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Antibodies to Pseudomonas testosteroni 3-oxosteroid Δ^4-Δ^5-isomerase.

M. Ferrez[+], M. Renoir[+], A. Alfsen[°], E.E. Baulieu[+] and H. Weintraub[+]

+ Lab. Hormones, 94270 Bicêtre, France.
° Lab. des Etats Liés Moléculaires, 45, rue des Saints-Pères, 75006-Paris.

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Summary. Antibodies to Pseudomonas testosteroni isomerase have been generated in rabbits immunized with the isomerase. Complexes between the isomerase and antibodies were characterized by double immunodiffusion and by enzymatic assays. The titre of antibodies was determined. The enzymatic activity was totally abolished with increasing antibody concentrations. Antibody-isomerase complexes were inactive. Total immunological identity was observed between the unpurified extract and the pure enzyme either free or bound to a competitive inhibitor. However, the denatured enzyme did not interact with antibodies. Antigenic identy could not be detected between the bacterial antibodies and the bovine adrenocortical isomerase from microsomes.

Introduction. Pseudomonas testosteroni 3-oxosteroid Δ^4-Δ^5-isomerase (isomerase, EC 5.3.3.1), promotes the isomerization of $\Delta^{5(6)}$ and $\Delta^{5(10)}$-3-oxosteroid substrates to the corresponding Δ^4-reaction product (1). The enzyme has been purified to homogeneity by affinity chromatography (2,3). To the best of our knowledge, there has been no immunological information on the production or characterization of isomerase antibodies reported so far. This paper describes the preparation of crude and monospecific antibodies, as well as of $F(ab')_2$ and Fc' fragments. Crude isomerase antibodies allow to monitor the purity of the enzyme at different steps of the purification. Specific isomerase antibodies provide also a very sensitive probe of the native state of the isomerase. Cross-immunodiffusion experiments have been made between the bovine adrenocortical isomerase (4) and the bacterial isomerase antibodies. Indeed, it was important to know, if these two enzymes, which promote the same proton transfer reaction and exhibit the same substrate specificity, share also any degree of immunological kinship.

Materials and methods.

Chemicals. The steroid substrate (Δ^5-androstene-3,17-dione) and the competitive inhibitor (estradiol) were given by Roussel-Uclaf and found pure by thin-layer-chromatography. Agarose, Indubiose A-37 was from l'Industrie Française (Paris). DEAE-cellulose (DE-52) was from Whatman (England). Activated sepharose-4B and sephadex G-150 were from Pharmacia (Uppsala Sweden). Freund's complete and incomplete adjuvants were Difco products (Detroit, Michigan).

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Antigen. The isomerase was purified to homogeneity (3) : The enzyme was extracted with 4mM potassium phosphate buffer (pH 7.2) and 20 % (w/v) glycerol, from an acetonic powder of Pseudomonas testosteroni bacteria cells, grown with progesterone (1). The supernatant, after centrifugation, termed "crude S extract", 0.25 % pure (114 units/mg), was dialyzed against a 2 mM potassium phosphate buffer solution (pH 7.2), 20 % glycerol and subjected onto a DEAE column chromatography. The isomerase was eluted with a step-wise gradient (2-100 mM) of potassium phosphate buffer (pH 7.2). The active fractions were subjected to an affinity chromatography column : an estradiol-17β-acetate-glutathione-sepharose-4B adsorbent. The isomerase, selectively bound at high ionic strength, was eluted with a water-glycerol 20 % (w/v) solution. Then, the enzyme (70 % pure), was precipitated with solid $(NH_4)_2SO_4$ at 25 % (w/v) and dialyzed against 30 mM potassium phosphate buffer (pH 7.2) and 20 % (w/v) glycerol. The homogeneity of the isomerase was assessed by the previously published purity criteria (1,2,5-7) and by the additional observation :
- Only one sharp precipitin band appeared by immunoelectrophoresis against a crude isomerase rabbit antiserum, obtained after immunization with the crude "S" extract.

Generation of antibodies. Five rabbits (adult females, Fauve de Bourgogne breed), were immunized by injection under the hind toepads of 50-150 µg homogeous or non homogeneous isomerase, emulsified in 1 ml of Freund's complete adjuvant. One month later, rabbits received in a similar manner, a booster injection of the same amount of antigen, emulsified in 1 ml of Freund's incomplete adjuvant. Two weeks after the second injection and every following week, rabbits were deprived of food for 16-20 h and bled by puncture of the marginal ear vein. A subsequent injection was made, if necessary, between 4-6 months after the second injection.

Preparation of IgG : Partially purified IgG were obtained by $(NH_4)SO_4$ precipitations at 40 % (w/v) dissolution of precipitates in 150 mM NaCl, and dialysis against the same saline solution and then, against PBS, (100 mM sodium phosphate buffered saline, 150 mM NaCl, pH 7.2) for 48 h. The monospecific IgG "Ms", were prepared using an immunoadsorbent column chromatography : Pure isomerase was coupled to activated sepharose-4B, according to Axen et al., (8). The non specific IgG "Ns", were washed out with PBS, whereas the monospecific "Ms" IgG anti-isomerase, selectively adsorbed, were eluted with 3 M NaSCN (9), further dialyzed against PBS and then concentrated. Preimmune sera, or IgG were stored at - 38° C. - F(ab')2 and Fc' fragments were prepared by peptic digestion (at 37° C for 24 h) of IgG anti-isomerase (10). F(ab')2 and Fc' fragments were separated by a chromatography step on a sephadex G-150 column, dialyzed against PBS and concentrated.

Detection and characterization of isomerase-antibody complexes.
Antibody-isomerase complexes were investigated by double immunodiffusion (11) and by immunoelectrophoresis (12).
- Glass microscope slides were covered with a thin layer of 1.5 % (w/v) agarose in water and dried at 80°C. The dried slides were overlaid with 1 % (w/v) agarose in 10 mM veronal buffer, pH 8.35. Electrophoresis running conditions at 4°C were : 1 h at 6 mA and 250-260 mV per 3 slides connected together by the gel. The tank buffer was 40 mM veronal, pH 8.35. Bromophenol blue was used as a tracking dye. After 24 h immuno-diffusion at room temperature, gels were washed for 48 h in PBS, dried and stained for 30 min with a 2.5 % (w/v) solution of Coomassie Brilliant Blue "R-250" in methanol-acetic acid water (45.4:9.2;45.4), followed by background destaining in the same dye-free solvent.

Enzymatic detection. To follow the formation of complexes between the isomerase and antibodies, loss of the enzymatic activity was monitored as a func-

tion of time at a fixed isomerase concentration and with increasing antibody concentrations. Experiments were repeated with different enzyme concentrations. Incubations of the isomerase in 30 mM potassium phosphate buffer, pH 7.2, with antisera in PBS, were made in the silica cells, in a final volume of 3 ml, at 4 and 20° C. At different periods of time, from 15 s to 48 h (to find the time necessary for complexes to reach equilibrium), the enzymatic activity was measured under standard conditions (1). Dixon plots (13) were used for K_I determinations ; experiments were made in the presence of 10 % (v/v) methanol (14).

Proteins: For antisera, IgG and the unpurified isomerase, protein concentrations were determined according to Lowry (15). For the pure enzyme, the absorbancy coefficient at 280 nm was used (3).

Results and discussion

The isomerase antibodies were identified in sera of rabbits immunized either with the non homogeneous or pure isomerase. Figure 1, shows immunoelectrophoretic precipitation patterns of a non homogeneous isomerase "S" on one hand, and of a pure isomerase solution (I) on the other hand, against anti-isomerase crude IgG. With the unpurified isomerase "S", 3 precipitin bands were detected, while with the pure enzyme (I), a single band only was revealed giving evidence of IgG specificity and the purity of the enzyme. The monospecific of IgG was shown in figure 2, since a single band only was observed by immunoelectrophoresis with a crude "S" extract or a pure isomerase (I). A total immunological reaction was observed between the crude "S" extract, and the pure isomerase (I) by cross-immunoelectrophoresis experiments (figure 3). With the denatured enzyme precipitin bands were not detected, neither by immunoelectrophoresis nor by immunodiffusion.

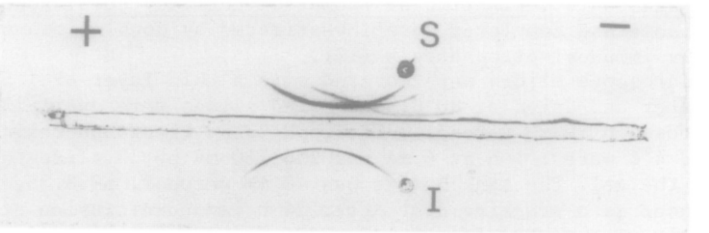

Figure 1. Existence of a specific anti-isomerase antiserum and demonstration of the purity of the enzyme by immunoelectrophoresis.
- 2 µl of the crude "S" extract (44 mg/ml) were placed in the upper-side well, whereas, 2 µl of the pure isomerase "I" (0.36 mg/ml) were added to the lower-side well. They were subjected to an electrophoretic migration. Then, 80 µl of the crude IgG preparation were immediately placed in the trough.

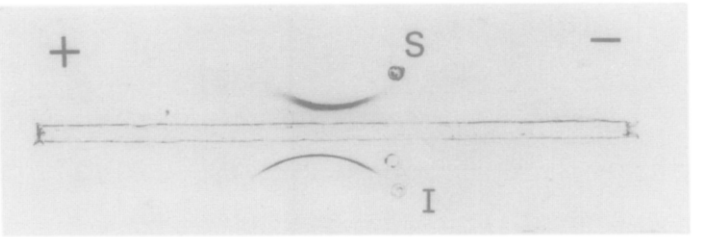

Figure 2. Immunoelectrophoretic evidence of monospecific anti-isomerase antibodies.
- 2 µl of the crude "S" extract (44 mg/ml) were placed in the upper-side well, whereas, 2 µl of the pure isomerase "I" (0.36 mg/ml), were added to the lower-side well. They were subjected to an electrophoretic migration. Then 80 µl of the IgG preparation (44 mg/ml), supposed monospecific, were placed in the trough.

The isomerase either free or bound to a competitive inhibitor, retains still the capacity to form complexes with antibodies. Indeed, precipitin bands appeared by immunodiffusion in agarose containing estradiol, incorporated into the gel (to avoid isomerase-estradiol complex dissociation, if any during immunodiffusion). Complete immunological identity was also detected by immunodiffusion between the isomerase either free or bound to a competitive inhibitor (estradiol). Suggesting that antibodies bind to the enzyme at a site(s), which differ(s) probably from the active binding site. The non competitive inhibition produced by antibodies on the enzymatic activity, as observed from Dixon plots (with a K_I value of 0.13 nM), could also arise

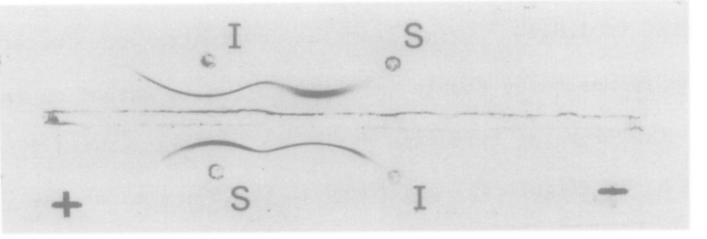

Figure 3. Immunological identity between the crude "S" extract and the purified isomerase "I", by cross-immunoelectrophoretic experiments.
- 2 µl of the crude "S" extract (44 mg/ml), were added to the right upper-side well, whereas, 2 µl of the purified isomerase "I" (0.36 mg/ml), were applied to the right lower-side well. They were subjected to an electrophoretic migration. Then, 2 µl of the same "S" extract and 2 µl of the same purified enzyme, were added into the left lower-side and left upper-side wells respectively. The right and left wells being separated by a distance of 1.8 cm. At the same time, 80 µl of antibodies, were added to the trough.

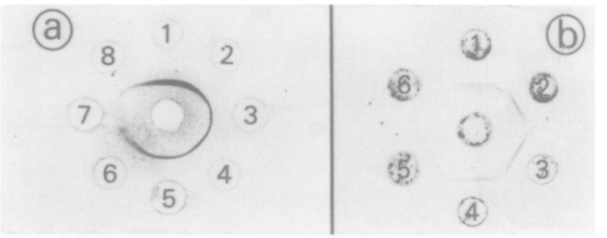

Figure 4a et b. Anti-isomerase antiserum efficiency (4a) and titre (4b) determined from immunodiffusion experiments.
Figure 4a. 10 µl of the IgG (44 mg/ml), were applied in the central well, whereas in the outer adjacent wells, proceeding clockwise, were added 10 µl of the following isomerase concentrations : (1) 154 µg/ml., (2) 77 µg/ml., (3) 51 µg/ml., (4) 38.5 µg/ml., (5) 31 g/ml., (6) 22 µg/ml., (7) 15.4 µg/ml., (8) 7.7 µg/ml.
Figure 4b. 10 µl of the pure isomerase "I" (9 µg/ml), were applied in the central well, whereas, 10 µl of two-fold serial dilutions of IgG, were added into the outer adjacent wells, beginnning at 12 o'clock (no dilution of IgG: (44 mg/ml) and proceeding clockwise.

from masking of the substrate binding site on the isomerase, leading to steric hindrance of the substrate to access to the catalytic site, owing to the large size of antibody molecules (150,000 d) compared with the isomerase (27,000 d). Conformational changes of the isomerase, or only of the active site area, caused by coupling with antibody : could also lead to the inhibition observed. The lowest concentration of the isomerase, which gave a visible precipitin band by immunodiffusion, against IgG (44 mg/ml), was found to be as little as 1200 units/ml, corresponding to an isomerase concentration of 7 µg/ml (figure 4a). However, it is possible to detect kinetically the presence of the isomerase, with only 1.10^{-2} unit/ml (3,1), corresponding to $1.8.10^{-4}$ µg/ml isomerase concentration. Therefore, detection of the isomerase by double immunodiffusion is rather an insensitive technique. The titre of isomerase antibodies was calculated from Ouchterlony precipitin bands (figure 4b) and found to be 290 µg/ml of the isomerase precipitated by 1 ml of antisera.

The isomerase activity was strongly decreased by incubation with increasing antibody concentrations (figure 5). Preimmune sera used as controls did not affect the enzymatic activity. These results demonstrate that antibody-isomerase complexes are inactive. Is the inactivation of the enzyme due to the

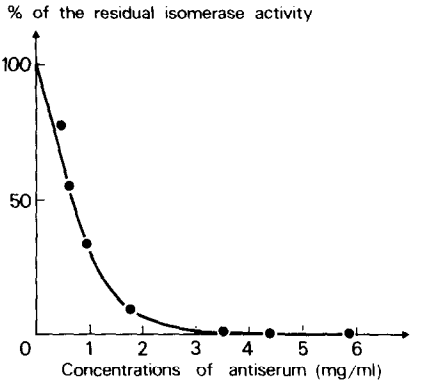

Figure 5. Inhibition of the isomerase activity by anti-isomerase antibodies. The isomerase (1.5 µg/ml), was incubated, in a final volume of 3 ml, in the silica cells, with increasing concentrations of antiserum, for 24 h at 4° C. Then the enzymatic activity was measured under standard conditions (1). The isomerization reactions were initiated by the addition of substrate.

specific interaction with antibodies, or does the inactivation of the isomerase occur because the enzyme can not react in a solid phase, the question remains open, since soluble isomerase-antibody complexes could not be evidenced. When cross-immuno reaction experiments were carried out between the mammalian microsomal isomerase solubilized in SDS 0.1 % w/v against crude or monospecific isomerase IgG from bacteria, precipitin bands appeared after a few hours at room temperature. However, preimmune rabbit IgG, produced identical precipitin bands. Another bovine detergent solubilized membraneous protein : the Folch-Pi apoprotein (16), gave similar results, whereas, bovine serum albumin, under the same solvent conditions, dit not form precipitin lines with the same IgG preparation. Therefore, $F(ab')_2$ and Fc' fragments have been prepared from IgG anti-isomerase. With the Fc' fragments, very visible precipitin lines appeared against the mammalian microsomal isomerase, wherease, with the bacterial enzyme precipitin bands were not detected. On the contrary, with the $F(ab')_2$ fragments precipitin bands appeared with the bacterial isomerase, while they were absent with the mammalian enzymatic fraction. The bacterial isomerase activity was strongly inhibited with increasing concentrations of $F(ab')_2$, whereas Fc' did not affect the enzymatic activity. These results, show the absence of any commun

antigenic determinants between these two enzymes. These findings are not surprising, Schultz et al. (17), have not detected either cross-immunological reactions between Pseudomonas testosteroni 3(17)β - hydroxysteroid dehydrogenase rabbit antibodies and human placental 17-β -estradiol dehydrogenase.

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