IS THE ESTROGEN RECEPTOR OF MAMMARY GLANDS A METALLO-PROTEIN?

G. Shyamala with the assistance of Yar-Fen Yeh

Department of Zoology and Cancer Research Laboratory

University of California, Berkeley, California 94720

Received March 10,1975

SUMMARY: The cytoplasmic estrogen binding proteins (EBP) of mammary glands were characterized using sucrose gradient centrifugation and Sephadex G-200 gel chromatography. The EBP obtained with ammonium sulfate precipitation tends to aggregate in both low and high ionic strength buffers in the presence of EDTA. If the ammonium sulfate precipitate is redissolved in buffers without EDTA, the EBP sediments as a discrete 8S molecule under low ionic conditions and as a 4S EBP with 0.4 M KCl. In addition to EDTA, the aggregation of EBP is caused by various structurally different metal chelators and this effect can be reversed by the addition of Zn^{++} or Mn^{++}. It is proposed that the cytoplasmic EBP of mammary glands may contain a bound metal ion which may be crucial for the confirmation of the protein favorable to the binding of the steroid.

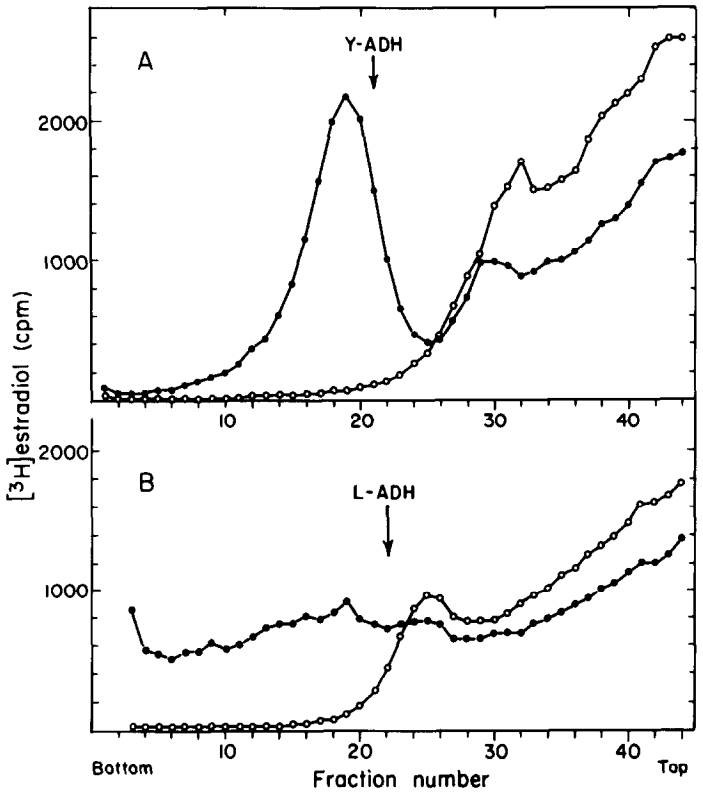
The target tissues of the estrogenic hormones contain within their cytoplasm specific estrogen binding proteins (EBP) also referred to as receptor proteins (1). The interaction of the steroid hormone with the receptor is believed to be an indispensable event in the course of its action in the target cell (2). The cytoplasmic EBP of the lactating mammary glands have been described previously and the binding characteristics are not discernably different from that of the uterus (3,4). However, the EBP of the mammary glands have not been characterized in detail. In order to understand the molecular mechanisms of hormone action by the receptor, it is necessary to resolve the physical basis for the interaction of the hormone and the receptor. To this extent, the present study was undertaken. During the course of this investigation, it became apparent that in the native state, the EBP of mammary glands may be associated with a metal ion which may be crucial in maintaining its configuration.

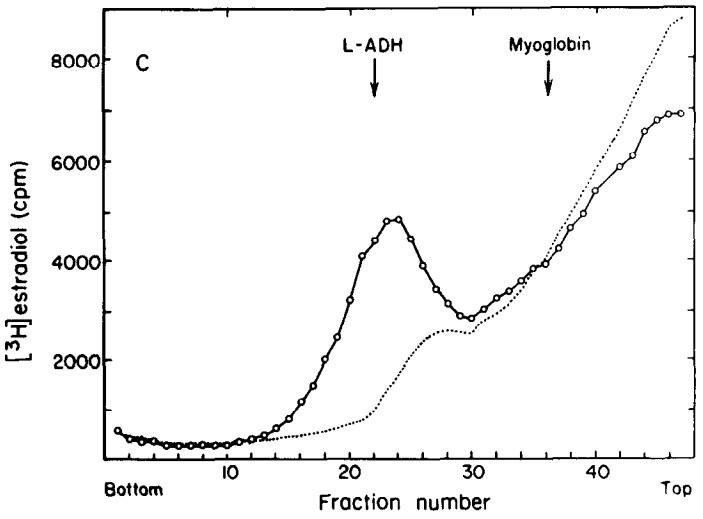
EXPERIMENTAL

BALB/cCrgl mice 5-7 months old were from our own colony. At the time of sacrifice, they had been lactating for 7-10 days. Tissue homogenates were prepared according to procedures described previously (3). The homogenate was centrifuged at 220,000 x g for 30 min to obtain a cytoplasmic extract that is referred to as the "cytosol". The [^{3}H] estradiol (95-100 Ci/mmole; New England Nuclear Corp.) was immediately added to yield a concentration of 2-20 nM and incubated at 4° C for 30 min. To obtain partially purified EBP, ammonium sulfate was added to the labelled cytosol to yield a final concentration of 20%. After 10 min the mixture was centrifuged at 10,000 x g for 10 min. The sediment was resuspended in various buffers containing 0.02 M thioglycerol and the undissolved material was removed by centrifugation at 10,000 x g for 30 min. Sucrose density gradient analyses were performed as described earlier (3). Beef liver catalase, yeast alcohol dehydrogenase (Y-ADH, 7.6 S) and liver alcohol dehydrogenase (L-ADH, 5S) and myoglobin (2S) were used as standards for sedimentation co-efficient estimations by the method of Martin and Ames (5). Radioactivity was measured in a Beckman LS-250 liquid scintillation spectrometer with a counting efficiency of 55% for [^{3}H].

RESULTS AND DISCUSSION

Initial studies on the cytoplasmic EBP of mammary glands indicated that the protein sediments in the region of 8S on sucrose density gradients in dilute buffers, and undergoes marked aggregation upon centrifugation in gradients containing 0.4 M KCl (Fig. 1A,B). Removal of EDTA from the buffer used in gradients results in the disaggregation of the EBP which now sediments in the region of 4S (Fig. 1C). To elucidate the phenomenon of aggregation, further studies were carried out with the partially purified EBP. The partially purified EBP tended to aggregate under both low and high ionic conditions (Fig. 2A,B) but only if the ammonium sulfate precipitate was redissolved in an EDTA containing buffer. In the absence of EDTA, the partially purified EBP sediments in the region of 8S (8.3 ± 0.05S) on sucrose gradients prepared in low ionic strength buffers (Fig. 2A) and in the region of 4S (4.5 ± 0.03S) on gradients containing 0.4 M KCl (Fig. 2C). In addition to EDTA, aggregation of EBP is also caused by various structurally different metal chelators such as O-phenanthroline, azide and EGTA. The degree of aggregation caused by the chelators depends on the concentration of metal chelator in the incubation mixture (Data not shown). Next, several metal ions were added along with the metal chelators to test their ability to reverse the aggregation.


A
Y-ADH
2000
1000
B
L-ADH
2000
1000
[3H]estradiol (cpm)
10
20
30
40
Bottom
Top
Fraction number


C
L-ADH
Myoglobin
8000
6000
4000
2000
[3H]estradiol (cpm)
10
20
30
40
Bottom
Top
Fraction number

The results of such experiments using O-phenanthroline as the metal chelator is shown in Figure 3. As may be seen, Zn^{++} is effective in reversing the aggregation caused by O-phenanthroline while Ca^{++} and Mg^{++} at the same concentrations are ineffective. Subsequent experiments indicate that Mn^{++} is as effective as Zn^{++} at 4 mM concentration while Co^{++} is without any effect (data not shown).

The partially purified EBP was also characterized on calibrated Sephadex G-200 columns, and the elution of the protein was carried out with both low and high ionic strength buffers without EDTA. The molecular weights were determined by the procedure originally described by Siegel and Monty (6) in which the molecular Stokes radius determined by gel chromatography was used with the sedimentation co-efficient determined by the sucrose gradient analyses. In low ionic buffers, the EBP has a molecular weight of approximately 200,000 while in the presence of 0.4 M KCl it is approximately 100,000. Detailed molecular parameters of these two EBP species will be described elsewhere.

The results from experiments using chelating agents suggest that bound metal ions may be crucial for maintaining the configuration of EBP. Among the metal ions so far tested, only Zn^{++} and Mn^{++} have the ability to reverse the aggregation of EBP caused by chelating agents. It also appears that the salt dissociated EBP is much more susceptible to chelation than the 8S EBP. The evidence for this is as follows. Although the 8S EBP can be readily identified

---

Figure 1. Sucrose gradient sedimentation of mammary gland cytosol labelled with 2 nM [^{3}H] estradiol (●—●—●—●) or [^{3}H] estradiol (2 nM) and unlabelled estradiol (200 nM) (o—o—o—o). (A) Gradients of 10-30% sucrose in 10 mM Tris, 1.5 mM EDTA, pH 7.4, were centrifuged at 155,000 x g for 16 hr at 4° C. (B) Gradients of 10-30% sucrose in 10 mM Tris, 1.5 mM EDTA, 0.4 M KCl, pH 7.4 were centrifuged at 174,000 x g for 23 hr at 4° C. (C) Cytosol labelled with 2 nM [^{3}H] estradiol (o—o—o—o) and cytosol labelled with 2 nM [^{3}H] estra-estradiol and 200 nM unlabelled estradiol (.....). Gradients of 10-30% sucrose in 10 mM Tris, 0.4 M KCl, pH 7.4 were centrifuged at 174,000 x g for 23 hr at 4° C. Y-ADH, L-ADH, catalase and myoglobin were used as standards to estimate sedimentation co-efficient.
Any binding of [^{3}H] estradiol observed in incubations containing an excess of unlabelled estradiol is due to non-specific binding and not due to EBP.

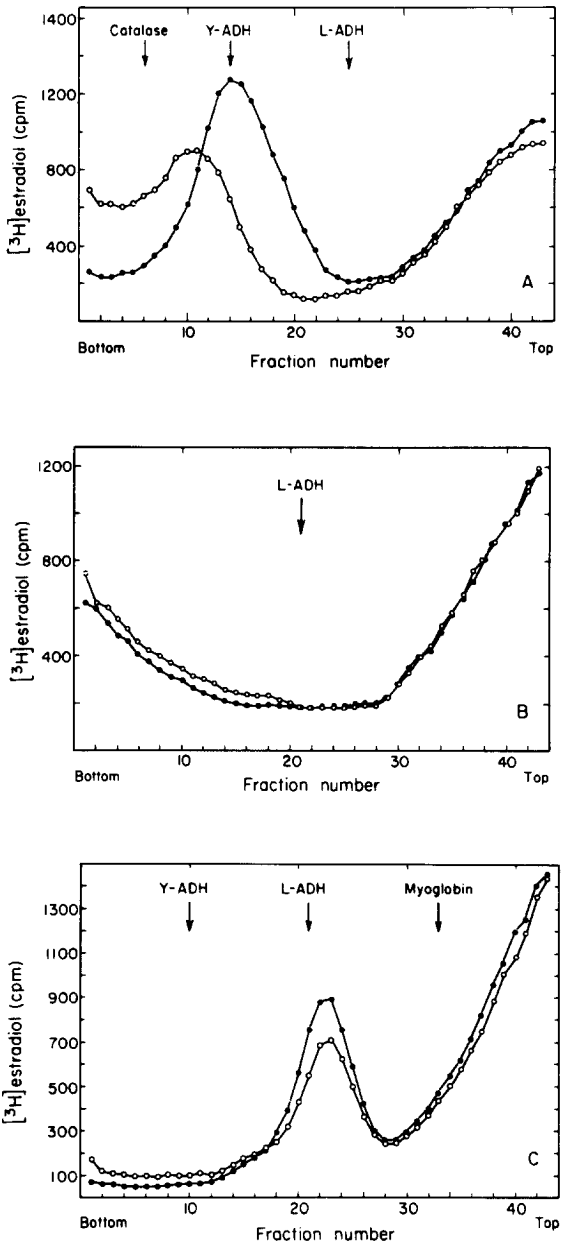


Figure 2. Sucrose density gradient sedimentation of partially purified EBP. (A) EBP was redissolved in 10 mM Tris, 1.5 mM EDTA, pH 7.4 (o—o—o—o) or in 10 mM Tris, pH 7.4 (●—●—●—●). Gradients of 10-30% sucrose in 10 mM Tris, 1.5 mM EDTA were centrifuged at 174,000 x g for 18.5 hr at 4° C. (B) EBP was redissolved in 10 mM Tris, 1.5 mM EDTA and centrifuged at 174,000 x g for 23 hr in gradients of 10 mM Tris, 0.4 M KCl, pH 7.4 (●—●—●—●) or 10 mM Tris, 1.5 mM EDTA, 0.4 M KCl, pH 7.4 (o—o—o—o). (C) EBP was redissolved in 10 mM Tris, pH 7.4 and centrifuged at 174,000 x g for 23 hr at 4° C in gradients of 10 mM Tris, 0.4 M KCl, pH 7.4 (●—●—●—●) or 10 mM Tris, 1.5 mM EDTA, 0.4 M KCl, pH 7.4 (o—o—o—o). Y-ADH, L-ADH, catalase and myoglobin were used as standards to estimate sedimentation co-efficient.

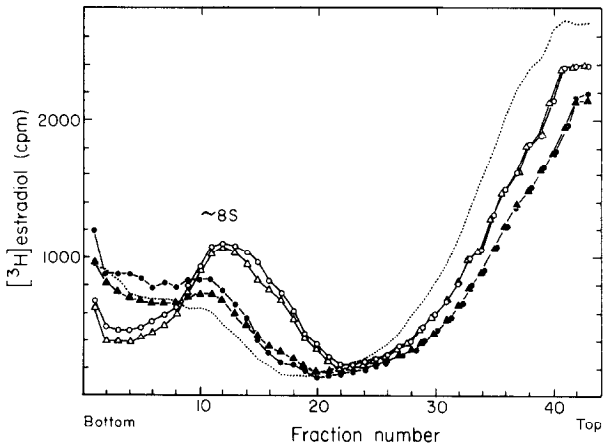


Figure 3. Sucrose gradient sedimentation of partially purified EBP. All gradients were centrifuged at 174,000 x g for 18.5 hr at 4° C. EBP was redissolved in 10 mM Tris, pH 7.4 (Δ—Δ—Δ—Δ) or 10 mM Tris, 1.5 mM O-phenanthroline, pH 7.4 (.....) or 10 mM Tris, 1.5 mM O-phenanthroline, 4 mM Zn^{++}, pH 7.4 (o—o—o—o) or 10 mM Tris, 1.5 mM O-phenanthroline, 4 mM Ca^{++}, pH 7.4 (▲—▲—▲—▲) or 10 mM Tris, 1.5 mM O-phenanthroline, 4 mM Mg^{++}, pH 7.4 (●—●—●—●). Y-ADH and catalase were used as standards to estimate sedimentation co-efficient.

in the original cytosol preparation containing EDTA, it undergoes marked aggregation with EDTA when analysed in the presence of 0.4 M KCl or after ammonium sulfate precipitation as reported here and elsewhere (7,8). Also once the partially purified EBP is recovered in the form of 8S, further exposure to EDTA by centrifuging on gradients containing EDTA does not cause aggregation. Therefore it is highly likely that the metal ion is more tightly bound to the 8S EBP oligomer or less exposed in low ionic environment. This leads us to speculate that the attachment of individual subunits of EBP that give rise to the various oligomers may depend on the orientation of the metal ion. In this context, it is necessary to point out that the 8S oligomer may consist of non-identical subunits of EBP and the exact role of the metal ion must await final identification and characterization of the individual subunits of EBP. It is also possible that the metal ions and the chelating agents may be exerting their effects on other proteins that are non-specifically associated with the EBP.

However, if it indeed turns out that Zn^{++} or a similar metal ion is associated with the EBP, it has implications in the general mechanism of steroid hormone action. It is known that after the binding of estradiol the EBP is associated with the chromatin and the DNA and more specifically to certain polydeoxynucleotides (9, 10-12). Several other steroid receptor complexes also translocate to the nucleus where they are believed to act as modulators of gene expression (2). Most nucleotidyl transferases analysed so far appear to be metalloenzymes with bound Zn^{++} (13) and it has been suggested that the initiator binding site on the terminal transferase may be the metal sensitive site (14). Similarly the presence of a bound metal ion and its exchangeability in the steroid receptor molecule may provide a new probe for the investigation of the molecular basis of hormone action.

ACKNOWLEDGEMENTS

I wish to thank Dr. R.D. Cole for his constant encouragement and helpful discussions during the course of this work. Helpful critique of the manuscript was provided by Dr. W.J. Rutter. I also wish to thank Emily Reid and John Underhill for the preparation of illustrations. This work was supported by National Institutes of Health grant CA-05388.

REFERENCES

1 Jensen, E.V., and DeSombre, E.R. (1972) Annu. Rev. Biochem., 41, 203-230.

2 Raspe, G., ed., Advances in the Biosciences, 7 (Pergamon-Vieweg, Braunschweig, Germany).

3 Shyamala, G., and Nandi, S. (1972) Endocrinology, 91, 861-867.

4 Wittliff, J.L., Gardner, D.G., Battema, W.L., and Gilbert, P.J. (1972) Biochem. Biophys. Res. Commun., 48, 119-125

5 Martin, R.G., and Ames, B.N.((1961) J. Biol. Chem., 236, 1372-1379.

6 Siegel, L.M., and Monty, K.J. (1966) Biochim. Biophys. Acta, 112, 346-362.

7 Erdos, T. (1968) Biochem. Biophys. Res. Commun., 32, 338-343.

8 DeSombre, E.R., Puca, G.A., and Jensen, E.V. (1969) Proc. Natn. Acad. Sci., 64, 148-154.

9 Shyamala, G., and Gorski, J. (1969) J. Biol. Chem., 244, 1097-1103.

10 Shyamala-Harris, G. (1971) Nature New Biology, 231, 246-248.

11 Toft, D. (1972) J. Steroid Biochem., 3, 515-552.

12 Slyuser, M., Evers, S.G., and Nijssen, T. (1974) Biochem. Biophys. Res. Commun., 61, 380-388.

13 Valenzuela, P., Morris, R.W., Faras, A., Levinson, W., and Rutter, W.J. (1973) Biochem. Biophys. Res. Commun., 53, 1036-1041.

14 Chang, L.M.S., and Bollum, F.J. (1970) Proc. Natn. Acad. Sci., 65, 1041-1048.