Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 787–789. Pergamon Press. Printed in Great Britain

ACTIVE FORM OF 17β-HYDROXYSTEROID DEHYDROGENASE OF PORCINE TESTES

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(*Received* 16 *November* 1976)

SUMMARY

17β-Hydroxysteroid dehydrogenase (E.C. 1.1.1.64) of porcine testes was centrifuged at 10° over a sucrose density gradient which contained androstenedione and NADPH, and then the gradient was fractionated. The amount of testosterone was quantitated in each fraction immediately after the centrifugation, and also after further incubation of each fraction at 37° for 3 h. From distributions of the testosterone over the gradients, it was concluded that the active form of the dehydrogenase occurs as its monomer with sedimentation coefficient, 3.11 *s* and molecular weight, 35,400 daltons.

INTRODUCTION

A method for the determination of active form of an enzyme has been proposed and called as active-enzyme-centrifugation [1], the principle of which is described as follows: in a centrifuge tube, enzyme solution is layered on the top of an appropriate medium, into which the substrate as well as the required cofactor are added. After centrifugation of the tubes at an appropriate temperature at which the enzyme reaction occurs to some extent, the active form of the enzyme is estimated from the distribution of the product formed during the centrifugation. By this method, the active unit of alcohol dehydrogenase of yeast was established as a tetramer [1], and the active form of glucose-6-phosphate dehydrogenase of yeast was shown to be a dimer [2]. 17β-Hydroxysteroid dehydrogenase has been purified to an apparently homogeneous state from the microsomal fraction of porcine testes in this laboratory [3], and its molecular weight was estimated as 35,000 daltons by gel filtration [3], and SDS-polyacrylamide gel electrophoresis [4]. In this paper, the molecular structure of active form of the 17β-hydroxysteroid dehydrogenase has been examined by means of active enzyme centrifugation.

EXPERIMENTAL

Materials. [4-^{14}C]-Androstenedione (S.A. 56.6 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, England). Radioactive purity of the steroid was confirmed by t.l.c. NADPH was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). The purified preparation of testicular 17β-hydroxysteroid dehydrogenase was employed, after its homogeneity was confirmed by SDS-polyacrylamide gel electrophoresis [4].

Centrifugation. An automatic density gradient maker was employed for preparation of a linear gradient with sucrose concentration from 5–20%. The gradient contained [^{14}C]-androstenedione (0.5 μCi and 200 nmol/tube) and NADPH (6 nmol/tube) uniformly, regardless of the sucrose concentration. Immediately before the centrifugation, 0.1 ml of the enzyme solution (0.1 mg of protein) was layered on the top of the gradient in the tubes for centrifugation (vol. 5 ml/tube). These were centrifuged at 38,000 rev./min (about 101,000 *g* at the top of tube and 181,000 *g* at the bottom) for 24 h in a swinging bucket-type rotor (RPS-42T2) at 10° in an ultracentrifuge (Model 65 P, Hitachi, Japan). After the centrifugation, every 11 drops were collected into a test tube from the bottom of the centrifuge tubes. As a reference protein, a solution of ovalbumin (0.1 ml, 1.5 mg of protein, molecular weight 43,000 daltons, 3.55 *s*) was layered on the top of the sucrose density gradient without the steroid and NADPH separately, and centrifuged simultaneously in the same rotor. Concentration of the ovalbumin in the gradient was measured by absorption at 280 nm.

Measurement of testosterone. Immediately after the fractionation of the contents of the tube, 3 ml of dichloromethane was added to each fraction and the steroids were extracted by vigorous shaking. Non-radioactive androstenedione and testosterone (300 nmol each/fraction) were added as carriers. An aliquot of the extract was chromatographed on thin layer plate of a mixture of silica gel G and GF (4:1, w/w, E. Merck, Darmstadt, Germany) in a system of benzene and acetone (4:1, v/v). Spots of the carrier steroids on the chromatogram were visualized under U.V. light (254 nm). The spot of testosterone was scraped from the plate, and the steroid was eluted with dichloromethane from the absorbent. The extract was evaporated and residue was dissolved in

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5 ml of the toluene solution, which contained 0.4% PPO and 0.01% POPOP. Radioactivity was determined with a liquid scintillation spectrometer, the counting efficiency of which was approximately 70% for radiocarbon. Production of testosterone (in %) was calculated from the radioactivity of testosterone formed from the radioactive androstenedione.

RESULTS

When the 17β-hydroxysteroid dehydrogenase of testicular origin was subjected to the sucrose density gradient centrifugation in the presence of the substrate and the required cofactor, the extent of migration of the active form of the enzyme was determined by measuring the amount of testosterone enzymatically formed during the sedimentation through the gradient. As shown in Fig. 1(A), the edge of the testosterone plateau was taken as the lower limit of the sedimenting enzyme with its active function and was localized between Fractions 11 and 13, when testosterone was measured immediately after the fractionation of the centrifuged gradient. In Figs. 1(A) and 1(B), location of the active form of the enzyme was determined as the position of peak of the testosterone which was newly produced by the further incubation at 37° for 3 h, or between Fractions 12 and 13, which corresponded to 3.11 *s*. By comparing this sedimentation coefficient with that of ovalbumin which was employed as a standard protein, the molecular weight of the protein located between Fractions 12 and 13

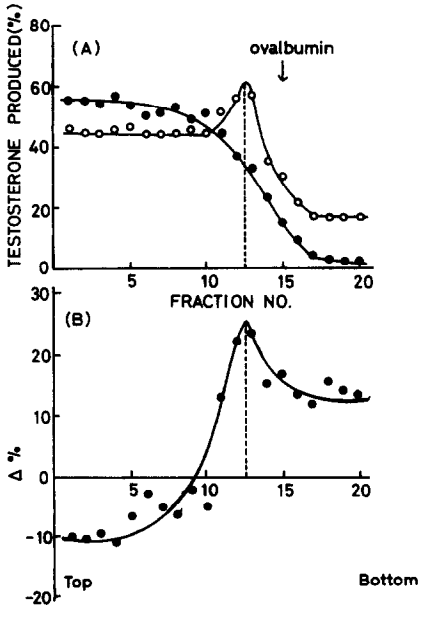

Fig. 1. Sedimentation of the testicular 17β-hydroxysteroid dehydrogenase in the presence of androstenedione and NADPH at 10°. (A) Testosterone was quantitated of each fraction, immediately after the centrifugation (●——●), and when the fractions were further incubated at 37° for 3 h after the fractionation of the centrifuged gradient (○——○). (B) Difference between the above two lines in Fig. 1(A).

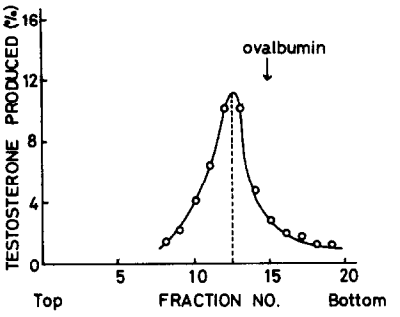

Fig. 2. Sedimentation of the 17β-hydroxysteroid dehydrogenase in the sucrose gradient in the absence of the steroid and the cofactor. After the centrifugation, the gradient was separated into fractions which were respectively incubated with androstenedione in the presence of NADPH. The amount of testosterone produced by incubation in each fraction was indicated (○——○).

was calculated as 35,400 daltons, according to the method reported by Martin and Ames [5].

On the other hand, 0.1 ml of the purified 17β-hydroxysteroid dehydrogenase solution was layered on the top of the sucrose gradient which contained neither substrate nor cofactor, and then centrifuged under the identical conditions to the previously stated one. Immediately after the centrifugation, every 11 drops were collected into an incubation tube from the bottom of the centrifuge tube and diluted with 1.0 ml of 5 mM phosphate buffer (pH 7.4) which contained 7 mM 2-mercaptoethanol, 1 mM EDTA and 20% (v/v) glycerol. [^{14}C]-Androstenedione (0.02 μCi, 0.35 nmol) was incubated with each fraction at 37° for 3 h in the presence of NADPH (1.2 nmol/tube). Testosterone produced by the 17β-hydroxysteroid dehydrogenase showed a single and symmetrical peak (Fig. 2) around Fractions 12 and 13. From these results, the sedimentation coefficient of the native enzyme molecule was determined as 3.11 *s* and accordingly its molecular weight was estimated as 35,400 daltons.

DISCUSSION

In the present experiments, an apparently homogeneous preparation of the testicular 17β-hydroxysteroid dehydrogenase was sedimented at 10° over the sucrose density gradient which contained androstenedione and NADPH. As expected, the enzymatic reduction of androstenedione occurred during the centrifugation. When the amount of testosterone formed during the centrifugation was taken into consideration, in order to locate the sediment of the active form of the enzyme, its active form was found around the location of Fractions 11, 12 and 13. Furthermore, increased production of testosterone observed by further incubation of the fractions at 37° indicated the location of the active form of the dehydrogenase in the gradient more precisely at the position between Fractions 12 and 13. From the sedimentation coefficient, the molecular weight of the

active form of the dehydrogenase was calculated as 35,400 daltons.

The molecular weight of the native 17β-hydroxysteroid dehydrogenase of porcine testes was estimated as 33,200–35,500 daltons by gel filtration [3], 36,500 daltons by SDS-polyacrylamide gel electrophoresis [4], and 34,000 daltons by disc electrophoresis of polyacrylamide gel [4]. Also, the molecular weight of the same enzyme which was obtained as 35,400 daltons by the ultracentrifugation method in the present work is in agreement with the values obtained by other methods in our reports [3, 4]. Under the present experimental conditions, no evidence of polymerization of the testicular 17β-hydroxysteroid dehydrogenase was obtained. When a linear gradient with sucrose concentration from 5–20% (w/v) in 5 mM KH_2PO_4, 7 mM 2-mercaptoethanol and 1 mM EDTA was employed for determination of the sedimentation behaviour of the enzyme, however, its sedimentation coefficient was estimated as 5.3 *s* [3], suggesting dimerization of the native dehydrogenase molecule in this medium. If the dimeric structure (molecular weight, about 70,000 daltons) is the active form of this dehydrogenase, production of testosterone in the gradient would level off from the top to the bottom of the tube, as the dimer would sediment at the bottom under the present conditions.

The molecular weight of human placental 17β-hydroxysteroid dehydrogenase was reported as 33,500–37,000 daltons by SDS-polyacrylamide gel electrophoresis [6, 7], but 67,700–72,000 daltons by the ultracentrifugation method in the presence of $NADP^+$ [7] or estradiol-17β [8]. The dimeric structure of the subunit was suggested as the native form, judged from the sedimentation behaviour, and dissociated into its monomer by treatment of SDS.

On the other hand, the active form of the porcine testicular 20α-hydroxysteroid dehydrogenase was reported as a monomer, the molecular weight of which was 35,000 daltons, suggesting its non-oligomeric nature [9]. The cytochrome *P*-450 related to cholesterol side chain cleavage isolated from bovine adrenocortical mitochondria consisted of 4, 8 and 16 subunits. By the active enzyme centrifugation method, the hexadecamer was revealed as the functional enzyme unit [10].

Acknowledgement—The authors wish to express their gratitude to Dr. H. Nagase, National Institute of Animal Industry, for his continuous encouragement during this work.

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