

Properties of an Antiserum Against *Streptomyces hydrogenans* 20 β -Hydroxysteroid Dehydrogenase

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20 β -Hydroxysteroid Dehydrogenase, Immunoprecipitation, Immunodiffusion, Molecular Weight, *Streptomyces hydrogenans*

Antiserum against crystallized 20 β -hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* was used for different immunodiffusion and immunoprecipitation tests to quantify the bacterial enzyme in cell-free supernatants of the microorganism. After immunoprecipitation and gel electrophoresis the molecular weight of the subunits of 20 β -hydroxysteroid dehydrogenase was calculated to be $27\,300 \pm 700$.

Introduction

After addition of various steroids to the growth medium the synthesis of 20 β -hydroxysteroid dehydrogenase increases significantly in *Streptomyces hydrogenans*^{1,2}. During this induction period the total content of RNA drops. Moreover, synthesis of total RNA slows down, although the production of mRNA-like nucleic acids increases³. However there is no clear evidence whether the increase in enzyme activity is caused by activation of a precursor of the enzyme or by true *de novo* synthesis.

Therefore, by precipitation with a specific antiserum against 20 β -hydroxysteroid dehydrogenase the enzyme should be quantified in the cell homogenates of the microorganism. The properties of both antiserum and enzyme, enriched by various immunological methods are described in detail.

Material and Methods

Chemicals

11 β ,21-Dihydroxy-4,17(20)-pregnadien-3-one was a generous gift from Dr. G. Neesemann, Hoechst AG, Frankfurt/M. Chemicals for gel electrophoresis, Rifamycin SV, Coomassie Brilliant Blue R 250 and Naphthalene Black B were purchased from Serva, Heidelberg. L-[3,5-³H]Tyrosine (60.3 Ci/mmol), [³H]amino acid mixture, and the tissue solubilizer Protosol were obtained from NEN New England Nuclear Corp., Dreieichenhain; Agar and Agarose from Behringwerke, Marburg, Freund adjuvant

from Difco Labor.; all other chemicals were of reagent grade (E. Merck AG, Darmstadt).

Cultivation of the microorganism

Streptomyces hydrogenans (ATCC 19631) was cultivated as described previously². Protein content was determined by the method of Lowry *et al.*⁴. DNA and RNA were estimated using the diphenylamine or orcinol reaction, respectively. Cells were harvested by suction and wet cell material was homogenized sonically using a Branson Sonifier S75 at a current of 4.5 A (power setting of 8)⁵. Soluble 20 β -hydroxysteroid dehydrogenase was determined in a S-100 supernatant obtained after 3 hours of centrifugation at $105\,000 \times g^2$.

Preparation of antiserum

20 β -Hydroxysteroid dehydrogenase was obtained either from Boehringer, Mannheim, or from cultures of *Streptomyces hydrogenans* following the procedure of Hübener *et al.*⁶ modified by an additional polyacrylamide gel electrophoresis. 0.5 mg of the pure enzyme as antigen were dissolved in 2 ml of saline and emulsified with an equal volume of complete Freund's adjuvant. Three areas of the skin, in the neck and on each side of the belly of three month old female inbred rabbits were shaved. 0.5 ml of antigen were s.c. injected into these 3 areas and 0.5 ml were injected in the hindleg between the foot pads. Every week the rabbits were boosted following the procedure above. After the fourth week test blood was taken from the central ear artery and the rabbits were boosted again. After six weeks the animals were anesthetized with a sublethal dosis of nembutal® and exsanguinated by puncture of the vena vaca and by cardiac puncture. About 120 ml blood could be obtained from one animal. The blood was clotted and after removal

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of the clot the resulting clear serum was made 3 mM with respect to sodium azide to prevent bacterial growth and stored in 1.5 ml batches at -20°C .

Immunodiffusion and immunoprecipitation

5 Parts of borate-saline buffer (100 mM boric acid, 25 mM sodium tetraborate, 75 mM sodium chloride, pH 8.5) were mixed with 95 parts of 154 mM sodium chloride solution containing 1% agarose. To get Ouchterlony plates, petri dishes (internal diameter 8 cm) were filled with 15 ml of this solution and wells of 7 mm diameter were cut out. Each well was filled with 0.1 ml of either antiserum or antigen solution.

Quantitation of antigens was performed on Mancini plates. Agarose buffer mixture as used for Ouchterlony plates was enriched with 1.33% antiserum. Wells of 3 mm diameter were filled with 10 μl of antigen solution. Formation of clear precipitation rings took place after two days at 37°C . Plates were kept in buffer for further two days to let diffuse soluble proteins out of the agar. Subsequently, the plates were dried and stained with 1% naphthalene blue in a mixture of 1 part of glacial acid and 9 parts of methanol.

Immunoprecipitation of 37°C for 1 hour was performed in Beckman tubes in a total volume of 0.4 ml. Precipitates were spun down in a Sorvall centrifuge using Hostafion adaptors made for use in the SM-24 rotor, washed twice with borate saline buffer and dissolved in 0.1 ml Protosol for 1 hour at 37°C . After addition of 5 ml of dioxan scintillator⁷ radioactivity was measured in a Packard Tri-Carb model 3375 (counting efficiency for ^3H : 21%).

SDS gel electrophoresis

Marker proteins or antigen-antibody precipitates were dissolved in 0.2 ml of a solution consisting of 24 mg Tris(hydroxymethyl)aminomethane, 30 mg dithiothreitol, 20 mg sodium dodecylsulfate (SDS), 0.2 ml glycerol and 1.8 ml H_2O by heating for 5 min at 100°C ⁸. 10% Standard serum protein gel was made 0.2% with respect to SDS according to Maurer⁹ in a Desaga gel slab apparatus. Separation in 8 cm long gel slabs using Tris-glycin-buffer brought to 0.2% SDS, pH 8.5, took about 2 hours at 60 mA constant current and 150 V. Gels were stained in 1% Coomassie Brilliant Blue in 7% acetic acid for 1 hour and destained electrophoretically. Conservation of the gels was achieved by covering them with a solution of 3.5 g gelatine dissolved in 47.5 ml water and 2.5 ml glycerol. Determination of radioactivity was described in a previous paper⁵.

Results

Immunization of rabbits yields an antiserum of high specificity and titer against 20 β -hydroxysteroid dehydrogenase. In Ouchterlony plates two precipitation bands are visible due to the dimer and tetramer of the enzyme (Fig. 1 *). Crossed immunoelectrophoresis¹⁰ reveals that there are at least six more antibodies against *Streptomyces* proteins. Their concentration, however, is too low to give rise to visible precipitates under immunoprecipitation conditions used with antigen in great excess. Antibodies from rabbits completely precipitate 20 β -hydroxysteroid dehydrogenase from the S-100 supernatant. Increasing the concentration of antiserum, enzymatic activity of 20 β -hydroxysteroid dehydrogenase in the supernatant decreases from 100 to 0% (Fig. 2). By comparison of incorporated

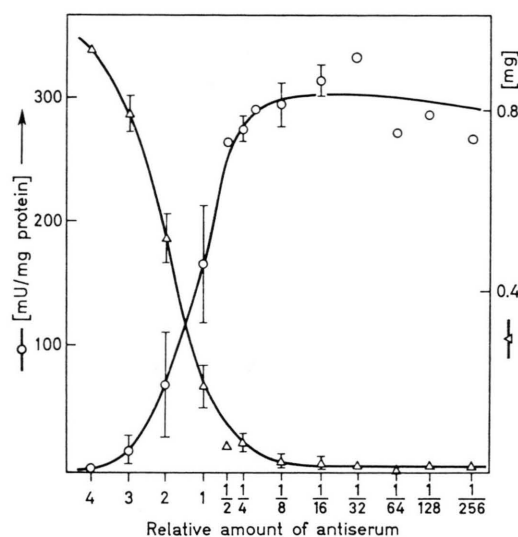


Fig. 2. Immunoprecipitation experiment with ^3H -labelled S-100 supernatant proteins from *Streptomyces hydrogenans*. 0.2 ml of undiluted or diluted S-100 supernatant were mixed with 0.2 ml of undiluted or diluted antiserum as shown on the abscissa. Enzyme activity in the resulting precipitate-free supernatants as well as the amount of protein in the spun down antigen antibody precipitates were measured. The values are the mean of 3 or 4 precipitations with their standard deviations or single determinations.

radioactivity in total acid precipitable proteins with that of antibody precipitated proteins after long term labelling in the presence of tritium labelled amino acid mixture followed by induction for further 4 hours we get the part of antibody pre-

* Fig. 1 see Plate on page 290 a.

capitable proteins in relation to total cell protein to $1.6 \pm 0.4\%$. This figure is in accordance with former results¹¹.

The radioactive antigen antibody complex can be dissociated and fractionated by gel electrophoresis in 10% acrylamide gel containing SDS. Two radioactive peaks can be seen corresponding to molecular weights of 27 000 and 54 000, respectively (Fig. 3). The relative mobilities of marker pro-

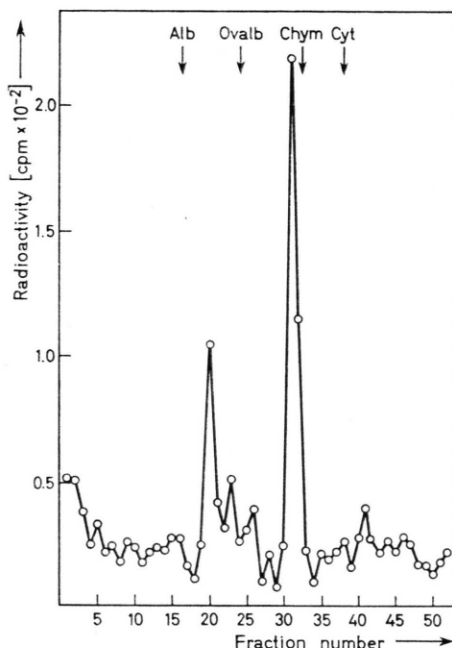


Fig. 3. Gel electrophoretic separation of ^3H -labelled, antibody-precipitated and dissociated 20 β -hydroxysteroid dehydrogenase. The precipitate of ^3H -amino acid-labelled S-100-supernatant proteins and antiserum was washed three times with borate saline buffer and dissociated in the presence of SDS and dithiothreitol by heating to 100 °C for 5 min. 50 μg of protein was applied on top of a 10% polyacrylamide gel containing 0.2% SDS and run at 60 mA per gel. The protein containing strip was sliced into 1.4 mm thick pieces, hydrolysed in 0.1 ml of 30% H_2O_2 , and counted in dioxan scintillator. The migration distances of albumin, ovalbumin, chymotrypsinogen, and cytochrome c are given by arrows.

teins and of the 20 β -hydroxysteroid dehydrogenase monomer can be plotted versus their molecular weights (Fig. 4). The values of three independent gels are given. The correlation coefficient for the linear is $r = 0.983$. The calculated molecular weight for the monomer of 20 β -hydroxysteroid dehydrogenase is $27\,300 \pm 700$ as the mean of seven determinations in three independent runs. In gels lacking

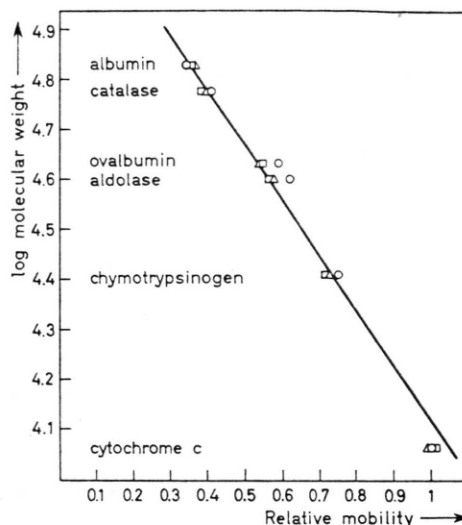


Fig. 4. Relationship between the molecular weights of different marker proteins and their relative electrophoretic mobilities. SDS-gel electrophoresis of marker proteins treated as the antigen-antibody precipitates and run in the same gel as shown in Fig. 3. The relative mobility of the marker proteins used, bovine serum albumin (molec. weight 68000), ovalbumin (43000), aldolase (40000), catalase (60000), chymotrypsinogen (25700), and cytochrome c (11000) in three different runs is given. Separation was performed in gel slabs consisting of 10% polyacrylamide containing 0.2% SDS.

SDS 20 β -hydroxysteroid dehydrogenase migrates as a particle twice as heavy. From Sephadex G-200, however, 20 β -hydroxysteroid dehydrogenase can be eluted with elution volumes corresponding to a molecular weight of about 60 000¹² and of more than 100 000. Probably, the native enzyme in the cell consists of four subunits which can easily be dissociated into two dimers. For complete dissociation into monomers these dimers require dissociating agents like SDS or dithiothreitol. Free subunits have a high tendency to aggregate to dimers at least. The dimer shows already full enzymatic activity *in vitro*. The values for the molecular weights are in accordance with those obtained in the analytical ultracentrifuge for the presumed tetramer¹³. Blomquist gives values of 111 000 for agarose filtration and 106 000 for density gradient centrifugation of the tetramer¹⁴. 3 α -Hydroxysteroid dehydrogenase¹⁵ and 3-ketosteroid isomerase¹⁶ from *Pseudomonas* show similar tendencies to form oligomeric proteins *in vivo*.

Fig. 5 shows that increasing amounts of radioactive proteins from S-100 supernatant added to a constant amount of antiserum form antigen-anti-

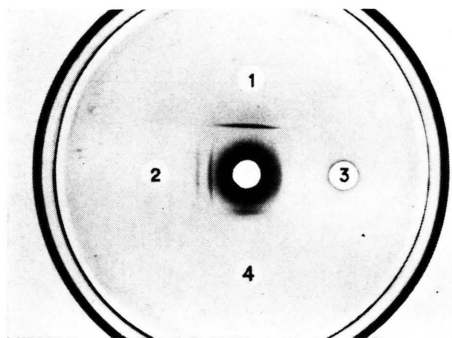


Fig. 1. Immunodiffusion experiment with commercially available 20 β -hydroxysteroid dehydrogenase, dissociated enzyme and S-100 supernatant from induced cells of *Streptomyces hydrogenans*. The center well contained 100 μ l of undiluted antiserum, the other wells were filled with 100 μ l of antigen solution of appropriate concentration of the enzyme as antigen (1) commercially available 20 β -hydroxysteroid dehydrogenase; (2) S-100 supernatant from induced cells of *Streptomyces*; (3) dissociated S-100 supernatant proteins of *Streptomyces*; (4) dissociated commercial 20 β -hydroxysteroid dehydrogenase.

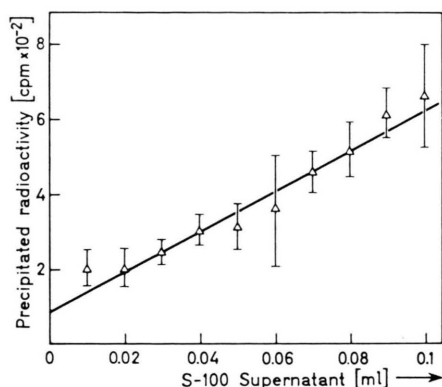


Fig. 5. Precipitation of ^3H -labelled 20 β -hydroxysteroid dehydrogenase of *Streptomyces hydrogenans* by an antiserum. Cells of *Streptomyces hydrogenans* were grown in the presence of ^3H -amino acid mix and a S-100 supernatant was prepared. The enzyme activity was 530 mU/mg protein. Increasing amounts of S-100 supernatant brought to the same volume by addition of buffer were mixed to an equal amount of antiserum. The values given are the mean of six determinations and the standard deviations.

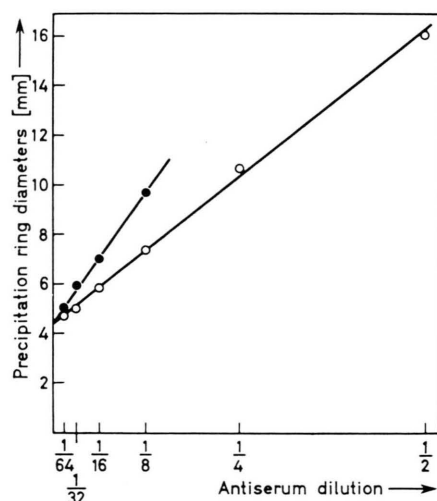


Fig. 6. Precipitation ring diameters in Mancini plates containing either 2% antiserum (○), or 1% antiserum (●) in 1% agarose were plotted versus antigen dilution.

body complexes, the radioactivities of which are proportional to the amount of radioactive proteins added. This relationship is significant on the 0.1% level and proves that there is an excess of antibody under the conditions used. Complete precipitation was obtained at 4 °C within one hour.

Fig. 6 demonstrates the relationship between the concentration of the antigen and the size of precipitation rings in two plates differing in the concentration of antiserum used for the assay. The resulting linears have correlation coefficients of $r = 0.997$ for the 2% antiserum plate and $r = 0.999$ for the 1% antiserum plate. The slopes of both linears differ by a factor of 1.91 ± 0.11 (theoretical value: 2).

Discussion

The experiments prove that 20 β -hydroxysteroid dehydrogenase can be quantified in the supernatant of the cell homogenate from *Streptomyces hydrogenans* by immunoprecipitation with high reproducibility. Moreover, the combined methods of immunodiffusion and gel electrophoresis permit a very exact determination of the size of the subunits of the enzyme. By precipitation of enzyme-forming polysomes with the specific antiserum a source of pure bacterial mRNA is available. Additionally the determination of half-lives of mRNA coding for 20 β -hydroxysteroid dehydrogenase is possible¹⁷. In this way further details of the mechanism of enzyme induction in *Streptomyces hydrogenans* can be clarified.

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