Endogenous ecdysteroid receptor binds non-specific DNA as a heteromer, the formation of which is enhanced by ligand and DNA

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Abstract. Chromatography of nuclear extracts from Calliphora vicina on heparin-agarose separates two fractions binding ponasterone A. One does not bind calf-thymus (ct) DNA, has a molecular mass of 100 kDa, and may represent an ecdysteroid receptor monomer. The other binds to ctDNA and is 180 kDa, indicating this to be a receptor complex of two or more heteromeric partners. The formation of this DNA-binding complex is promoted by the presence of high-affinity ligand and of DNA itself.

INTRODUCTION

The ecdysteroid receptor (EcR) from Drosophila melanogaster was recently cloned (Koelle et al., 1991) and shown to be a member of the steroid receptor superfamily (Evans, 1988). EcR belongs to the type II subclass of steroid receptors, according to the half-site sequence of the response element to which it binds on DNA. In common with other type II receptors such as the thyroid hormone, retinoic acid, and vitamin D, receptors, EcR binds to its response element as a heterodimer, in Drosophila its dimer partner is ultraspirecle (USP) (Yao et al., 1992; Thomas et al., 1993), the Drosophila homologue of retinoid X receptor (Oro et al., 1990). The details of heterodimer formation and its binding to DNA are not well understood, although recent work has demonstrated a degree of interdependency between EcR, USP, and the ecdysteroid ligand (Yao et al., 1993). We have determined that in extracts of another fly species, Calliphora vicina, two proteins binding ponasterone A are in monomeric and heteromeric states. Their possible identity with ecdysteroid receptors is discussed.

MATERIALS AND METHODS

Ecdysteroids

20-Hydroxyecdysone was purchased from Simes (Milan, Italy). 26-[125I]iodoponasterone A (specific activity 2,175 Ci/mmol) was synthesised from 26-mesylinokosterone (from S.-S. Lee) by radioiodination, according to the method of Cherbas et al. (1988 and Peter Cherbas, pers. comm.). [3H]Ponasterone A (sp. act. 134 Ci/mmol) was synthesised as described previously (Terentiev et al., 1993). The tritiated radioligand was used at a radiochemical purity of at least 98%. This was achieved by reversed-phase hplc purification (column: Merck LiChroCART 250 x 4 mm cartridge, LiChrospher 100RP-18, 5 µm; mobile phase: methanol : water, 1:1 [v/v]; isocratic, 0.9 mL/min). After initial synthesis and purification, the radio-iodinated tracer was used for up to three months.

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Insects

Receptor extracts were prepared from 3rd instar larvae of Calliphora vicina (blue blowfly), which were cultured as described (Koolman & Karlson, 1975). The larvae were taken on the 8th day after hatching, as late as possible (1–3 h) before the onset of pupariation.

Isolation of ecysteoid receptor

Ecysteoid receptor was prepared by isolation and salt extraction of nuclei from whole Calliphora larvae, essentially following the method of Lehmann & Koolman (1988). Such crude receptor preparations are able to metabolise the radioligand used to detect and measure the receptor; therefore it was necessary to purify the receptor partially in order to obtain a preparation for further analysis and this was achieved by anion-exchange chromatography (Lehmann & Koolman, 1988). The nuclear salt extracts (approx. 25 mL from 100g larvae) were dialysed against 3 x 800 mL buffer B (10 mM Tris-HCl, pH 7.4, with 1.5 mM EDTA), each time for 1 h. Precipitated proteins were removed by centrifugation (12,000 g, 10 min.) and the soluble fraction was loaded onto a 10 mL column of DEAE-Sephael (Pharmacia), which had been equilibrated in buffer B, at 15 mL/h. After washing with buffer B, the receptor was eluted in the reverse direction with B + 250 mM KCl and dialysed overnight with 3 x 800 mL buffer B. Dithiothreitol was added to 7 mM, and the receptor was stored in 1 mL aliquots at −70°C.

Receptor assays

Two types of assay were used to assess the amount of receptor in samples. For routine measurement, specific binding of [3H]ponasterone A (10 nM) was determined by a dextran-coated charcoal assay (Lehmann & Koolman, 1988), with 20-hydroxyecdysone (30 μM) being used to estimate non-specific binding. For samples containing lower levels of receptor, the specific binding of 26-[3H]iodoponasterone was measured using a filter assay: 5 μL (approx. 530,000 cpm) radioligand was added to 215 μL receptor sample, and incubated for 2 h at 20°C. Samples were then cooled on ice, and 100 μL aliquots were applied to Whatman GF/A filters. After two minutes, the filters were washed three times with ice-cold buffer B, including 7 mM dithiothreitol, each time for 5 min. The filters were then transferred to tubes for counting in a gamma counter. Again, non-specific binding was determined by inclusion of 30 μM 20-hydroxyecdysone.

Heparin and DNA separations

Heparin-agarose was used either in 1 mL pre-packed “HiTrap” columns (Pharmacia), or purchased separately (from Sigma) and packed as a volume of 5 mL in chromatography columns (Pharmacia). Calf-thymus DNA-cellulose was purchased from Pharmacia and packed either as approx. 1 mL in polystyrene disposable columns (Pierce), or as 5 mL, as for heparin-agarose.

Separations with pre-equilibrated receptor were carried out as follows: receptor samples were incubated to equilibrium with 10 nM [3H]PoA for 2 hours at 20°C in buffer B with 7mM dithiothreitol. Where appropriate, deoxyribonuclease I (Type IV from bovine pancreas, 1,500–2,500 Kunitz units per mg protein; Sigma) was added to a final concentration of 0.1 mg/mL. The samples were put on ice for at least 5 min., and all subsequent steps were carried out at 4°C with ice-cold buffers. Excess free radioligand was removed on a de-salting column (NAP-5, NAP-10 or PD-10 for sample volumes 0.5 mL., 1.0 mL, or 2.5 mL respectively), and the void volume was applied immediately to the equilibrated heparin or DNA column. Fractions were collected, and aliquots taken for radioassay.

The stock buffer for the heparin and DNA separations, buffer A, was 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, pH 7.4 (after Sorger et al., 1989). This was used with added ammonium sulphate, denoted A, where x is the millimolar concentration of ammonium sulphate.

Size-exclusion chromatography

All separations were carried out at 4°C on a Superose 12 10/30 pre-packed column (Pharmacia), eluting with 200 mM K2HPO4 containing 400 mM KCl, pH 7.2 (Landon et al., 1988) at 0.5 mL/min. using a Pharmacia P-500 pump. Samples were applied with a V7 sample injection valve. Fractions of 250 μL were collected in mini-scintillation vials (Packard), 2 mL scintillation cocktail (Rotiszint, Roth) was added, and radioactivity was determined in a liquid scintillation counter.

The column was calibrated with dextran blue (V₀, 7.6 mL), DNP-alanine (V₁, 40.7 mL), and eight standard proteins of known molecular mass, with detection by absorbance at 280 nm.
RESULTS AND DISCUSSION

Heparin-agarose chromatography of ecdysteroid receptor preparations which had been pre-equilibrated with radioligand showed two separable species when eluted with a linear gradient of A0 to A600 (buffer A with 0 to 600 mM ammonium sulphate; see Methods). Separation of the two receptor species in a ratio of approx. 1:1 was achieved by stepwise elution with A215 and A600 (Fig. 1a). Non-specifically bound radioligand eluted in the washing steps, and both peaks of radioactivity were shown to be associated with a macromolecular fraction by their elution in the void volume of de-salting columns. The high salt peak did release some free radioligand, but this had probably dissociated from the receptor during elution, as the interaction between the receptor and the ligand is very salt-labile (e.g. Dinan, 1985). It appears then that ligand-bound receptor exists in at least two states which show differing affinity for heparin.

Pre-equilibrated receptor bound to calf-thymus DNA-cellulose, and eluted as one fraction with A105 after the non-binding radioactivity had been washed out (Fig. 1b). If the A105 material eluting from ctDNA was diluted to 50mM ammonium sulphate and applied directly to heparin-agarose, it eluted only with A600; no significant labelled material was eluted with A215 (Fig. 2b). In contrast, heparin chromatography of the non-binding material from the DNA column showed both the A215 and A600 peaks in a ratio of 1:1 (Fig. 2a), as seen without the prior DNA-binding step. If the heparin A215 material was diluted to 50mM ammonium sulphate and applied to ctDNA, a negligible amount bound to the DNA.

These separations show that, of the two receptor species identified from heparin, the high affinity species (denoted HHA) was in a state able to bind DNA. The lower affinity form (HLA) was unable to bind DNA. The presence of a considerable amount of HHA in the DNA non-binding material was not due to overloading of the DNA column, as higher loadings allowed greater binding on the same column, although the amount of liganded receptor which bound to the ctDNA column was one third that eluting from heparin as A600, with equivalent loadings. This may be explained if HHA were already associated with a fragment of DNA from the extraction procedure (see below), thereby preventing its interaction with the ctDNA of the column, so that the ratio of HLA to HHA after DNA
binding would be expected to be 1 : 0.66. That the ratio was maintained as 1 : 1 was possibly due to conversion of some HLA to HHA following the removal of the HHA, and in the continued presence of sufficient free hormone, which also did not bind to DNA. This indicates that an equilibrium may exist between the two forms, which is upset by the removal of HHA, causing more to be produced; the conversion of HLA to HHA must therefore be rapid, and possible at low temperature. Such a view would be supported by previous work in this laboratory, which showed that several cycles of incubation of ecdysteroid receptor with radioligand and ctdNA are required to remove all DNA-binding receptor (Lehmann & Koolman, 1989). The presence of the hormone is important in the conversion of HLA to HHA: heparin chromatography was performed of receptor preparations in the absence of ligand, followed by radioligand assay using 26-[125I]iodoestosterone. This showed specific binding of 9474 cpn (SD = 996, n = 2) for HLA, compared with 1720 cpn (SD = 359, n = 2) for HHA, giving a ratio of HLA : HHA of 5.5 : 1, as opposed to the 1 : 1 ratio in the presence of radioligand (Fig. 1a). This shows that ligand-binding promotes the existence of DNA-binding form.

Molecular mass analysis of the two species eluting from heparin at A215 and A600 showed molecular mass of 100 kDa and 180 kDa, respectively. We believe that the 100 kDa HLA, which does not bind ctdNA, is an ecdysteroid receptor monomer; this size is in close agreement with earlier measurement of the Calliphora receptor in our laboratory (105 kDa; Lehmann & Koolman, 1988), and with that of the Drosophila EcR isoforms A and B1 (105 kDa; Talbot et al., 1993). The 180 kDa DNA-binding HHA (Fig. 3a) is probably a heterodimer, comprising the receptor and possibly the Calliphora homologue of the ultraspiracle protein. The presence of a USP-like protein in our extracts has been indicated by immunological detection on Western blots of a 50 kDa species (Drosophila USP is 55 kDa) using antibodies against Drosophila USP (J. Garwood, pers. comm.). Another possibility is that the partner in the heterodimer is not an ultraspiracle homologue, but a homologue of another member of the steroid receptor superfamily which has been identified in Drosophila (see Segraves, 1991), but for which no function is yet known. It has recently been shown that in vitro-translated Drosophila EcR and USP are able to form an unstable
an unstable complex which is stabilised by ligand-binding (Yao et al., 1993). Our results from heparin chromatography in the presence and absence of [H]PoA confirm the ligand-induced stability of a putative heterodimer with endogenous Calliphora receptor. In addition, we find that DNA may also be important for stabilization of the complex. If, prior to heparin chromatography, a receptor preparation was treated with DNase, the ratio of HLA to HHA in the presence of radioligand was altered from 1:1 to 5:1; SEC chromatography confirms this change by exhibiting a loss of the peak at 180 kDa on DNase treatment of liganded receptor (Fig. 3b), as well as the removal of a large (> 2 x 10^6 kDa) aggregate, seen in Fig. 3a, probably held together by pieces of DNA. This indicates that the heterodimer requires DNA for its assembly; the DNA fragment may be retained in the heparin chromatography as an integral part of the 180 kDa species, and may be responsible for allowing only one third of HHA to bind to the ctDNA column, as some exchange would probably be required between the bound endogenous DNA fragment and the unspecific ctDNA. It has not been possible to determine whether the HHA which binds to the ctDNA column shows a slightly lower molecular weight to the HHA eluting as A600 from heparin.

The DNA-binding studies described here have all used non-specific calf-thymus DNA rather than the specific ecdysteroid response element to which EcR binds specifically and with high affinity. Many sequence-specific DNA-binding proteins show affinity for ctDNA, from which they are eluted at low salt concentration (Sorger et al., 1989), and this may reflect a physiological situation in which the protein attaches itself loosely to non-specific DNA sequences as part of a process of scanning the DNA for its unique binding site sequence. If even such a loose interaction was able to promote the formation and/or stability of a liganded receptor heterodimer, then the specific response element would be able to receive the ready-assembled complex. In addition, although the response element binds tightly, it may not be responsible for holding the complex together (a function possibly precluded by its short length), as this could be achieved by adjacent non-specific sequences.

The assembly of the ecdysteroid receptor-DNA complex appears to be a much more involved process than was expected, with interaction between at least four components.
being necessary for its stability. This provides the basis for a number of control mechanisms to regulate the eventual success of the ecdysteroid response, and it is this potential for such precise mediation of the hormonal signal which is under increasingly intense study.

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REFERENCES


