Physico-chemical properties of the hydroxysterol binding protein of human lymphocyte cytosol

Effects of high salt concentrations and molybdate

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Side chain-hydroxylated derivatives of cholesterol (OH sterol) inhibiting lymphoblastic transformation bind with high affinity and specificity to a hydroxysterol binding protein (OHSBP) in the cytosol of human lymphocytes. These binding properties of OHSBP suggested some analogies with that of steroid hormone receptors. The observation of a nuclear binding of 25-OH[^{3}H]cholesterol prompted us to apply to the cytosolic OH sterol–OHSBP complex the physico-chemical treatments known to 'activate' the steroid hormone receptors. A change of sedimentation coefficient from 8.3 to 4.3 S was observed in hypertonic buffer (0.4 M KCl) but the resulting 4.3 S complex dissociates easily whereas the 'native' 8.3 S form does not. Moreover, molybdate did not prevent the 8.3⟶4.3 S transformation induced by KCl and neither ammonium sulfate precipitation nor increasing temperature had any effect on the sedimentation coefficient of the 8.3 S complex. Thus, several physico-chemical features differentiate the OH sterol–OHSBP complex from steroid hormone receptors.

Hydroxysterol *Human lymphocyte* *Specific binding protein* *Cell division*

1. INTRODUCTION

We showed previously that human lymphoblastic transformation was specifically inhibited by several side chain-hydroxylated cholesterol derivatives [1,2]. This effect does not seem to be explained solely by the HMG-CoA reductase inhibition observed with these compounds [2]. We have recently been able to demonstrate the presence of a cytosolic protein in human lymphocytes which binds specifically side chain-hydroxylated cholesterol derivatives [3]. This protein was characterized by a sedimentation coefficient of 8 S, limited binding capacity, high affinity (demonstrated by indirect probes) and strict specificity for the side chain-hydroxylated sterols selectively inhibiting lymphoblastic transformation.

Here, we report that substantial amounts of labelled 25-OH cholesterol specifically bound to proteins can be extracted from the nuclei of lymphocytes incubated with 25-OH [^{3}H]cholesterol. This prompted us to investigate the ability of the

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Abbreviations: HMG-CoA, hydroxymethylglutaryl coenzyme A; OH sterol, hydroxysterol; OH-SBP, hydroxysterol binding protein; PBS, phosphate-buffered saline; cholesterol, cholest-5-ene-3β-ol; 25-hydroxycholesterol, cholest-5-ene-3β,25-diol; 23-hydroxycholesterol, cholest-5-ene-3β,23-diol; 25-hydroxycholecalciferol, 9,10-seco-5,7,10(19)-cholestatriene-3β,25-diol; 1,25-dihydroxycholecalciferol, 9,10-seco-5,7,10(19)-cholestatriene-1α,3β,25-triol; estradiol, 1,3,5(10)-estratriene-3,17β-diol; progesterone, 4-pregnene-3,20-dione; dexamethasone, 9α-fluoro-16β-methyl-11α,17β,21-trihydroxypregna-1,4-diene-3,20-dione

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cytosolic hydroxysterol–hydroxysterol binding protein complex (OH sterol–OHSBP) to be activated by analogy with that of the steroid hormone-binding protein complex. Activation of a steroid hormone receptor is generally defined as structural changes of the binding protein in the presence of high salt concentrations or increasing temperature; these modifications are accompanied by a decrease in the dissociation rate of the ligand and an increased ability to bind to DNA or other polyanions. Such is the case for glucocorticoid or estradiol receptors [4–10]. Here we report the effects of temperature, high salt concentrations and molybdate on the sedimentation coefficient and the rate of dissociation of the tritiated 25-OH cholesterol–protein complex.

2. MATERIALS AND METHODS

2.1. *Materials*

25-Hydroxy-[26,27-^{3}H]cholesterol (87 Ci/mmol) (New England Nuclear) was checked for radioactive purity by thin-layer chromatography [two successive elutions in chloroform–methanol–ethanol (80:2:1.5 then 80:1:0.5, v/v)].

Unlabelled sterols: 25-hydroxycholesterol was kindly donated by Roussel-Uclaf (Romainville, France) and 23(*R*)- and 23(*S*)-hydroxycholesterol by Dr J.E. van Lier (Sherbroke, Canada). 25-Hydroxy- and 1,25-dihydroxycholecalciferol were from Hofmann La Roche (Basel, Switzerland), dexamethasone from Sigma, progesterone and estradiol from Steraloids.

The sources of other reagents were: DL-saccharose, KCl, Carlo Erba; Tris, proteinase K, Merck; molybdic acid (sodium salt), aprotinin (5000 kIU/ml), DNase I (2000 IU/mg), Sigma; Dextran T70, Ficoll 400, Pharmacia; charcoal Norit A, Prolabo; sodium metrizoate, Nyeggard.

Culture medium was sterile RPMI 1640 (Gibco) buffered with 40 mM Hepes (Sigma), pH 7.4, supplemented with gentamycin sulfate (142 mg/l) (Unilabo).

Buffers: PBS – 10 mM Na phosphate buffer (pH 7.4), containing 0.15 M NaCl; buffer A – 10 mM Tris (pH 7.4), 1 mM $MgCl_2$, 1 mM $CaCl_2$; buffer AM – buffer A + 20 mM sodium molybdate; buffer B – buffer A + 0.4 M KCl (or 0.15 M when indicated); buffer BM – buffer AM + 0.4 M KCl; buffer C – 10 mM Tris (pH 7.4), 3 mM $MgCl_2$, 0.25 M saccharose; buffer D – 10 mM Tris (pH 7.4), 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 0.15 M KCl.

2.2. *Methods*

Circulating lymphocytes from normal volunteer donors were separated on Ficoll–metrizoate gradients as in [11]. Incubation of lymphocytes for OHSBP labelling, cytosol preparation and analysis were performed as in [3]. Briefly (0.8–2) $\times$ 10^8 cells in 2–4 ml RPMI 1640 medium were incubated 15 min at 37°C in the presence of 20 nM tritiated 25-OH cholesterol with or without an excess (500 $\times$) of unlabelled sterol. The following steps were performed at 4°C: the cells were washed 3 times with PBS then lyzed by hypotonic shock in buffer A or AM. In all experiments aprotinin was added to the homogenate giving a final concentration of 60 kIU/ml. Crude nuclei were pelleted at 400 $\times$ *g* for 5 min. When necessary the KCl concentration was adjusted to the desired level in the 400 $\times$ *g* supernatant (S400). S400 was submitted to ultracentrifugation at 105000 $\times$ *g* for 60 min: the 105000 $\times$ *g* supernatant is referred to as prelabelled cytosol. After brief treatment with dextran-coated charcoal (15 min at 4°C, final concentration of charcoal 0.625%; of dextran T70, 0.0625%), prelabelled cytosol was analysed by ultracentrifugation on a linear sucrose gradient (5–20%) (200000 $\times$ *g*, 15 h, Beckman, SW50.1 rotor).

In some experiments prelabelled cytosol prepared in buffer A was fractionated by ammonium sulfate precipitation; the proteins precipitated between 25 and 40% ammonium sulfate saturation were redissolved in buffer A or B and analysed by ultracentrifugation on sucrose gradient.

Dissociation experiments were performed by incubating an excess of non-radioactive 25-OH cholesterol with prelabelled cytosol: the non-radioactive sterol was added in ethanolic solution (final concentration of ethanol, 5%) at a concentration 300-fold greater than that of the cytosolic tritiated 25-OH cholesterol concentration evaluated after charcoal-dextran treatment. Incubation times and temperature are indicated in section 3.

Effects of temperature on the OH sterol–OHSBP complex were tested in cytosols ob-

tained from entire cells incubated with 25-OH [^{3}H]cholesterol at 4°C for 3 or 15 h. Prelabelled cytosols were then heated at 37°C for 15 min in the presence of aprotinin and submitted to ultracentrifugation on a linear sucrose gradient.

Nuclear extracts were prepared as follows: after cell lysis in hypotonic buffer A, prelabelled nuclei were sedimented at 400 × *g* and washed twice in buffer C at 4°C. Nuclei were then incubated in buffer D with 1000 IU DNase per 0.5 ml during 30 min at 4°C. The supernatant obtained after centrifugation (500 × *g*, 10 min) of the incubation mixture is referred as S_1. The nuclear pellet was submitted to extraction by buffer B (0.4 M KCl) during 30 min and the supernatant obtained by centrifugation (200000 × *g*, 1 h) was denoted S_2. S_1 and S_2 were then analysed by ultracentrifugation on linear sucrose gradients as described for prelabelled cytosol.

3. RESULTS

3.1. *Specific binding of 25-OH cholesterol to nuclear extracts of human lymphocytes*

Fig.1a shows the binding pattern of 25-OH [^{3}H]cholesterol in supernatant S_1 obtained directly after chromatin digestion by DNase. Fig.1b shows the binding pattern in supernatant S_2 obtained by 0.4 M KCl extraction of the residual nuclear pellet. A 25-OH [^{3}H]cholesterol–macromolecule complex appeared in a zone corresponding to a sedimentation coefficient of 3.7 S for S_1 and 4.6 S for S_2. The labelling disappeared partially (S_1) or totally

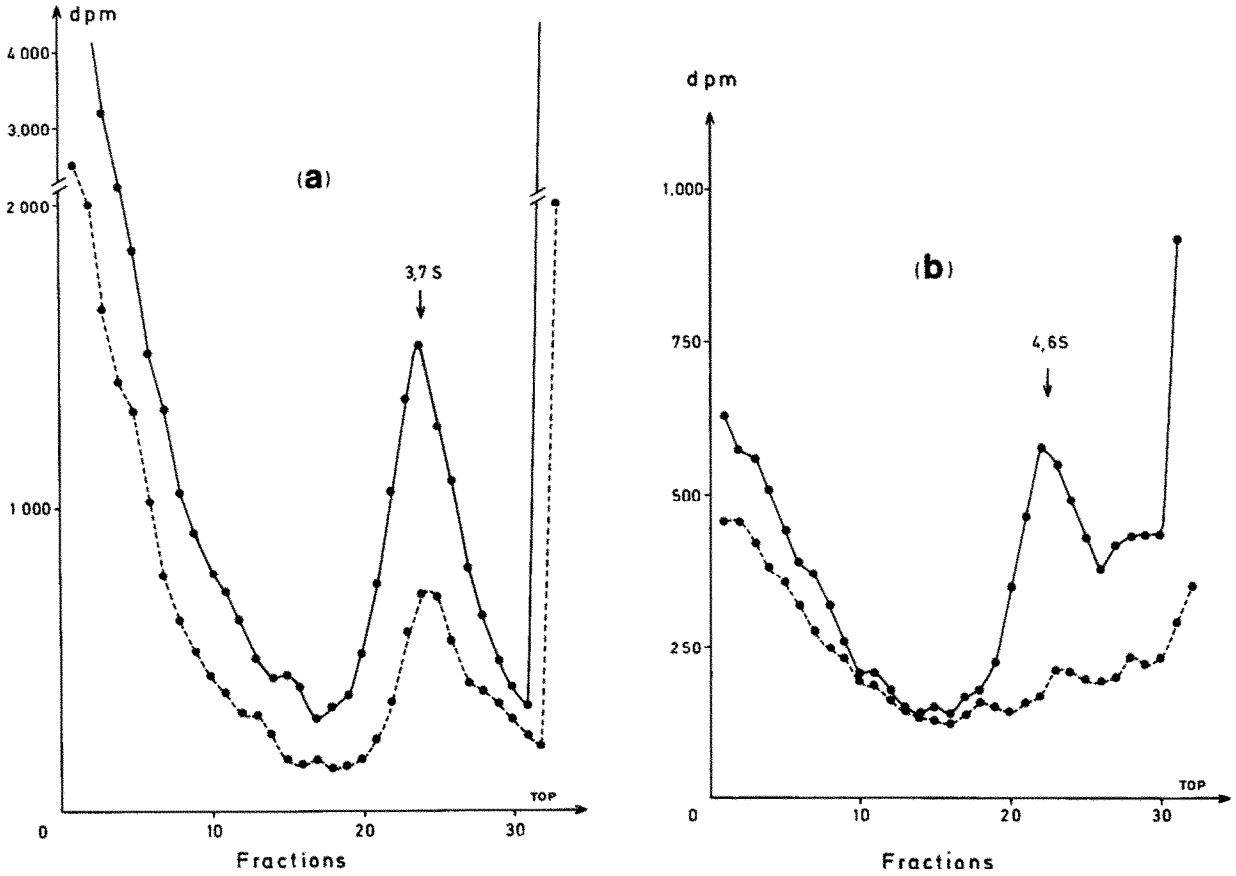

Fig.1. Binding pattern of tritiated 25-OH cholesterol to nuclear extracts of human lymphocyte: 0.5 × 10^8 lymphocytes were incubated at 37°C for 15 min with 10 nM 25-OH [^{3}H]cholesterol with (●---●) or without (●—●) an excess of unlabelled sterol (1000 ×). S_1 (a) and S_2 (b) were prepared and analysed by ultracentrifugation on sucrose gradients as described in section 2.

(S_2) when lymphocytes were incubated in the presence of an excess of unlabelled sterol (1000 ×). No radioactive peaks were observed when S_1 and S_2 were treated with proteinase K for 30 min at 18°C indicating the proteinaceous nature of the binding components extracted from the nucleus.

In another set of experiments we compared the specific binding in cytosol and nuclear extracts (S_1 and S_2) from lymphocytes incubated 10 or 60 min in the presence of labelled 25-OH cholesterol. Table 1 shows the increase of the nuclear binding between 10 and 60 min with concomitant decrease of the cytosolic binding.

3.2. *Influence of temperature on the cytosolic tritiated 25-OH cholesterol–OHSBP complex*

After labelling of whole cells with 25-OH [^{3}H]cholesterol at 37°C for 15 min we obtained a labelled OH sterol–OHSBP complex with a sedimentation coefficient of 8.3 S [3]. When cells were labelled at 4°C (3 or 15 h), the sedimentation coefficient of the complex remained unchanged as did the amount of 25-OH [^{3}H]cholesterol specifically bound to the protein (approx. 63 fmol/mg protein). Heating at 37°C for 20 min did not change the sedimentation coefficient of the complex previously obtained at 4°C. The binding however disappeared completely after heating at 56°C for 30 min or 100°C for 5 min. This result indicates that the OH sterol binding remaining after heating at 37°C for 20 min cannot be related to some unspecific interaction with denatured protein.

Table 1

Comparison of the specific 25-OH [^{3}H]cholesterol binding in cytosol and nucleus after labelling of entire lymphocytes at 37°C

Incubation time (min)	Cytosolic binding (fmol/mg P)	Nuclear binding (fmol/mg P)		
		S_1	S_2	S_1+S_2
10	50	249	150	399
60	18	318	115	433

Lymphocytes were incubated at 37°C for 10 or 60 min in the presence of 10 nM 25-OH [^{3}H]cholesterol with or without an excess of unlabelled sterol (1000 ×). Cytosol and nuclear extracts were analysed by sucrose gradient ultracentrifugation. Quantitative estimation of binding as in [3]

3.3. *Effects of increasing KCl concentrations on the sedimentation coefficient of the cytosolic 25-OH cholesterol–OHSBP complex*

When prelabelled cytosol was prepared and submitted to sucrose gradient ultracentrifugation in hypotonic buffer, the 25-OH cholesterol–OHSBP complex always sedimented with a coefficient of 8.3 S. In the presence of increasing amounts of KCl, the complex was differently distributed between two fractions of 8.3 and 4.3 S according to the experimental conditions. When prelabelled cytosol was obtained in hypotonic buffer A and layered on a 0.4 M KCl gradient (buffer B), the distribution was: complete transformation into 4.3 S, 24% of the experiments; partial transformation, 38%; no transformation, 38%. When prelabelled cytosol was obtained in buffer B containing 0.4 M KCl, the radioactivity of the complex was recovered in the 4.3 S zone in most experiments (86%) whereas in 0.15 M KCl, it was recovered in the 4.3 S zone in 60% of the experiments only.

Fig.2a,b shows results from a typical experiment demonstrating the complete change of sedimentation coefficient of the complex when S400 was adjusted to 0.4 M KCl. Transformation was accompanied by a sharp decrease in the number of binding sites (mean value, 42 fmol/mg protein in buffer B *vs* 92 in buffer A).

In none of the experiments did 20 mM sodium molybdate prevent the change of sedimentation coefficient induced by addition of 0.15 or 0.4 M KCl in cytosol (fig.2). Nevertheless, in hypotonic buffer an increase of the number of binding sites was observed: this did not occur in hypertonic buffer either at 37°C (fig.2a,b) or at 4°C (not shown).

3.4. *Study of the 4.3 S complex dissociation*

We previously reported [3] that the 8.3 S complex was not dissociable when incubated either 13 h at 4°C or 3 h at 37°C in the presence of a 500-fold excess of unlabelled 25-OH cholesterol added to prelabelled cytosol. Similar dissociation experiments were carried out with the 4.3 S complex obtained in the presence of 0.4 M KCl.

When prelabelled cytosol prepared in buffer B or BM was incubated with an excess of unlabelled

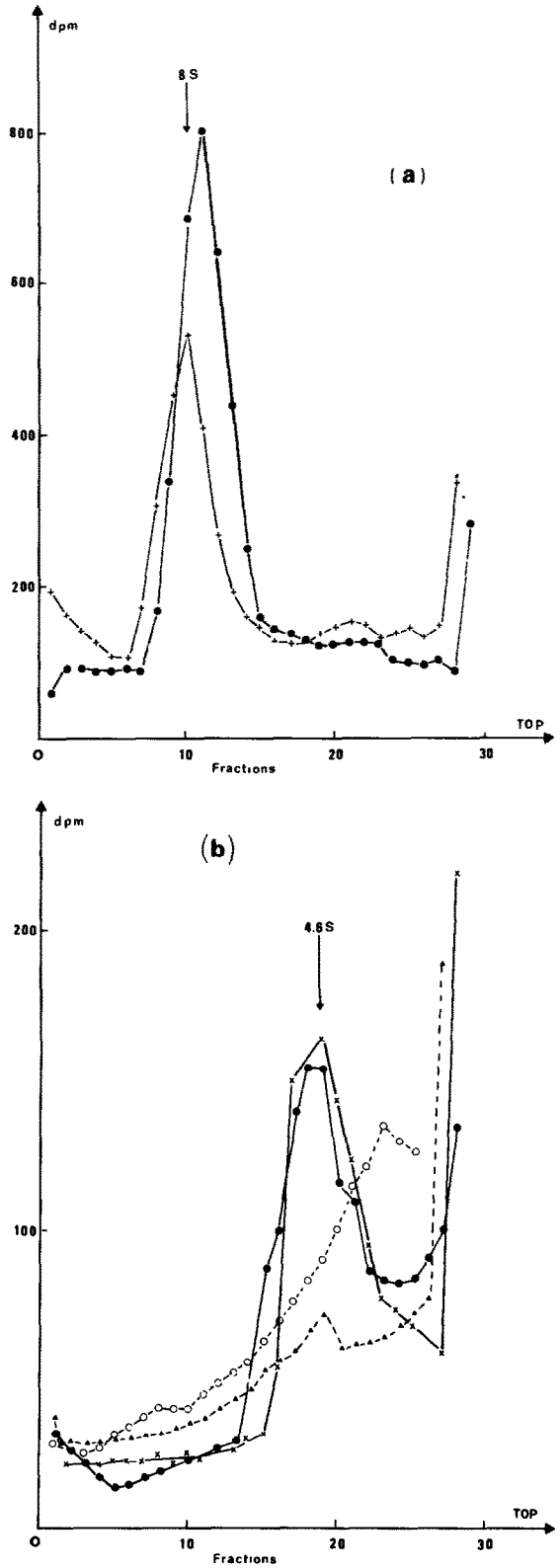

Fig.2. Binding pattern of tritiated 25-OH cholesterol to cytosolic proteins of human lymphocyte: 0.5×10^8 cells were incubated at 37°C for 20 min with 20 nM 25-OH [^{3}H]cholesterol; cytosol was prepared in hypotonic buffer A (+—+) or AM (●—●) (a) or in hypertonic buffer B (+—+) or BM (●—●) (b) and left in the presence of aprotinin 2 h at 37°C before ultracentrifugation. Protein concentration, 0.3 mg/ml; cytosolic 25-OH [^{3}H]cholesterol concentration, 0.5 nM. Specific binding of 25-OH cholesterol (fmol/mg P), 96 (buffer A), 145 (AM), 30 (B or BM). (b) Also shows the dissociation of the complex incubated in the presence of an excess (500 ×) of cold 25-OH cholesterol for 2 h at 37°C (buffer B, ▲---▲; buffer BM, ○---○).

25-OH cholesterol (500 ×) only 20% of the initial binding remained after 17 h at 4°C and complete dissociation of the 4.3 S complex was observed after 2 h at 37°C (fig.2b). Sodium molybdate (20 mM) did not prevent the dissociation of the 4.3 S form (fig.2b).

Table 2 shows the specificity of the exchange of unlabelled hydroxysterols with the 25-OH [^{3}H]cholesterol bound by the 4.3 S complex: the 4.3 S transformed form retained the ability to bind specifically the same OH-sterols as the 8.3 S form, in particular the (*R*) and (*S*) epimers of 23-OH cholesterol are discriminated.

3.5. *Ammonium sulfate precipitation effects*

The 25-OH [^{3}H]cholesterol–OHSBP complex was precipitated with ammonium sulfate (25–40% saturation) (fig.3a). No detectable binding occurred in the 0–25 and 40–80% fractions. A noticeable increase of specific binding activity of OHSBP was observed: 18 fmol/mg P in native cytosol as opposed to 154 fmol/mg P in the 25–40% fraction. When the precipitate was redissolved in hypotonic buffer, the complex sedimented in a 8.3 S zone (fig.3a). Redissolution in 0.4 M KCl buffer did not induce any modification of the sedimentation coefficient but decreased the number of binding sites (fig.3b). The 8.3 S complex obtained by ammonium sulfate precipitation was dissociated neither at 0 nor at 28°C when incubated for 2 h in the presence of an excess of unlabelled 25-OH cholesterol (fig.3b).

Table 2

Effect of an excess (× 500-fold) of several unlabelled steroids on the binding of 25-OH [^{3}H]cholesterol to the 4.3 S form

Unlabelled steroid (500-fold excess)	Residual binding of 25-OH [^{3}H]cholesterol (%)
None	100
25-OH cholesterol	12[a]
23(R)-OH cholesterol	83
23(S)-OH cholesterol	40
Cholesterol	100
25-OH cholecalciferol	81
1α,25-diOH cholecalciferol	54
Dexamethasone	100
Progesterone	86
Estradiol	86

[a] Mean of 4 experiments

Prelabelled cytosol was prepared in the presence of 0.4 M KCl as described in section 2 and then incubated with or without an excess (500-fold) of cold sterol for 2 h at 37°C. Cytosol was centrifuged on sucrose gradient and the 25-OH [^{3}H]cholesterol residual binding on the 4.3 S form was evaluated

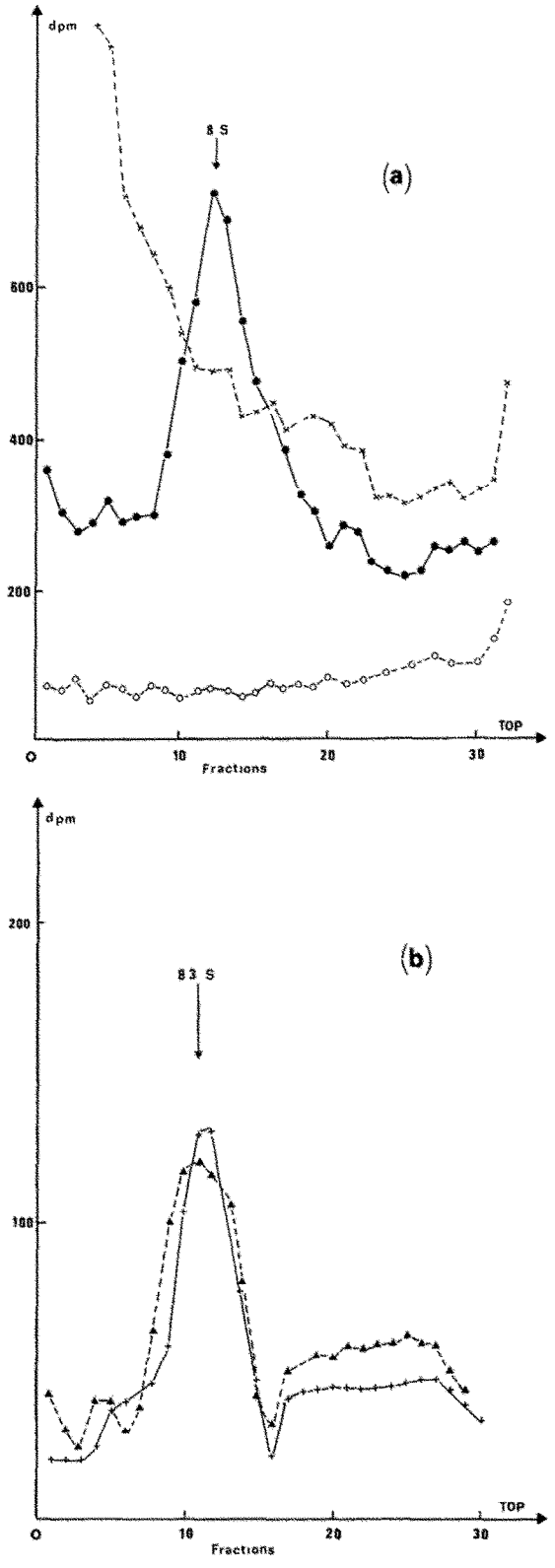

Fig.3. Effect of ammonium sulfate precipitation on the conformation and stability of 25-OH [^{3}H]cholesterol–OHSBP complex. (a) Prelabelled cytosol prepared in hypotonic buffer A was fractionated by successive precipitations with $(NH_4)_2SO_4$. Each precipitate of proteins was redissolved in buffer A and submitted to ultracentrifugation on a linear sucrose gradient = 0.25% precipitate, ○---○; 25–40%, ●——●; 40–80%, +---+. Initial binding, 18 fmol/mg P; 25–40% binding, 154 fmol/mg P. (b) Prelabelled proteins precipitated between 25–40% were redissolved in hypertonic buffer B and incubated in the presence (▲---▲) or absence (+---+) of an excess of unlabelled sterol (500 ×) for 2 h at 28°C before ultracentrifugation on linear sucrose gradient. Specific binding of 25-OH cholesterol, 86 fmol/mg P.

4. DISCUSSION

Oxygenated sterol binding proteins have been described in L cells [12–14] and in CHO cells [15]. Those experiments, however, were not focused either on the nuclear binding of 25-OH cholesterol or on the effects of high salt concentrations and of molybdate on the physico-chemical properties of the OHSBP.

The present data show the presence in human lymphocytes of nuclear protein(s) able to bind 25-OH cholesterol with a limited capacity and a sedimentation coefficient in the range of 4 S. The 25-OH [^{3}H]cholesterol–OHSBP complex can be extracted from the nuclei as early as 10 min incubation of living lymphocytes at 37°C in presence of labelled ligand. Whether the OH-sterol nuclear binding sites are preexistent in the nucleus before the incubation as described for estradiol receptors [16,17] remains to be elucidated. The presence of OH-sterol binding proteins both in cytosol and nucleus also raises the question of a possible activation and translocation of the cytosolic OHSBP. Some physico-chemical treatments known to activate steroid hormone receptors were applied to the cytosolic 25-OH [^{3}H]cholesterol–OHSBP complex.

All the present experiments were performed on prelabelled cytosols obtained from whole lymphocytes incubated with tritiated 25-OH cholesterol (in vivo assays). This procedure is unusual for studying the physico-chemical properties of a steroid receptor although it is used in studies of the androgen receptors of sex skin fibroblasts [18]. The difficulties encountered in direct labelling of cytosol with tritiated OH sterol (in vitro assays [3]) have not yet been overcome.

When the 25-OH cholesterol–OHSBP complex was treated with increasing concentrations of KCl, the 8.3 S complex was transformed into the 4.3 S form. The most efficient way to induce this transformation was to add KCl at a high concentration (0.4 M) and at an early step of cytosol preparation (i.e., to the S400). When KCl was only added to the gradient buffer, transformation of 8 S into 4.3 S was not consistently observed. This explains why authors in [12] mainly observed an 8 S complex though using KCl: these authors used only 0.3 M KCl, and this was added only at the step of sucrose gradient analysis. This transformation, 8.3⟶4.3 S, is in agreement with the well-known transformation of steroid hormone receptors in the presence of high salt concentrations, for instance, glucocorticoid receptor [4,5] or estradiol receptor [19]. The reversibility of the transformation could not be tested owing to the small amount of material available after sucrose density gradient ultracentrifugation and to the lower stability of the 4.3 S complex; indeed, the transformation 8.3⟶4.3 S is accompanied by a loss of binding sites and proteolytic cleavage cannot be excluded.

Some other physico-chemical properties of OHSBP are different from those of steroid hormone receptors:

(i) The 4.3 S form of steroid hormone-receptor complexes is generally considered as an activated form unable to dissociate [9]. In contrast, we observe a decrease of the 4.3 S 25-OH [^{3}H]cholesterol–OHSBP complex when incubated at 37°C for 2 h in the presence of an excess of unlabelled steroid (table 2). This could result from dissociation, denaturation or proteolysis of the complex. Though inactivation processes could not be excluded, an actual dissociation is likely since the results of table 2 are expressed as percent of the control treated under the same conditions but in the absence of an excess of unlabelled steroid.

(ii) Ammonium sulfate precipitation is known to activate glucocorticoid or estradiol receptors [7,10]: this had no effect on the sedimentation coefficient or on the dissociation of the labelled OH sterol–OHSBP complex. This result was observed even when the precipitate was redissolved in 0.4 M KCl containing buffer. This explains the observation of an 8 S OH-sterol–OHSBP complex after cytosol preparation involving ammonium sulfate precipitation [14]. It should be observed that ammonium sulfate treatment resulted in a partial purification of the OHSBP and can be considered as a useful tool for in vitro assays.

(iii) Heating at 37°C of the 8.3 S complex obtained after cell labelling at 4°C did not result in the transformation into a 4.3 S form as it could be expected from the properties of glucocorticoid receptor [5].

(iv) In the same way, molybdate which is known to stabilize the glucocorticoid receptor in an inactivated form [10] did not prevent the transformation induced by KCl or the rapid dissociation of the 4.3 S complex. Nevertheless molybdate seems

to protect the 8 S complex from degradation at 37°C (fig.2). Phosphatase inhibition could be involved in this process since we observed a rapid disappearance of the labelled 8 S complex after incubation with alkaline phosphatase (not shown).

In conclusion, with the exception of the 8.3 to 4.3 S transformation in presence of 0.4 M KCl, the physico-chemical characteristics of the lymphocyte OHSBP reported here are very different from those of a classical steroid hormone receptor.

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