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AFFINITY CHROMATOGRAPHY OF 3α-HYDROXYSTEROID DEHYDROGENASE FROM *PSEUDOMONAS TESTOSTERONI*

USE OF *N,N*-DIMETHYLFORMAMIDE TO PREVENT HYDROPHOBIC INTERACTIONS BETWEEN THE ENZYME AND THE LIGAND

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Summary

1. The 3α-hydroxysteroid : NAD^+-oxidoreductase (EC 1.1.1.50) from *Pseudomonas testosteroni* (ATCC 11996) has been purified by affinity chromatography on Sepharose 4B using glycocholic acid as ligand covalently bound through its carboxyl group to the ethylenediamine spacer.

2. The attachement of the enzyme to the substrate-containing matrix is greatly enhanced by the presence of NAD^+ suggesting that this enzyme has a compulsory ordered mechanism where NAD^+ binds to the enzyme before the steroid.

3. A NAD^+-independent interaction between the enzyme and the ligand was also found. This interaction was mainly hydrophobic and interfered with the NAD^+-dependent binding. The NAD^+-independent interaction was reduced by *N,N*-dimethylformamide.

4. By using the affinity column in the presence of 10% *N,N*-dimethylformamide, highly purified enzyme, as judged from polyacrylamide gel electrophoresis, could be obtained in one step from crude bacterial extracts.

Introduction

The 3α-hydroxysteroid : NAD^+oxidoreductase (EC 1.1.1.50) from *Pseudomonas testosteroni* (ATCC 11996) [1,2] may be used for determination of

Abbreviations: glycocholic acid, 3α,7α,12α-trihydroxy-24-glycyl-5β-cholanic acid; deoxycholic acid, 3α,17α-dihydroxy-5β-cholanic acid; androsterone, 3α-hydroxy-5α-androstane-17-one; epiandosterone, 3β-hydroxy-5α-androstane-17-one; testosterone, 17β-hydroxy-4-androsten-3-one; GA-Sepharose, Glycocholic acid-Sepharose 4B.

3α-hydroxysteroids in various biological fluids [3—10]. It has been difficult to achieve a complete separation of the enzyme from the 3β- and 17β-hydroxysteroid dehydrogenases (EC 1.1.1.51) [3,11,12]. Affinity chromatography has been used for the purification of several enzymes having steroids as substrates [13], e.g. Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) and 3β- and 17β-hydroxysteroid dehydrogenases [14—16].

The present report describes for the first time the preparation of highly purified 3α-hydroxysteroid dehydrogenase by use of affinity chromatography. The purification system is based on the affinity of the enzyme towards glycocholic acid. When relatively impure preparations of the 3α-hydroxysteroid dehydrogenase are chromatographed on glycocholic acid-containing Sepharose 4B, the inactive proteins pass through and after washing the column, highly purified enzyme can be obtained. The binding of the enzyme to the column is, however, influenced by several factors, such as the presence of NAD^+ and hydrophobicity-peturbing agents like *N,N*-dimethylformamide.

Materials

NAD^+ was obtained from Sigma Chem. Co., St. Louis, Mo., U.S.A. CNBr-activated Sepharose 4B was purchased from Pharmacia AB, Uppsala, Sweden, the glycocholic acid and the deoxycholic acid from Steraloid, Pawling, N.J., U.S.A. and the androsterone and epiandrosterone from Ikapharm, Ramat Gan, Israel. Glyco[1-^{14}C]cholic acid (spec. act. 17 Ci/mol) was obtained from Radiochemical Centre, Amersham, England. *N,N*-Dimethylformamide was from Merck, Darmstadt, Germany. Buffer A was 0.02 M phosphate buffer (pH 7.0), 1 mM in EDTA and 0.1 mM in dithiothreitol (Cleland's reagent, Sigma Chem. Co.).

Methods

Enzyme source. Preparation of bacterial extracts from *Ps. testosteroni* (ATCC 11996), grown on a testosterone-containing medium, was performed as described earlier [12]. A crude enzyme preparation was prepared by heat treatment, $(NH_4)_2SO_4$ precipitation and gel filtration on Sephadex G-100 [12]. The activity measured with androsterone as substrate of such 3α-hydroxysteroid dehydrogenase preparations varied between 10 and 25 units/mg of protein. The amount of 3β- and 17β-hydroxysteroid dehydrogenases present was usually very low (less than 0.0005 units/mg when measured with epiandrosterone and testosterone as substrate). The crude enzyme preparation was stored frozen in buffer A with the addition of 20% glycerol.

Preparation of glycocholic acid-Sepharose 4B (GA-Sepharose). Glycocholic acid was attached covalently to Sepharose 4B [17,18] using ethylenediamine coupled to CNBr activated Sepharose 4B. *N*-Ethyl-*N*-(3-dimethylaminopropyl)-carbodiimide was used for the formation of a peptide bond between the ethylenediamine NH_2-group and the side-chain carboxyl group (glycine part) of the glycocholic acid molecule (Fig. 1).

The GA-Sepharose contained about 1 μmol of glycocholic acid per ml of wet gel as estimated by adding a small amount of glyco[1-^{14}C]cholic acid to

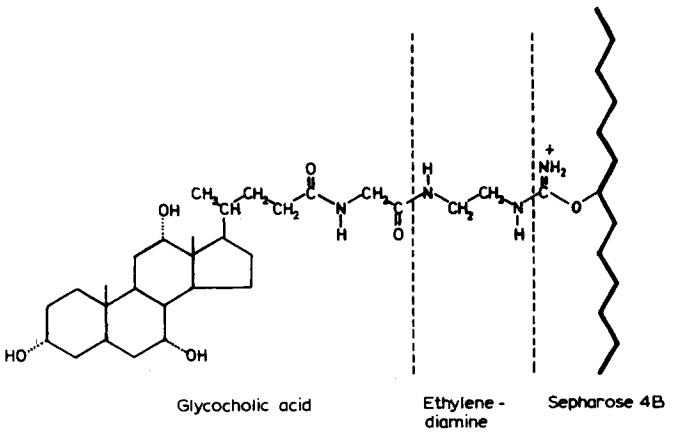

Fig. 1. The probable structure of the glycocholic acid ethylenediamine-Sepharose 4B complex.

the unlabelled glycocholic acid before attachment to the Sepharose 4B. The GA-Sepharose formed was washed with 50% *N,N*-dimethylformamide in water until no radioactivity could be detected in the eluate and then equilibrated with appropriate buffer before use. The experiments to be described were either performed with small columns containing approx. 3 ml of wet gel or as batch experiments in small plastic tubes where the enzyme solutions were added to preweighed amounts of wet GA-Sepharose, incubated (with occasional stirring) and centrifuged at $1000 \times g$ for 10 min. The enzyme activity was then determined in the supernatant using androsterone as substrate.

Polyacrylamide gel electrophoresis. Gels (7.5%) of pH 8.7 for analytical runs were prepared according to the general methods of Ornstein [19] and Davies [20] using an apparatus from Buchler Instruments (Fort Lee, N.J., U.S.A.). Runs were performed with 2.5 mA per tube and stopped when the marker dye had reached about 2 mm from the lower edge of the gel. The procedure for staining of proteins and detection of enzymatic activity in the gels (zymography) has been described in detail elsewhere [12].

Determination of protein in solutions was performed by use of the fluorescamine method of Weigele et al. [21,22].

Measurement of enzyme activity. Spectrophotometric assays were performed in Ultrocil (Hellma, Germany) cuvettes of 1 cm light path using a recording Zeiss spectrophotometer PQM 11 at 25°C. The assay system in a final volume of 3 ml contained: 200 μmol of $Na_4P_2O_7$ (pH 9.0), 0.5 μmol of NAD^+, 0.5 mg of bovine serum albumin and 0.02 ml of enzyme solution. The reaction was initiated by adding the steroid substrate in methanol (0.01 ml) to give a final concentration of 35 μM. Androsterone and deoxycholate were used as substrates. Epiandrosterone and testosterone were used for the detection of the 3β- and 17β-hydroxysteroid dehydrogenases. Absorbance at 340 nm was read against a blank containing all substances except the steroid. The reaction velocities were calculated from the slopes of the initial, linear part of the plot of absorbance against time. One unit of enzyme activity represents the amount that will convert 1 μmol of substrate per min at pH 9.0 and 25°C.

Results and Discussion

The effect of NAD$^+$

Partially purified 3α-hydroxysteroid dehydrogenase was applied to a small GA-Sepharose column, equilibrated with buffer A. The enzyme was immediately eluted by buffer A. Fig. 2 shows that the enzyme was slightly retarded by the column but that a significant tailing occurred.

It has been shown that the 3α-hydroxysteroid dehydrogenase probably exhibits an ordered bi-bi reaction mechanism, where the cofactor NAD$^+$ binds before the steroid substrate [23]. The low degree of retardation could be explained if NAD$^+$ must be present before the enzyme-ligand complex could be formed. The experiment described above was therefore repeated after adding 1 mM NAD$^+$ to the enzyme solution and to the equilibrating buffer. The results are given in Fig. 3. The arrow indicates when the elution with buffer A without NAD$^+$ was started. Most of the protein, but no enzyme, was eluted from the column when NAD$^+$ was present. On removing the NAD$^+$, however, the enzyme was eluted from the GA-Sepharose. The role of NAD$^+$ in the binding of the enzyme to the GA-Sepharose supports the results obtained from kinetics experiments with 3α-hydroxysteroid dehydrogenase [23]. Our results are also in accordance with those obtained with affinity chromatography used to study the mechanism of dehydrogenases such as the 3β- and 17β-hydroxysteroid dehydrogenases [16], and the lactic acid dehydrogenase [24]. During a series of batch experiments (results not shown) it was found that the binding of the enzyme in the presence of NAD$^+$ was not significantly influenced by changes in pH between 5.0 and 9.5 or temperatures from 4 to 37°C and that maximum binding was obtained after 90 min of incubation. Maximum binding of the enzyme to the GA-Sepharose was obtained at concentrations of NAD$^+$ higher than 0.1 mM (Table I). In order to avoid marginal conditions, 1 mM of NAD$^+$

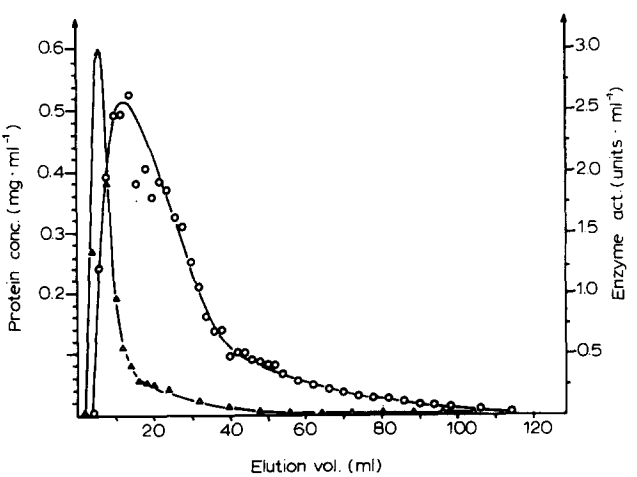

Fig. 2. Affinity chromatography of 3α-hydroxysteroid dehydrogenase on GA-Sepharose without NAD$^+$. A 2-ml solution of the enzyme containing 2.4 mg of protein/ml representing 48.0 units of activity (androsterone) was applied to a column having a bed volume of 3 ml at pH 7.0 and 25°C. Elution was performed at a rate of 10 ml/h. △———△, protein; ○———○, enzyme activity.

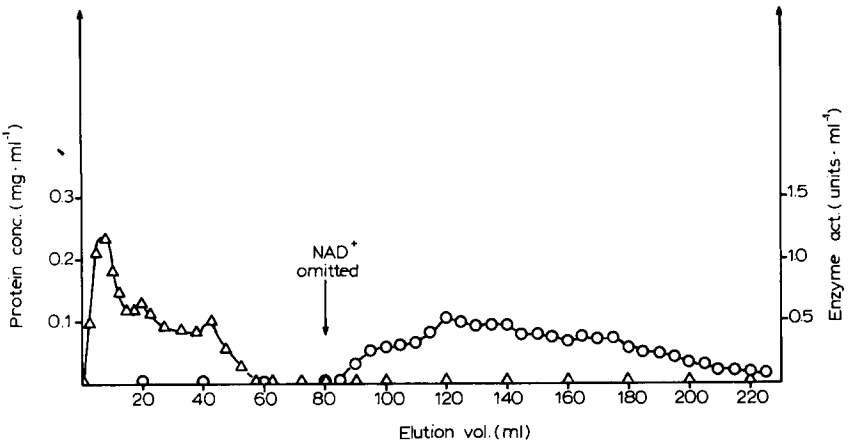

Fig. 3. Affinity chromatography of 3α-hydroxysteroid dehydrogenase on GA-Sepharose in the presence of 1 mM NAD^+. 2 ml of enzyme solution containing 42.3 units/ml and 2.8 mg of protein per ml were applied to the column (bed volume 3 ml) at pH 7.0 and 25°C. Elution was performed at a rate of 10 ml/h. The arrow indicates when the elution with buffer A without NAD^+ was started. △———△, protein; ○———○, enzyme activity. The content of protein in the fraction 80—220 ml was less than 5 μg/ml which is the lowest amount of protein detectable with the method used.

was used throughout all later experiments performed at pH 7.0 and 25°C where the NAD^+ is fairly stable.

Elution of the enzyme from the GA-Sepharose column

The experiments shown in Figs. 2 and 3 demonstrated a substantial tailing in the enzyme elution. This indicated that NAD^+-independent interaction between the column and the enzyme was also involved. Control experiments using either the matrix, Sepharose 4B (not CNBr-activated) or ethylenediamine-Sepharose 4B, showed that the NAD^+-independent interaction was only present when the glycocholic acid molecule was bound to the spacer. A main task was therefore to examine whether it was possible to inhibit this NAD^+-independent interaction without influencing the NAD^+-dependent binding of the enzyme to the GA-Sepharose.

The effect of electrolytes and hydrophobicity perturbing agents

The attachment forces between the steroid-ligand matrix and the enzyme may be of different types, both of hydrophilic and hydrophobic character. If hydrophilic, they could be counteracted by high ionic strength, while the hy-

TABLE I

THE EFFECT OF VARIOUS CONCENTRATIONS OF NAD^+ ON THE AFFINITY OF 3α-HYDROXYSTEROID DEHYDROGENASE TO GA-SEPHAROSE

The experiment was performed at pH 7.0 and at 25°C. Incubation time was 90 min.

GA-Sepharose added (mg)	0	36.9	35.1	36.2	36.3	35.3	35.9
Final concentration of NAD^+ (mM)	0	0	10^{-5}	10^{-3}	10^{-1}	1	10
Percent of enzyme activity remaining in supernatant	100	26	30	12	2	2	2

drophobic attraction could be inhibited by the presence of organic compounds like butanol or glycerol and increased by the presence of most electrolytes [25—28].

The following experiments were therefore performed: to three samples of enzyme were added 1 M KCl, 5% *n*-butanol and 20% glycerol, respectively, followed by pre-weighed amounts of GA-Sepharose. After 90 min of incubation at 25°C, the tubes were centrifuged and the enzyme activity in the supernatant was determined using androsterone as substrate. Controls without GA-Sepharose showed that the enzyme was not influenced by the three compounds during the incubation period. The results are given in Table II. 1 M KCl increased, while both 5% *n*-butanol or 20% glycerol decreased the NAD^+-independent binding of the enzyme to the GA-Sepharose. This indicated that hydrophobic attachment forces directed towards the glycocholic acid molecule itself were the most probable explanation for the NAD^+-independent adsorption of the enzyme to the ligand. Since the enzyme was destroyed by concentrations of *n*-butanol higher than 5%, and since the enzyme is inhibited by glycerol [23], another hydrophobicity perturbing agent, i.e. *N,N*-dimethylformamide, was tried. To samples of the enzyme solution were added increasing amounts of *N,N*-dimethylformamide up to a final concentration of 25% in order to investigate the possible influence of this agent on the enzyme activity. The enzyme remained fully active for 1 week at room temperature in buffer containing 25% of *N,N*-dimethylformamide. To two series of enzyme samples, one with NAD^+ present and one without, preweighed amounts of GA-Sepharose were added, followed by varying amounts of *N,N*-dimethylformamide. After 90 min of incubation at pH 7.0 and 25°C, the tubes were centrifuged and samples withdrawn for determination of enzyme activity with androsterone as substrate. The results are shown in Fig. 4. When the concentration of *N,N*-dimethylformamide reached 10%, no adsorption of enzyme to the GA-Sepharose could be detected if NAD^+ was not present, and the NAD^+-dependent attachment was hardly affected by the hydrophobic agent.

In order to reduce the hydrophobic interaction between ligand and the enzyme, the column experiment was repeated in the presence of 10% *N,N*-

TABLE II

THE INFLUENCE OF KCl, GLYCEROL AND *n*-BUTANOL ON THE NAD^+-INDEPENDENT ADSORPTION OF 3α-HYDROXYSTEROID DEHYDROGENASE TO GA-SEPHAROSE

To 2-ml samples of enzyme, GA-Sepharose was added in the presence of the given compounds at pH 7.0. After 90 min of incubation at 25°C, the tubes were centrifuged and the activity in the supernatant determined using androsterone as substrate. None of the compounds showed any influence on the initial activity of the enzyme at the concentrations given.

GA-Sepharose added (mg)	—	36.8	36.5	37.0	37.2	37.0
KCl (M)	—	1	—	—	—	—
Glycerol (%)	—	—	20	—	—	—
n-Butanol (%)	—	—	—	5	—	—
NAD^+ (mM)	—	—	—	—	1	—
Percent of enzyme activity remaining in supernatant	100	13	68	70	2	48

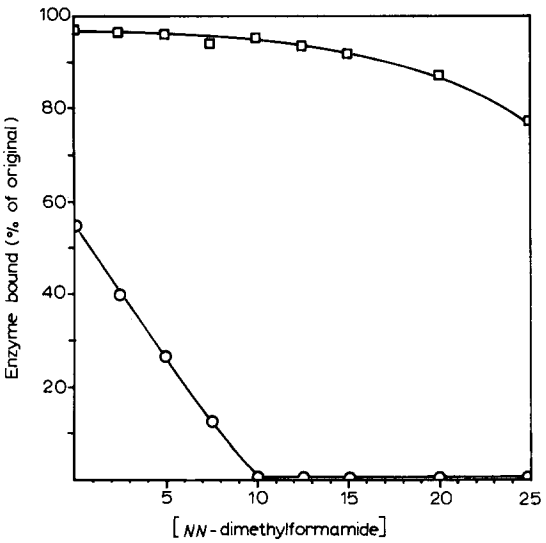

Fig. 4. The effect of *N,N*-dimethylformamide on the NAD^+-dependent (□———□) and the NAD^+-independent (○———○) affinity of 3α-hydroxysteroid dehydrogenase towards GA-Sepharose. Different samples of enzyme to which equal amounts of GA-Sepharose have been added, were divided into two series (with and without NAD^+ added) and then various amounts of *N,N*-dimethylformamide were added. After 90 min of incubation at 25°C, the tubes were centrifuged and the enzyme activity in the supernatant determined with androsterone as substrate.

dimethylformamide. The result obtained, is shown in Fig. 5. The tailing tendency, although detectable, is significantly decreased compared with the results given in Fig. 3. The enzyme eluted was free of any detectable non-enzymatic

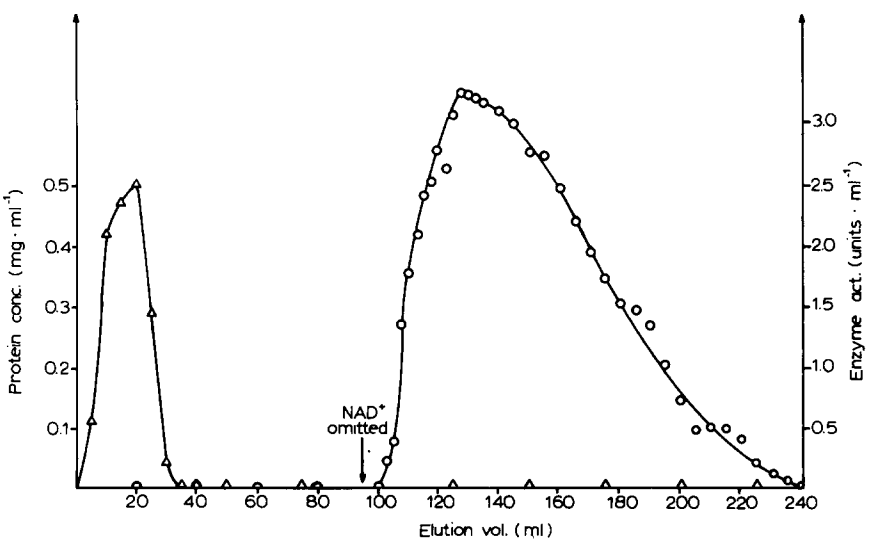

Fig. 5. Affinity chromatography of 3α-hydroxysteroid dehydrogenase on GA-Sepharose in the presence of 10% *N,N*-dimethylformamide. 20 ml of enzyme solution containing 14.1 mg of protein, representing approx. 200 units of activity (androsterone), were applied to a column (bed volume 3 ml) at pH 7.0 at 25°C. The buffer used contained 1 mM NAD^+. After washing of the column with this buffer, elution was started as indicated by the arrow with buffer without NAD^+ at the rate of 10 ml/h. △———△, protein; ○———○, enzyme activity.

components as judged from disc electrophoresis in polyacrylamide gels as shown in Fig. 6. The enzyme had a specific activity of about 300 units mg and was thus purified about 20 times in this single step. From Fig. 6 it is also seen that possibly five isoenzymes, and not three as reported earlier [29], seem to be present. The two most acid species apparently being present in very low concentrations, may, however, be artifacts due to deamidation of the other isoenzymes.

As high concentrations of organic compounds may precipitate water-soluble proteins, we tested whether treatment with *N,N*-dimethylformamide and subsequent affinity chromatography could be used in making a highly purified enzyme preparation from a crude bacterial extract in one step. To 25 ml of a crude bacterial extract (about 275 mg of protein and 220 units of 3α-hydroxysteroid dehydrogenase activity) was added *N,N*-dimethylformamide to give a final concentration of 10%. After incubation for 12 h at room temperature, precipitated proteins were removed by centrifugation. NAD^+ was added to the clear, yellowish supernatant to give a final concentration of 1 mM and applied to a GA-Sepharose column. The column was washed with several volumes of NAD^+- and *N,N*-dimethylformamide-containing buffer before NAD^+ was omitted from the buffer in order to elute the enzyme. From Table III it can be seen that approx. 33% of the activity present in the original extract was eluted. The specific activity of the 3α-hydroxysteroid dehydrogenase had increased about 300 times. Polyacrylamide disc electrophoreses as described above revealed that the eluate contained only proteins with 3α-hydroxysteroid dehydrogenase activity. A pattern identical to that shown in Fig. 6 was obtained.

Table III also shows that 3β- and 17β-hydroxysteroid dehydrogenases present in the crude extract are inhibited or denatured by the presence of 10% *N,N*-dimethylformamide and that they are not present in the highly purified preparation of the 3α-hydroxysteroid dehydrogenase.

Hydrophobic interactions are reported to cause problems in many affinity chromatography systems [30]. It has been shown that some affinity chroma-

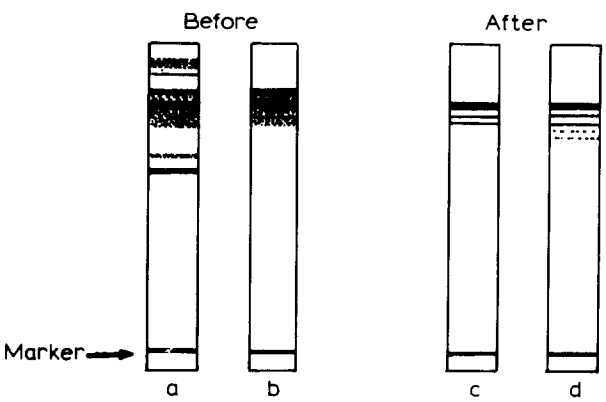

Fig. 6. Polyacrylamide gel electophoresis of 3α-hydroxysteroid dehydrogenase before and after affinity chromatography on GA-Sepharose in the presence of 10% *N,N*-dimethylformamide. Gels a and c were stained for protein, and gels b and d developed by zymography using androsterone as substrate. About 100 μg of protein were applied on each gel.

TABLE III

PURIFICATION OF 3α-HYDROXYSTEROID DEHYDROGENASE PRESENT IN CRUDE EXTRACT OF *Ps. TESTOSTERONI* BY USE OF AFFINITY CHROMATOGRAPHY ON GA-SEPHAROSE

25 ml of the extract containing about 25 mg of protein and about 220 units of enzyme activity were applied to the column in the presence of 10% *N,N*-dimethylformamide and 1 mM NAD^+ in buffer A (pH 7.0). Some data for the 3β- and 17β-hydroxysteroid dehydrogenases are also given. For details, see Methods.

	3α-hydroxysteroid dehydrogenase			3β- and 17β-hydroxysteroid dehydrogenases	
	Spec. act. (munits/mg)	Recovery (%)	Purification (fold)	Spec. act. (munits/mg)	Recovery (%)
Crude extract	990	100	—	375	100
After incubation 12 h in 10% *N,N*-dimethylformamide	935	71	—	18	3.5
Eluted from the GA-Sepharose	300 000	33	300	—	—

tography systems thought to be biospecific are actually based on unspecific hydrophobic interactions between the protein and the hydrophobic spacer holding the ligand out from the matrix. It may be possible to avoid such unwanted hydrophobic interactions by substituting hydrophobic spacers with more hydrophilic ones [31]. However, in our case, as may be the case in many other systems, it is not the spacer, but rather the ligand itself which causes the unwanted hydrophobic interactions. Obviously it is usually not possible to substitute ligands in the same way as it is possible to replace spacers. This illustrates the need for more general ways of inhibiting hydrophobic interactions. Here hydrophobic cosolvents such as *N,N*-dimethylformamide or ethylene glycol may prove to be general tools. The use of ethylene glycol as a weakener of hydrophobic interactions is already well known [25,26]. The present paper illustrates how *N,N*-dimethylformamide inhibits unwanted hydrophobic interactions, and thereby permitting both the adsorption of the 3α-hydroxysteroid dehydrogenase to the column and the desorption from it to be biospecific.

In conclusion, the results obtained in this study indicate that 3α-hydroxysteroid dehydrogenase in absence of NAD^+ forms a complex with GA-Sepharose which is essentially based on hydrophobic binding forces. The strength of such bonds can be reduced or even eliminated by the presence of hydrophobicity-perturbing agents such as *N,N*-dimethylformamide. When NAD^+ is present, some conformational changes seem to occur with the enzyme molecule, reducing the importance of hydrophobic bonds in the complex formation between enzyme and ligand. Thus, affinity chromatography, besides being superior to most other purification methods, represents a very interesting tool in the general study of steroid protein interactions.

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