AFFINITY PARTITIONING OF STEROID-BINDING PROTEINS. THE USE OF POLYETHYLENE OXIDE-BOUND ESTRADIOL FOR PURIFYING $\Delta_{5\rightarrow4}$ 3-OXOSTEROID ISOMERASE

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1. Introduction

The purification of steroid high-affinity binding proteins such as 'transport' plasma proteins and intracellular 'receptors', encounters severe difficulties. They are rather unstable, and present only in low concentration in most soluble extracts [1]. The high affinity of these proteins for their ligands makes possible their purification by affinity chromatography [2]. However, the results obtained so far have not yet been very satisfactory in most cases, essentially because of contamination by polymer-adsorbed ligand, non specific interactions of the bulk of proteins with the matrix, and unstability of the steroid-binding proteins during the elution from biospecific adsorbants [3].

The use of polymer aqueous two-phase systems for the partition of various macromolecules has been thoroughly investigated by Albertson [4] and especially the polyethylene oxide–dextran duplex. The latter system has been used successfully to study steroid-binding plasma proteins [5] and the interactions of steroid receptors with DNA [6]. Unfortunately, for purification purposes, the efficiency of this technique is hampered by its lack of specificity. However, if a ligand with a high affinity for the steroid-binding protein is covalently-bound to one of the polymers of the phase system the partition technique is expected to acquire the specificity it otherwise lacks [7–10]. Since this polymer can be almost completely restricted to one phase, it should be possible, by transforming it into a 'macroligand', to attract the protein to be purified into its phase, whereas the other proteins should be directed as completely as possible to the other phase by means of pH or salts effects.

In order to purify high-affinity estrogen-binding proteins, we covalently coupled an estradiol derivative [3] to polyethylene oxide. We report studies of the affinity material thus obtained, with $\Delta_{5\rightarrow4}$ 3-oxosteroid isomerase (EC 5.3.3.1) (isomerase) extracted from *Pseudomonas Testosteroni.* This enzyme has been already used as a model of steroid–protein interaction [11], and estradiol is a competitive inhibitor of the Δ_5-androstene-3,17-dione isomerization, with $K_i \sim 1$ μM under usual conditions [12].

2. Materials and methods

2.1. *Materials*

Polyethylene oxide (PEO 6000, mol.wt. 6000) was obtained from Roth, W. Germany. Dextran T80 (80 000) was supplied by Fluka, Switzerland. 19-Nortestosterone from which was prepared the ligand, and the isomerase-containing acetonic powder were obtained from Roussel-Uclaf, France.

2.2. *Soluble extracts from the acetonic powder*

The cultures of *Pseudomonas Testosteroni* and the preparation of the acetonic powder were prepared according to Boyer et al. [13]. Isomerase was

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extracted in adding 100 ml of Tris-HCl buffer 0.02 M, pH 7.2, at 20°C to 10 g of the powder [14,15]. After 3 h stirring in the cold room and high speed centrifugation for 1 h, the supernatant was collected. The activity of isomerase, measured with Δ_5-androstene-3,17-dione as substrate, was 700 u/ml. Total protein concentration, measured by a modified Lowry method, involving preliminary precipitation by trichloroacetic acid [4], was 12 mg/ml. This extract is indexed S.

2.3. *Two-phase systems and partition coefficients*

Two-phase systems (volume ratio 1/1) were prepared by mixing together phosphate 0.03 M buffer solutions of PEO 6000 (30% w/w) and Dextran T 80 (20% w/w) with desired amounts of polyethylene oxide-bound estradiol (PEO-E_{II}, see latter), KCl, buffer and extract S, so that the final concentrations of the polymers in the mixture are 7% w/w and 12% w/w, for PEO and dextran, respectively.

After 30 inversions and 1/2 h decantation, the phases are separated by centrifugation at approx. 1500 *g* for 10 min. Aliquots of both phases are collected and assayed for isomerase activity and proteins. Partition coefficients are calculated from the enzymatic activity and protein concentration ratios between the top and the bottom phases. All experiments were carried out at room temperature.

3. Results

3.1. *Synthesis of polyethylene oxide-bound estradiol*

When not granted by Roussel-Uclaf, estradiol-7α-butyric acid (R in fig.1) was synthetised according to Truong et al [3]. To bind this derivative to polyethylene oxide, the terminal hydroxyl functions of this polymer were initially reacted with thionyl chloride to yield the dichloro derivative. The latter was subsequently transformed by reaction with 1,3-diaminopropane, and finally coupled to estradiol-7α-butyric acid by a mixed anhydride method involving amide bond formation [16]. The polyethylene oxide-bound estradiol, PEO-E_{II} thus obtained (fig.1) was purified by extensive washing with ether in a Soxhlet-type liquid–liquid extraction apparatus. Since the macroligand is water-soluble, each step of the synthesis was easily controlled by microanalysis and spectrophotometric measurements. This point is

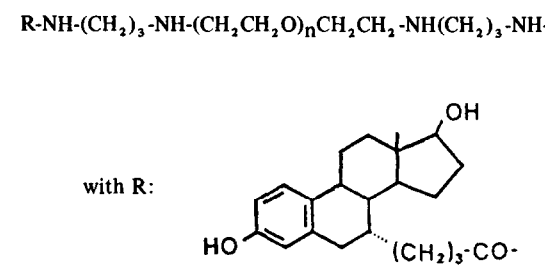

Fig.1. Polyethylene oxide-bound estradiol (PEO-E_{II}). The two forms PEO-R and PEO-2R are probably both present in the material (PEO-E_{II}) used in experiments described in this paper (see text).

of interest, knowing the major problems brought up, in affinity chromatography, by free-ligand contamination and the difficulty to evaluate it [17]. PEO-E_{II} thus obtained, contained about 2.2×10^{-4} mol of estradiol per g, corresponding to 1.32 mol of estradiol per mol of polyethylene oxide. Its K_i for isomerase, measured according to Dixon [18], was found to be approx. 5 μM in phosphate buffer, pH 7.0.

3.2. *Influence of PEO-E_{II} on partition*

PEO and PEO-E_{II} behave similarly in terms of partition between the 2 phases and it turns out, from

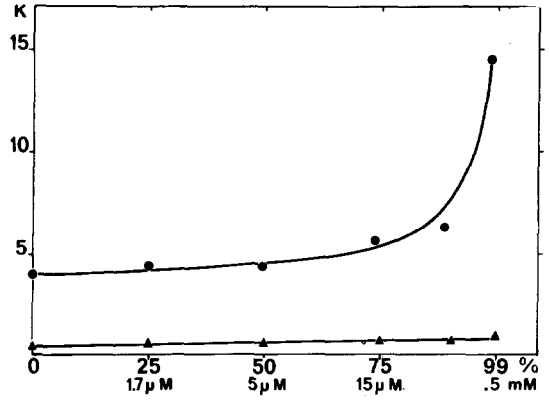

Fig.2. Partition coefficient (*K*) of isomerase (•) and total proteins (▲) as a function of the theoretical percentage of complex formed by affinity between isomerase and PEO-E_{II} and of the molar concentration of PEO-E_{II} present in the system. (PEO 6000, 7% w/w, dextran T80, 12% w/w, phosphate buffer 0.03 M pH 7.0, PEO-E_{II} up to 12 mg and extract S 0.4 ml) (total weight 4g).

the phase diagram [4] that more than 95% of the macroligand should be restricted to the top phase. Then, if isomerase forms a high-affinity complex with PEO-E_{II}, it should be progressively shifted to the PEO-rich top phase as the amount of PEO-E_{II} is increased. Furthermore, it follows that, with a concentration of macroligand enough to insure that 99% of isomerase is bound, $\sim$ 95% of the enzyme should be in the top phase.

Fig.2 represents the variation of the partition coefficients of isomerase and total proteins, plotted as a function of the concentration of PEO-E_{II}, added to the system and of the theoretical percentage of complex formed between isomerase and PEO-E_{II}. This percentage is calculated from the law of mass action, using the concentration of PEO-E_{II} and the K_i determined before. As the amount of PEO-E_{II} is increased, more enzyme is found in the top phase. When 99% of isomerase is expected to be complexed, its partition coefficient is 15.4, corresponding to about 95% in the top phase. Conversely, total proteins are only slightly affected by increasing amounts of PEO-E_{II}, their partition coefficient evolving from 0.4 to 0.5. It is probable that some of the proteins exhibiting low-affinity interaction with estradiol are attracted to some extent into the top phase.

From these results, it appears that the difference between partition coefficients of isomerase and of other proteins in the PEO-E_{II}-containing system might be useful for the purification of the enzyme. Thus, pH and salt effects were studied in order to improve the separation of isomerase from the bulk of proteins.

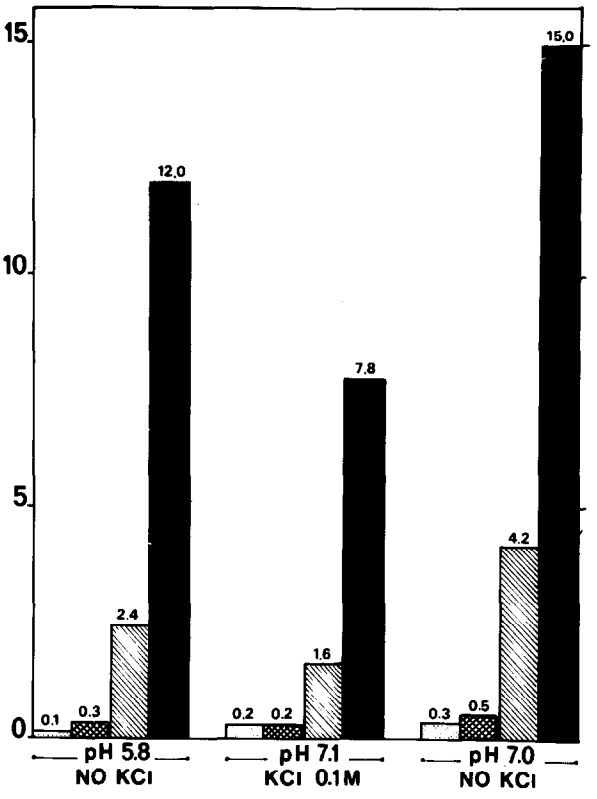

Fig.3. Effect of PEO-E_{II} 0.5 mM on the partition coefficient of isomerase and total proteins under various pH and KCl conditions. Partition coefficients of proteins: dotted columns, no PEO-E_{II}; cross-hatched columns, with PEO-E_{II}. Partition coefficient of isomerase: single-hatched columns, no PEO-E_{II}; solid columns, with PEO-E_{II}.

3.3. *Partial purification of isomerase*

3.3.1. pH effects on partition coefficients of isomerase and proteins

Isomerase is stable between pH 5.8 and 7.8 for at least 12 h at room temperature. Since the p*I* of isomerase is 4.75 [19,20], it is logically observed [4] that its partition coefficient increases with pH from $\sim$2.5 to 7.0. Between the same 5.8–7.8 pH range, the partition coefficient of total proteins increases from 0.1 to 0.5.

When PEO-E_{II} 0.5 mM is added to the pH 5.8 system, the partition coefficient of isomerase is increased from 2.4 to 12, while the one of total proteins shifts from 0.1 to 0.2 (fig.3).

3.3.2. Salt effects on partition coefficients of isomerase and proteins

Influence of salts on partition in PEO–dextran systems has been widely studied by Johansson [21]. Changes depend on the global net charge of proteins and on the type of ions. Potassium chloride has been tested at pH 7.0. The partition coefficient of isomerase decreases when KCl concentration increases up to 0.1–0.2 M from $\sim$ 4 down to $\sim$ 1. However, when KCl concentration increases more, a salting-in effect reestablished the partition coefficient to approx 3 at 0.6 M KCl concentration. The partition coefficient of the total proteins does not vary very much, but follow the same trend within a range of 0.4–0.3, the minimum being reached by 0.1 to 0.2 M KCl.

Table 1

	Isomerase activity (u/ml)	Proteins (mg/ml)	Isomerase specific activity (u/mg of proteins)	Purification ratio	Partition coefficient of proteins	Partition coefficient of isomerase
extract S 1 ml	700	12	58			
T_1	206	0.75	274	4.7		
					0.3	12.5
B_1	16.5	2.3	7			
T_2	198	0.33	600	10.3		
					0.8	12.3
B_2	16.1	0.41	39			
T_3	195	0.11	1770	30		
					0.8	11.8
B_3	16.5	0.13	127			
T_4	176	0.07	2510	45		
					1.2	11.9
B_4	14.7	0.06	245			

Four-step extraction with bottom phase in phosphate buffer 0.03 M, pH 5.8. Total weight: 4 g, volume ratio $\sim$ 1/1, extract S 1 ml, concentration of PEO-E_{II} 0.5 mM. (T and B respectively stand for top and bottom phases).

When PEO-E_{II} 0.5 mM is added to the 0.1 M KCl-containing system, the partition coefficient of isomerase shifts from $\sim$ 2 to 7.8, while proteins remain almost unaffected (fig.3).

3.3.3. Multi-step extractions

After a first partition as described in Materials and methods, the top-phase is collected and mixed with an equal weight of fresh bottom phase. Then, after mixing by inversions, the separation follows. This procedure is repeated 3 times with a new bottom phase, and protein and enzymatic determinations are performed systematically. Two series of experiments were conducted with 2 selected combinations of pH and salt, pH 5.8 in absence of salt, and pH 7.1 in presence of 0.1 M KCl (tables 1 and 2). In both cases, the first partition leads to loose 40% of isomerase. This is due to precipitation of proteins at the interface after the first partition from extract S. (With a further-purified extract, no precipitation occurs and isomerase activity is fully recovered in the two phases). However, in the following stages of both series of multi-step partitions, it can be seen that less than 20% of isomerase is lost in the next 3 steps. It can be also observed that the partition coefficient of isomerase is remarkably reproducible, step after step, and with the same values for the partition in presence of extract S (first stage) as in the presence of low amounts of proteins (following steps). On the other hand, for the total proteins, the partition coefficient, even it is the same as in results reported in sections 3.3.1 and 3.3.2 for the first partition, does not remain constant and increases up to 1.2 while the steps are successively performed. This means that a fractionation of proteins takes place during this process, and that the system is progressively enriched in proteins favouring the top phase. It has been postulated that these proteins have a positive net charge under the experimental conditions [4], and therefore, to attract them into the bottom-phase, it became logical to use a higher pH and to decrease the ionic strength.

A new experimental series was then performed. The two first extractions were carried out at pH 7.1, 0.1 M KCl, as in the experiment reported in table 2. Then, for the 3 next partitions, the top phase is exposed to a new fresh bottom phase, pH 7.8 and not

Table 2

	Isomerase activity (u/ml)	Proteins (mg/ml)	Isomerase specific activity (u/mg of proteins)	Purification ratio	Partition coefficient of proteins	Partition coefficient of isomerase
extract S	700	12	58			
T_1	200	0.75	266	4.5	0.3	6.8
B_1	29.4	2.82	10.5			
T_2	173	0.36	480	8.3	0.8	7.2
B_2	24	0.43	56			
T_3	171	0.20	855	14.7	1.3	7.8
B_3	22	0.15	147			
T_4	154	0.09	1710	30	1.3	8.1
B_4	19	0.07	270			

Four-step extraction with bottom phase in phosphate buffer 0.03 M, pH 7.1, KCl, 0.1 M. Total weight: 4 g, volume ratio $\sim$ 1/1, extract S 1 ml, concentration of PEO-E_{II} 0.5 mM. PEO and dextran concentrations as usual).

containing KCl (same concentration of dextran as usual). Therefore, in steps 3, 4 and 5, the system acquires progressively higher pH and lower ionic strength, with approx. pH 7.7 and KCl 0.01 M at the end. The results after the 5 steps are presented in table 3. The partition coefficient of isomerase has reached 25 and, quite interestingly, the partition coefficient of proteins has gone down from 1.3 to 0.4, confirming the working hypothesis. Finally, such a succession of pH and salt combinations has led to a purification of about 170, indicating that the changes in experimental conditions may produce substantial modification in the partition of proteins and therefore in the yield of purification.

In conclusion, the data which has been presented indicate the successful synthesis and use of an affinity material for estradiol-binding proteins which may be used for purification purposes. The versatility of the system is indicated by the salt and pH effects which can be adapted to any particular case, taking into

Table 3

	Isomerase activity (u/ml)	Proteins (mg/ml)	Isomerase specific activity (u/mg of proteins)	Purification ratio	Partition coefficient of proteins	Partition coefficient of isomerase
extract S 1 ml	700	12	58			
T''_5	150	0.015	10 000	170	0.4	25
B''_5	6	0.042	143			

Fifth extraction of the five-step procedure with pH varying from pH 7.1 to $\sim$ 7.7 and KCl from 0.1 M to $\sim$ 0.01 M. Total weight of the phases: 4 g, volume ratio, 1/1, extract S 1 ml. Concentration of PEO-E_{II} 0.5 mM, PEO and dextran concentrations as usual.

account the characteristics not only of the binding protein and of its complex with the ligands, but also the properties of other proteins contained in the biological extract.

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