inhibitory to the enzymic action, glycerol is protective for 20α-hydroxysteroid dehydrogenase, preventing loss of the enzyme activity during storage (data not shown above).

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