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EFFECTS OF UREA AND MOLYBDATE ON THE CHICK OVIDUCT PROGESTERONE RECEPTOR

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1. The chick oviduct cytosol progesterone receptor, when complexed with ligand, can be exposed to urea concentrations as high as 3 M (at 0°C) without loss of steroid binding capacity. The ligand dissociation rate is increased $\geqslant$ 10 fold under these conditions.

2. The "native" 8 S form of the receptor is progressively converted to a 4 S species by urea (> 2 M) as seen in ultracentrifugation analysis. This conversion is inhibited by Na_2MoO_4 (5-50 mM) suggesting that molybdate stabilizes the 8 S molecule by direct interaction.

3. At urea concentrations above 2 M, the ligand-free receptor looses progressively its binding capacity. The "transformed", 4 S receptor was less stable than the 8 S species, and could not be protected by molybdate.

Previous studies (1-3) on the process of "activation" ("transformation") of the chick oviduct progesterone receptor have led us to the hypothesis that activation may be equivalent to the separation of the subunits (sedimentation coefficient $\sim$ 4 S) present in the non-transformed ("native") 8 S molecule. The latter can be stabilized by molybdate (5-50 mM) in a form sedimenting at $\sim$ 8 S (4, 5). Similar effect of molybdate (as well as of vanadate and tungstate) can be seen with most or all steroid hormone receptors. The mechanism of action of these anions is not clear at present ; it has been suggested that it may due to inhibition of phosphatase activity (6, 7), or to direct interaction with the receptor molecule (8, 9). In this paper we report data showing that urea can convert the 8 S-progesterone receptor molecule to a 4 S form and that molybdate inhibits this process, a finding that suggests a direct interaction and stabilization of the 8 S species.

MATERIALS AND METHODS

Chemicals : [1,2,6,7-^{3}H]progesterone (80-110 Ci/mmol) was from CEA (Saclay, France). Progesterone was a gift from Roussel-Uclaf (Romainville, France). Other chemicals (analytical grade) were from usual commercial sources.

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Animals and tissue fractionation : Chickens (5 days old ; Warren strain) were primed with 10 daily injections of oestradiol benzoate in sesame oil (1 mg/day). Before being killed, they received additionnally three to five daily injections of the oestrogen (5). The magnum portion of the oviduct was excised and homogenized in 4 vol. of Tris (10 mM)/EDTA (1.5 mM) buffer containing 12 mM-thioglycerol and 10 % glycerol, pH 7.4 (TE buffer), or in the same buffer containing 20 mM-Na_2MoO_4 (TE-M buffer), by using an Ultra-Turrax tissue disintegrator. This and all further operations were carried out at 0-4°C. Phenylmethanesulphonyl fluoride was added to the homogenate to a final concentration of 0.3 mM. The cytosol was obtained by ultracentrifugation at 100 000 g for 60 min.

Activation of progesterone-receptor : Cytosol in TE buffer was incubated at 0°C for 16 h with 0.3 M KCl (1). When necessary, KCl was subsequently removed by gel filtration on Tris-acryl GF05 at 0°C in the TE buffer.

Labelling of receptor : Receptor-hormone complexes were usually formed by incubation with 20 nM-[^{3}H]progesterone for 1-4 h at 0°C. Under these conditions, 80-90 % of all receptor binding sites are saturated. Cortisol (1 µM) was included in the incubation medium to eliminate binding of labelled progesterone to contaminating plasma transcortin and glucocorticosteroid receptor (10).

The unbound hormone was removed by charcoal (0.25 %)/dextran (0.025 %)/gelatin (1 mg/ml) adsorption (10 min at 0°C) and centrifugation (5 min at 0°C)).

Non-specific binding was always monitored by parallel incubations with a 100-fold excess of unlabelled progesterone. In all cases, non-specific binding was < 10 % of the total [^{3}H]progesterone binding.

To determine the concentration of the binding sites, 20 µl of sample were added to tubes containing either 200 µl of 20 nM [^{3}H]progesterone (to measure total binding) or 200 µl of 20 nM [^{3}H]progesterone plus 2 µM unlabelled progesterone (to measure non-specific binding). The steroid solutions were made in TE-M buffer. Incubation was carried out for 16 h at 0°C and the protein-bound radioactivity was determined by the charcoal adsorption technique as described above (5). The incubates contained urea at concentrations up to 0.6 M ; we have verified that this did not affect the results. In fact, even the presence of 3 M urea did not cause a detectable underestimation of the concentration of the receptor, probably because the on-rate of the complex formation is much faster than the urea-dependent receptor inactivation.

Dissociation kinetics : [^{3}H]progesterone-receptor complexes were formed as described above ; urea and KCl concentrations were adjusted according to the experimental design. Dissociation of the radioactive ligand was initiated by adding a 500-fold excess of radioinert progesterone in ethanol (10 µl/ml of receptor containing solution). Incubation was carried out at 0°C. At differents times, aliquots (100 µl) were removed and the receptor-bound radioactivity was determined as described above. Decrease of the receptor binding capacity was studied in parallel incubations where addition of the unlabelled hormone was omitted.

Sucrose density gradient ultracentrifugation ; Samples (0.2 ml) were layered onto discontinuous sucrose gradients (5-20 %) made in TEK-M buffer (TE buffer + 0.15 M KCl + 50 mM Na_2MoO_4), and ultracentrifuged in an SW 60 rotor at 0°C at 50,000 rev/ min for 15-16 h. [^{14}C]BSA (2,000 cpm) was included in the samples as internal standard. Fractions (two drops) were collected manually after piercing the bottom of the tube. The receptor was identified by direct counting of the collected fractions.

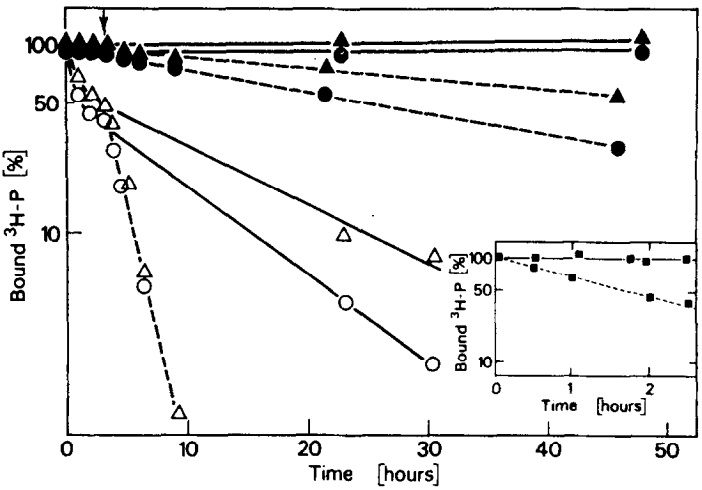


Figure 1. Effect of 3 M urea and 3 M urea plus 0.3 M KCl on stability and dissociation kinetics of the [^{3}H]progesterone-receptor complexes.

Cytosol was prepared in the TE buffer and incubated with 20 nM [^{3}H]progesterone. To one portion of this preparation Na_2MoO_4 was added to a concentration of 20 mM (●, ○), and KCl concentration was adjusted to 0.3 M in both samples. The mixtures were incubated at 0°C for 16 h. Urea 3 M was then added to aliquots of each solution and the incubation was continued for another 3 h. The solutions were again divided in portions for the study of the rate of dissociation of the [^{3}H]progesterone-receptor complexes. Control of stability of the complexes was carried out in parallel (for more details see Materials and Methods). The results are expressed as log (percent of [^{3}H]progesterone bound to receptor at zero time).

Circles : samples containing 20 mM Na_2MoO_4 ; triangles : samples without molybdate ; solid symbols : 0.3 M KCl alone ; open symbols : 0.3 M KCl + 3 M urea ; dashed lines : dissociation kinetics ; solid lines : stability controls ; arrow : time of addition of excess unlabelled progesterone to initiate dissociation of [^{3}H]progesterone.

Cytosol was prepared in the TE-M buffer and incubated with 20 nM [^{3}H]progesterone. Urea was then added to a concentration of 3 M and the rate of dissociation of the [^{3}H]progesterone-receptor complexes was measured (■---■). Control of stability of the complexes (■——■).

RESULTS

Stability and dissociation kinetics of the receptor-ligand complexes

In the first series of experiments (Fig. 1-insert) we established that incubation with as much as 3 M urea for several hours at 0°C does not decrease the concentration of the pre-formed [^{3}H]progesterone-receptor complexes. However, under these conditions urea has a destabilizing effect on the receptor molecule as can be judged by the fact that the half-life of dissociation of the ligand is ∿ 2 h at 0°C (vs ∿ 20 h for the 8 S non-transformed and ∿ 50 h for the 4 S, transformed progesterone receptor (1, 5)). When 0.3 M KCl was included in the incubation mixture together

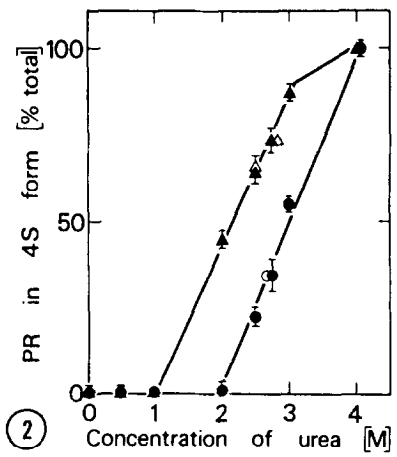
with 3 M urea, about 40 % of the [^{3}H]progesterone binding was lost within 2 h at 0°C (Fig. 1). Afterwards, the binding capacity continued to decrease but with a lower rate so that the denaturation curve shows two components. Under these conditions, molybdate (20 mM) did not protect the [^{3}H]progesterone-receptor complex against denaturation. Dissociation kinetics in the presence of 0.3 M KCl and 3 M urea was similar to that observed in 3 M urea alone. Molybdate had no effect on the rate of dissociation under any of these conditions (Fig. 1). Incubation of the [^{3}H]progesterone-receptor complex with 3 M urea + 0.3 M KCl resulted in a complete transformation of the complex to the 4 S-sedimenting species within 3 h at 0°C, irrespective of the presence or absence of 20 mM Na_2MoO_4 (data not shown).

8 S → 4 S transformation

We next examined the effect of 1 h incubation at 0°C with various concentrations of urea in the presence or absence of 20 mM Na_2MoO_4 on the sedimentation coefficient of the [^{3}H]progesterone-receptor complex. Here, a clear protective effect of molybdate was seen (Fig. 2) : the half-effective concentration of urea (i.e. that causing 50 % of the [^{3}H]progesterone-receptor complex to be converted into the 4 S form) was 2.2 M in the absence and 3.0 M in the presence of molybdate. It is to be noted that in neither case did < 3 M urea cause any loss of total (8 S + 4 S) receptor-bound [^{3}H]progesterone. Molybdate concentrations between 5 and 50 mM were equally effective when tested with 2.75 M urea (data not shown). In some experiments the conversion of the 8 S to 4 S form by urea was studied in the absence of ligand. The results obtained were the same as with the receptor-hormone complex (Fig. 2).

Inactivation of unoccupied receptor binding sites

These experiments were carried out with two sorts of receptor preparation. In one series, the cytosol was adjusted to 0.3 M KCl and incubated for 16 h at 0°C in order to "activate" the receptor (1) ; KCl was subse-



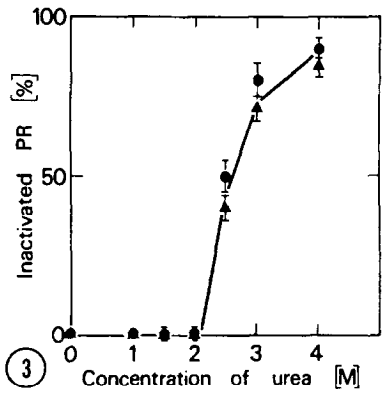


Figure 2. 8 S → 4 S transformation of the [^{3}H]progesterone-receptor complexes by urea.

Cytosol was prepared in the TE buffer and incubated with [^{3}H]progesterone. The effect of 1 h incubation at 0°C with various concentrations of urea in the presence (circles) or absence (triangles) of 20 mM Na_2MoO_4, on the sedimentation coefficient of [^{3}H]progesterone-receptor complexes (solid symbols) was studied by ultracentrifugation in sucrose gradients. The concentration of Na_2MoO_4 was adjusted in all samples to 20 mM, prior to being layered on the sucrose gradients. Samples containing urea were diluted with buffer so that the urea concentration was < 1 M in the solution deposited onto the gradient.

For the experiments with ligand-free receptor (open symbols), the samples were incubated for 1 h at 0°C with buffers containing urea and then labeled with [^{3}H]progesterone.

Figure 3. Inactivation of progesterone binding sites of the unoccupied transformed progesterone-receptor by urea.

Cytosol was prepared in the TE buffer and KCl was added to a concentration of 0.3 M. After incubation for 16 h at 0°C, the sample was chromatographed on a desalting column (Tris-acryl GF 05). The void volume fraction was pooled and incubated with various concentrations of urea for 1 h at 0°C in the presence (●) or absence (▲) of 20 mM Na_2MoO_4. The residual progesterone binding capacity of the progesterone-receptor was tested as described in Materials and Methods.

quently removed on a desalting column. Another series of data was obtained with the non-activated receptor, i.e. kept at 0°C and at low ionic strength. These two kinds of cytosol were incubated for 1 h at 0°C with various urea concentrations, in the absence or presence of 20 mM Na_2MoO_4, prior to determination of the concentration of the receptor binding sites. The results of these experiments are represented in Figure 3 and 4. Molybdate had no noticeable protective effect on the transformed receptor binding sites (Figure 3) (half-effective concentration of urea $\sim$ 2.6 M). The non-transformed receptor was more resistant to inactivation (Figure 4a) (half-effective urea concentration $\sim$ 3 M). In this case, molybdate had none

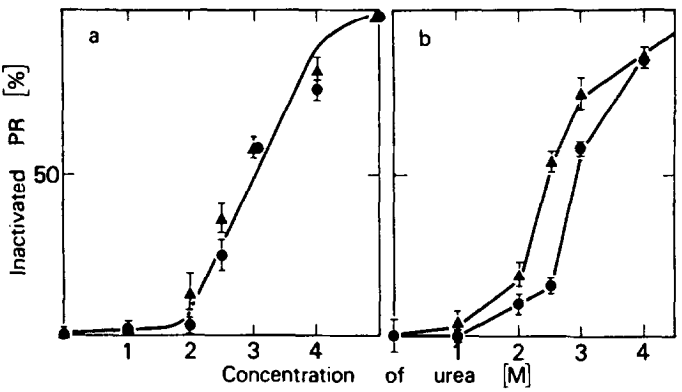


Figure 4. Inactivation of progesterone binding sites of the unoccupied non-transformed progesterone-receptor.
Cytosol was prepared in the TE buffer and incubated at 0°C with various concentrations of urea for 1 h (a) and 2 hs (b) in the presence (●) or absence (▲) of 20 mM Na_2MoO_4. The residual binding capacity of the progesterone-receptor was tested as described in Materials and Methods.

or only marginal effect during 1 h incubation. However, when the same experiment with non-transformed receptor was carried out for 2 h at 0°C (Fig. 4b), the effect of molybdate became more apparent, the half-effective concentration of urea being ∿ 2.5 M without molybdate, ∿ 2.75 M with molybdate. Increasing the concentration of molybdate (up to 750 mM) did not result in a greater protection of the binding sites against urea inactivation (data not shown). The inactivation curve observed for 2 h incubation without molybdate is the same as that for the transformed receptor (Fig. 3), and it was verified that under these conditions (absence of ligand), ∿ 70 % of the receptor was converted to the 4 S species within 1 h at 0°C in 2.5 M urea (Fig. 2).

DISCUSSION

The studies of denaturation of oligomeric proteins have the advantage that they permit the detection of early interactions with urea, i.e. probably those which influence the functional domains of the protein. Such interactions are probably formed late in the course of protein folding, and are the first to be disrupted when exposed to a denaturing agent. A possible hierarchy of protein folding has been suggested (11).

The experiments described here allow to drawn certain conclusions concerning the structure of the chick oviduct progesterone receptor and its interaction with molybdate.

1. Molybdate probably interacts directly with the receptor molecules. It is difficult to explain otherwise the fact that it inhibits the 8 S → 4 S transformation induced by urea. The interaction is stronger with the non-transformed than with the transformed receptor. An attractive hypothesis is that the molybdate ions intercalate between neighbouring portions of the subunits forming the 8 S complex and tighten their association by introducing an additional bond, since molybdate is known to form at neutral pH, labile coordination complexes where two Mo atoms binds light radicals such as -SH or $-NH_2$ groups or H_2O molecules (vanadate and tungstate can form similar structures). High urea concentrations (> 3 M) can overcome the forces holding the 8 S complex together, probably by unfolding the native protein configuration. Raising the ionic strength of the medium amplifies the effect of urea (a fact known for other systems) ; this may be a consequence of destabilization of the ionic bonds implicated in the tertiary and quaternary structure of the protein.

2. There may be a difference in the configuration of the binding domain (the binding site itself plus the neighbouring regions of the receptor molecule) of the non-transformed and transformed receptor as inactivation of the binding site of the former occurs at a higher urea concentration. Alternatively, the native configuration of the binding site may be stabilized by inter-chain bonds, absent in the transformed receptor. Molybdate does not protect the binding site directly since it has no effect in the case of the 4 S form. Protection by molybdate of the non-transformed receptor results probably from stabilisation of the quaternary structure of the 8 S molecule.

3. Receptor complexed with a ligand is more resistant against inactivation of the binding site by urea than unoccupied receptor. One interpretation of this finding is that progesterone-receptor interaction may

involve hydrogen bond(s). In fact, existence of two H-bonds was suggested for the binding of progesterone to the receptor from rabbit uterus, in order to account for the free enthalpy change associated with the process of dissociation of the complex (12). (Urea probably attacks the receptor molecule at several points, but only those within the binding domain could be detected in our experiments).

Another interpretation of the stabilizing effect of ligand in urea inactivation studies is a change of the receptor conformation upon binding. The two alternatives are not mutually exclusive, and at this moment neither of them can be refuted. The presence of a ligand has been known to protect the binding site of steroid hormone receptors against denaturation (1, 13) ; this however may be simply a consequence of strengthening of the protein structure at the binding domain by all the bonds (ionic, hydrogen, van der Waals, hydrophobic) involved in ligand binding. In contrast, the 8 S → 4 S transformation by urea is not influenced by the presence of ligand at the binding site.

Beside the chick oviduct progesterone receptor, the estrogen receptor from mammalian uterus (14, 15) and avian oviduct (16) nuclei have also been shown to be stable at concentrations of urea as high as 4 M. These observations have also practical implications. Since the receptor-ligand complex resists 3 M urea, urea can be included in the buffers used at receptor purification by affinity chromatography. We have obtained preliminary results showing usefulness of this procedure (manuscript in preparation).

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REFERENCES

1. Yang, C.R., Mester, J., Wolfson, A., Renoir, J.M. and Baulieu, E.E. (1982) Biochem. J. 208, 399-406.
2. Yang, C.R., Seeley, D.H., Mester, J., Wolfson, A. and Baulieu, E.E. (1983) Biochim. Biophys. Acta 755, 428-433.

3. Yang, C.R., Renoir, J.M., Mester, J. and Baulieu, E.E. (1982) "64th Annual Meeting of the Endocrine Society", June 16-18, San Francisco, California. Abstract N°251.
4. Nishigori, H. and Toft, D.O. (1980) Biochemistry 19, 77-83.
5. Wolfson, A., Mester, J., Yang, C.R. and Baulieu, E.E. (1980) Biochem. Biophys. Res. Commun. 95, 1577-1584.
6. Nielsen, C.J., Sando, J.J., Vogel, W.M. and Pratt, W.B. (1977) J. Biol. Chem. 252, 7568-7578.
7. Leach, K.L., Dahmer, M.K., Hammond, N.D., Sando, J.J. and Pratt, W.B. (1979) J. Biol. Chem. 254, 11884-11890.
8. Murakami, N., Quattrocchi, T.M., Szocik, J.F. and Moudgil, V.K. (1982) Biochim. Biophys. Acta 719, 257-272.
9. Housley, P.R., Dahmer, M.K. and Pratt, W.B. (1982) J. Biol. Chem. 257, 8615-8618.
10. Mester, J. and Baulieu, E.E. (1977) Eur. J. Biochem. 72, 405-414.
11. Schulz, G.E. (1977) Argew. Chem. (Int. Ed. Engl.) 16, 23-32.
12. Seeley, D.H., Wang, W.Y. and Salhanick, H.A. (1980) Biochim. Biophys. Acta 632, 536-543.
13. Rochefort, H. and Baulieu, E.E. (1971) Biochimie 53, 893-907.
14. Stancel, G.M., Leung, K.M.T. and Gorski, J. (1973) Biochemistry 12, 2137-2141.
15. Notides, A.C. and Nielsen, S. (1974) J. Biol. Chem. 249, 1866-
16. Geynet, C. and She Wei Ming, (1983) unpublished results.