The Ultraspiracle protein (USP), a orphan nuclear receptor, is the insect ortholog of the vertebrate retinoid X receptor (RXR). Like RXR, it belongs to the superfamily of nuclear receptors (NR) (1), which are intracellular receptors regulating target gene expression upon binding of small, hydrophobic molecules like steroids, retinoids, thyroid hormones, and vitamin D3. In insects, steroid hormones, the ecdysteroids, control insect development, molting, metamorphosis, and reproduction (2–4). The weak binding of 20-hydroxyecdysone, the biological activity of this steroid hormone is mediated by two nuclear receptors: the ecdysone receptor (EcR) and the Ultraspireacle protein (USP). The crystal structure of the ligand-binding domain from the lepidopteran Heliothis virescens USP reported here shows that the loop connecting helices H1 and H3 precludes the canonical agonist conformation. The key residues that stabilize this unique loop conformation are strictly conserved within the lepidopteran USP family. The presence of an unexpected bound ligand that drives an unusual antagonist conformation confirms the induced-fit mechanism accompanying the ligand binding. The ligand-binding pocket exhibits a retinoid X receptor-like anchoring part near a conserved arginine, which could interact with a USP ligand functional group. The structure of this receptor provides the template for designing inhibitors, which could be utilized as a novel type of environmentally safe insecticides.

The major postembryonic developmental events happening in insect life, including molting and metamorphosis, are regulated and coordinated temporally by pulses of ecdysone. The biological activity of this steroid hormone is mediated by two nuclear receptors: the ecdysone receptor (EcR) and the Ultraspireacle protein (USP). The crystal structure of the ligand-binding domain from the lepidopteran Heliothis virescens USP reported here shows that the loop connecting helices H1 and H3 precludes the canonical agonist conformation. The key residues that stabilize this unique loop conformation are strictly conserved within the lepidopteran USP family. The presence of an unexpected bound ligand that drives an unusual antagonist conformation confirms the induced-fit mechanism accompanying the ligand binding. The ligand-binding pocket exhibits a retinoid X receptor-like anchoring part near a conserved arginine, which could interact with a USP ligand functional group. The structure of this receptor provides the template for designing inhibitors, which could be utilized as a novel type of environmentally safe insecticides.

Unlike its vertebrate homolog RXR for which a ligand is known, the 9-cis-retinoic acid (9-cis-RA), no hormone ligand has been identified for USP up to now. Juvenile hormone (JH), an esterified sesquiterpene, has been put forward as the candidate hormone ligand of USP. In fact, it has been known for a long time that JH prevents metamorphosis by modulating the ecdysteroid action at the outset of the ecdysteroid rise for the molt (3, 12–14). The hypothesis that JH might act through a NR relies on its chemical analogy with the vertebrate terpenes, represented by the retinoic acid. The idea that USP could be the receptor of JH or any of its derivatives raises the attractive possibility that JH might directly modulate the activity of the EcR/USP complex (15). In addition, some evidence was given that JH can bind to USP and stimulate oligomerization of USP in vitro and in yeast cells (16). However, this is largely debated and still awaits further substantiation (2). In particular, the dissociation constant for binding of JH to USP was measured to be about 0.5 μM (16). Compared with the typical affinity of hormones for their nuclear receptor (in the nanomolar range), this low affinity questions whether such concentrations might be meaningful at a physiological level, where other cellular molecules might compete with JH for USP binding.

NRs are modular proteins possessing a highly conserved DNA-binding domain and a moderately conserved ligand-binding domain (LBD) (17). The LBD confers specificity to ligand binding and is responsible for ligand-dependent transactivation. Crystallographic investigations of the LBD structures of several NRs indicate a conserved fold described as an antiparallel α-helical sandwich (18) composed of 11 α-helices (H1, H3–H12) and two short β-strands (s1–s2). Most importantly, the crystal structures of the LBDs of unliganded (apo) (18) and liganded RXR (19) and of other liganded NRs (for a review, see Refs. 20 and 21) have allowed us to gain insight into the molecular mechanisms that underlie the dramatic structural reorganization that accompanies the ligand binding to the LBD. This conformational rearrangement mostly affects the N-terminal part of H3, H11, and H12, which carries the autonomous activation function (AF-2) (18, 19, 22). Upon binding of an agonist ligand, the corepressors are released, and the LBD adopts a unique conformation that generates an interaction surface for the coactivators, which then recruit multiprotein complexes and lead to the activation of responsive genes (23). In contrast, antagonist ligands induce a transconformation of
the LBD that does not allow binding of coactivators. Several antagonist conformations have been observed that can be considered variations around a common theme (24–27). In these cases, the activation helix H12 lies in a groove similar to the binding site of the helical NR-box module of nuclear coactivators (the so called antagonist groove) (25, 28, 29). In the case of partial agonist/antagonist ligands, helix H12 also lies in this groove, even though the ligands do not sterically preclude the agonist position of H12. However, in contrast to the full antagonist ligands, a weak but clear transcriptional AF-2 activity is predicted for USP LBDs using the canonical structure of NR LBDs (22%); and trace amounts of C17:0, C18:0, C18:1, and C18:2 are detected as well. In the case of phosphatidylglycerol molecules, the major species detected by Time-of-flight mass spectrometry (745 ± 3 Da) correspond to a molecule with a tail made of C16:0 and C18:1 esterified fatty acids, fully consistent with the results of the organic solvent extraction and with the electron density maps.

RESULTS AND DISCUSSION

Structure-based Sequence Alignment—The present crystallographic investigations of hvUSP were restricted to the LBD, which comprises 264 residues starting at Val-205. The sequence of the hvUSP LBD is shown in Fig. 1 and is aligned to the sequences of other USP LDBs of the insect orders Lepidoptera and Diptera and to sequences of RXR of isotypes α, β, and γ. The sequences of USP LDBs altogether are rather well conserved with respect to those of RXR LDBs (between 34 and 42% sequence identity). However, while the conservation is highly pronounced within the whole RXR family, the USP sequences are highly conserved only inside the lepidopteran family (83–97% sequence identity) those of the diptera family being much less conserved when compared with each other (between 46 and 54% sequence identity) and to the sequences of the lepidopteran USP LDBs (between 40 and 49% sequence identity). On the basis of this sequence alignment, the secondary structure elements, 11 helices and a β-sheet can be predicted for USP LDBs using the canonical structure of NR LBDs.
The crystal structure of hvUSP LBD supports these predictions, and the secondary structure elements are represented schematically in Fig. 1, together with those of hsRXRα LBD. As readily shown in this figure, the helix H3 of hvUSP LBD is one turn longer compared with its counterpart in the RXR crystal structure. This figure also indicates that most of the conserved residues between lepidopteran USPs and RXRs are located in the helices, in particular in those forming the core of the LBD as well as within the signature region (17). Divergence between USPs and RXRs is observed mainly for two loops that connect helix H5 to the β-turn (s1) (H5-s1) and helix H1 to helix H3 (L1–3). The loop H5-s1 is longer for USPs than for RXRs. Its length also varies inside the USP family, and it shows rather poor sequence conservation. On the other hand, the length of L1–3 is rather similar for USPs and RXRs. Its sequence is poorly conserved between the two families. However, it is
highly conserved inside the family of lepidopteran USPs to which hvUSP belongs. This is remarkable, because within the whole NR superfamily L1–3 is usually found to be extremely variable in length and in sequence, consistent with its nature of a rather flexible and loosely structured region. In contrast, the crystal structure of hvUSP LBD presented here shows that L1–3 behaves as a rather stiff region due to strong interactions with several key secondary structure elements of the LBD.

Architecture of USP LBD—The overall architecture of USP LBD exhibits the canonical NR fold (18) with 11 α-helices (H1, H3–H12) and two short β-strands (α1–α2). In the following, the structure of USP LBD will be compared with two other crystal structures that bear the major features of NRs and are closely related to it: the agonist-bound (holo) RXRα (hsRXRα/9-cis-RA) (19) (Fig. 2A) and the RXRα in an antagonist conformation (msRXRα/oleic acid) (26) (Fig. 2B) LBDs. The superimposition of the USP LBD to the structure of holo-RXRα LBD was done by a least square fit (using the LSQ options of O (32)). Overall, the secondary structure elements of the USP LBD superimpose rather well with those of holo-RXRα LBD.

FIG. 2. Stereo views showing the superimposition of the crystal structure of USP with those of agonist (A) and antagonist-bound (B) RXRα LBDs. α-Helices are represented as cylinders and β-sheets as arrows. The overall structure of USP LBD is colored in dark yellow. Helices are labeled accordingly. The structure of RXRα/9-cis-RA (19) is depicted in blue (A) and that of RXRα/oleic acid (26) in green (B). Notice that for these two RXRα structures helix H3 is represented by two unconnected tubes to better account for the considerable bending of the N and C termini toward the protein core. This figure as well as Figs. 3 and 4 were generated with the program SETOR (41).
square deviation (r.m.s.d.) is 1.22 Å for 183 matched Co's atoms out of 246. Seven helices match rather well (r.m.s.d.: 1.13, 0.88, 0.57, 1.18, 0.67, 0.69, 0.75 Å for H4, H5, H7–H11, respectively). The helices H1, H3, H6 and the β-sheet show larger deviations. The activation helix H12 adopts a conformation similar to that observed in RXRa in the antagonist conformation. However, USP harbors at the same time features characteristic of agonist-bound NR LBDs, namely the length of H11 closer to that of agonist-bound RXRa and the positioning of the phenylalanine residues at its C-terminal part. The coexistence of features related to both agonist and antagonist-bound NR LBD structures is a unique and remarkable property of USP, which will be discussed below in more detail.

The helices H1 and H3 contribute to the outermost shell of the LBD. They are less coplanar for USP than for RXRa, the angle between their helical axes being 12.1° larger than the corresponding value in RXRa. This is clearly correlated to the path adopted by the loop L1–3 connecting H1 to H3. It induces a considerable difference in the positioning of the N- and C-terminal parts of H3 compared with agonist and antagonist-bound RXRa LBDs, resulting in a more straight helix, as depicted in Fig. 2. The N-terminal region of H3 (Pro-240 to Cys-250) is displaced outwards the protein core in a substantial manner. It is tilted by about 24° with respect to the same region in holo RXRa. This position is intermediary between the positions of the N-terminal region seen in the apo RXRa and the holo RXRa LBD structures (data not shown). The outward bending of the C terminus of H3 (by about 10°) has repercussions on the positioning of the neighboring loops, L3–4 and L8–9. The loop L3–4, which is part of the signature region of NRs (17), is displaced laterally by about 1.8 Å and bent in the direction of L8–9, which itself is pushed outwards by about 1.5 Å.

The conformation adopted by the connecting loop L1–3 is unusual and essential for the stabilization of the actual structure of USP LBD. This contrasts with the observation that this loop usually behaves, in most NRs, as a very flexible region. For hsRXRa, the crystal structures of both apo (18) and holo (19) conformations show substantial differences in the region connecting helices H1 and H3. In the holo-RXRα LBD structure, L1–3 consists of an extended loop passing above the β-sheet. The apo form exhibits an additional helix in this region, which unfolds in the holo form. In the apo to holo transition, L1–3 also moves substantially. As suggested from the comparison of the apo-RXR and the holo-RXR and RAR LBD structures, L1–3 might act as a molecular spring accompanying the conformational changes which take place upon ligand binding (19, 22). For ligand-bound RARγ LBD, the conformation of L1–3 is similar to that of holo-RXRα, except that it contains a C-terminal region forming a so-called Ω-loop (22). Interestingly, for ER LBDs, L1–3 follows a different path than in the retinoic acid LBD, the conformation of L1–3 is similar, except that it contains a C-terminal region forming a so-called Ω-loop (22).

For the USP LBD, L1–3 adopts none of these conformations. Its path (Val-220 to Pro-239) was unambiguously inferred from electron density maps, as demonstrated in Fig. 3A. Only a few residues at the beginning of the loop, Asp-222, Pro-223, and Ser-224, were included as alanines due to the weak electron density of their side chains. Accordingly, the temperature factors of these residues are higher (60–64 Å²) than those of the other amino acids of L1–3 (36 Å²) in average over L1–3. The first residues composing L1–3 form a path that crosses the region of H3 forming Gln-256 to Val-262. The next residues (Glu-226 to Pro-234) delineate an extended loop that runs along H3. Finally, the last five residues of L1–3 (Asp-235 to Pro-239) form a loop rather similar to the Ω-loop observed in the RARγ LBD (22). L1–3 adopts a rather tensed conformation, which allows it to establish direct contacts with residues in helices H3, H11, and H12 and stabilize them in their actual position (see Fig. 3B). This is important, because these helices are the structural elements that are shown to undergo the largest conformational changes upon ligand binding (19).

The observed conformation of L1–3 is relevant to the physiological state of the receptor, since no direct crystal contacts that could induce this conformation are observed in the region of L1–3. Furthermore, in a region farther away from this loop, the USP LBD and one of its symmetry-related molecules interact through their β-sheets. This contact can most certainly take place, because L1–3 does not occupy this region in solution. If L1–3 was forced to swing during crystallization and move from a conformation similar to that of RXRa to its actual conformation, then several secondary structure elements would need to move dramatically. This drastic reorganization of the whole LBD is very unlikely to happen, especially because L1–3 is located in a region of the LBD where it makes very specific interactions with neighboring secondary structure elements involving many conserved residues (see below).

The USP Ligand and the Ligand-binding Pocket—The unexpected ligand molecule, which was copurified and cocrystallized with USP LBD, is a phospholipid. A phosphatidylglycerol and a phosphatidylethanolamine are consistent with the crystallographic data, and the results of the mass spectroscopic and chemical analysis. In a similar way, recent crystallographic investigations of the heterodimer RARα/RXRb LBD reveal an E. coli-endogenous oleic acid (C18) or a closely related compound (stearic (C18) or palmitic (C16) acid) in the RXRa subunit (26). It is important to notice that even though this molecule is not the true ligand for this vertebrate NR, it induces and stabilizes an antagonist AF-2 conformation, which is most probably significant of the structure of the antagonist-bound RXRa LBD. In our case, the best fit of the electron density was obtained with a phospholipid with a tail composed of two fatty acids of a length corresponding to 18 and 16 carbon atoms, respectively. The longer fatty acid has a rather twisted shape with two major kinks, while the other fatty acid, bent inside the pocket, has a more regular shape. The tail of the phospholipid is buried inside the ligand-binding pocket, whereas its head is located at the very entrance of the cavity, as shown in Fig. 4A. The residues interacting with the phospholipid ligand are denoted by blue dots in Fig. 1. van der Waals contacts are observed between the ligand and residues in helices H3, H5, H7, and H11 in the β-sheet and in the loops L1–3, L6–7, and L11–12. One polar residue, Gln-338 (H6) is hydrogen-bonded to the ethanolamine moiety in the case of a phosphatidylethanolamine and to the phosphoryl-glycerol moiety in the case of phosphatidylglycerol. Almost all of the residues interacting with the ligand are conserved among the lepidopteran USPs. Exceptions are Phe-242 replaced by Tyr for other lepidopteran USPs, Met-325 replaced by Ile for hmUSP, and Ser-431 replaced by Cys in msUSP. The ligand-binding pocket is lined with a few other polar residues, which do not make contacts with the phospholipid ligand, but which might be crucial in the interaction with other types of ligands. These residues, shown in green in Fig. 4A, are located close to the C-terminal side of H5 (Gln-256 in H3 and Asp-297 in H5), in the vicinity of H12 (Asn-287 in H5, Ser-428, and Ser-431 in H10 and His-434 in H11), and at the entry of the pocket (Ser-435 and Gln-338 in H6).

It is interesting to notice that among the 16 residues of RXRa LBD, which are reported to interact with 9-cis-RA (19) (denoted by green dots in Fig. 1), only 4 of the sequence equivalent residues of hvUSP interact with the phospholipid (Leu-249,
Fig. 3. The loop L1–3 connecting helices H1 and H3. A, stereo view of the 1.65-Å resolution 2Fo − Fc electron density map contoured at 1.0 S.D., showing the quality of the data in the C-terminal region of the loop L1–3. B, detailed view of the specific interactions of the connecting loop L1–3 with helices H3 and H11. Protein atoms are colored in light gray for carbon, blue for nitrogen, and red for oxygen. In B the protein backbone is colored in yellow, water molecules are drawn as red spheres, and hydrogen bonds are depicted as green dotted lines. A few key residues are labeled accordingly.
FIG. 4. The ligand-binding pocket and the USP ligand. A, detailed view showing the phospholipid ligand and the residues inside the pocket. Residues closer than 4.0 Å from the ligand are depicted in blue and labeled accordingly. Polar residues inside the cavity, which do not interact with the ligand, are shown in green and indicated with green labels. The phospholipid ligand is colored in gray for carbon, red for oxygen, and green for phosphor atom. The protein backbone is colored in dark yellow. H3, H5, H6, H7, H10, and L1–3 are indicated. B, view showing the location of the phospholipid molecule in the ligand-binding pocket of USP relative to that of 9-cis-RA in the RXR cavity. The phospholipid ligand and the 9-cis-RA are colored in gray and blue. The conserved arginine belonging to H5 is also shown in this figure as a ball and stick representation. The color scheme for the atoms is: gray, carbon; red, oxygen; green, phosphor; blue, nitrogen. C, a stereo view of the superimposition of USP/phospholipid and RXRa/9-cis-RA in the region of the conserved arginine belonging to helix H5. The protein backbones of USP and RXRa are represented by orange and blue ribbons, respectively. The phospholipid ligand is depicted in yellow, and the 9-cis-RA is colored in light gray for carbon, blue for nitrogen, and red for oxygen. Residues belonging to RXRa are shown in blue, while those of USP are colored in light gray for carbon, blue for nitrogen, and red for oxygen. Water molecules are drawn as red spheres, and hydrogen bonds are depicted as green dotted lines. A few key residues are labeled in blue for RXRa and in black for USP.
lignand-binding pocket, as calculated by VOIDOO (37), is about a factor 2.5 larger than that of haRXRa LBD (1256 Å (3) for hvUSP compared with 489 Å (3) for hsRXRa (19) but comparable with the size of the peroxisome proliferator-activated receptor-γ ligand cavity (~1300 Å (3)) (28). The large size of the ligand-binding pocket of USP is a consequence of its unusual topology. In fact, the USP cavity is composed of a part that is similar to the ligand-binding pocket of RXRa, although slightly wider, and a bulky tube, which extends to the solvent-exposed region between L1–3, H3, H6, and H11. For RXRa, the ligand-binding pocket is closed by essentially three hydrophobic residues (corresponding to Val-246 (H3), Val-341 (L6–7), and Phe-439 (H11)) in hvUSP, while for USP, this region is occupied by the ligand. The USP cavity is widely open with a large cleft between H3 and H6. For RXRa and other NRs, this region forms tight contacts with the connecting loop L1–3. Notice that a largely open lignad cavity has also been observed for the ER complexed to the antagonist hormone relaxifene (24).

The binding of the ligand in the USP LBD is mostly responsible for the conformation of the N-terminal part of helix H3, which is significantly displaced outwards from the protein core compared with the ligand-bound RXRa. This shows the adaptation of the N-terminal end of H3 accompanying the binding of the ligand and suggests the mechanism of the ligand entry in the cavity. For RXRa, the comparison of liganded versus unliganded conformations indicates a substantial movement of H3 upon ligand binding in an induced-fit mechanism to clamp the ligand and lock it inside the pocket (19, 22). In the case of USP, the displacement of the N terminus of H3, which accompanies the binding of the bulky phospholipid ligand, results in a more widely open clamp. As it is apparent from the structure, the phospholipid most likely penetrates the ligand cavity from the channel formed by helices H3, H6, H11, and loops L1–3 and L6–7. This is in agreement with the path followed by the 9-cis-RA when entering the receptor cavity as described for RXRa (19, 22, 38). According to this dynamic model, the ligand would enter the cavity through a cleft formed by the breathing of H3 and H10/H11 and then be trapped in the observed antagonistic conformation.

The Antagonist Conformation of USP LBD—The structure of the USP LBD reveals that the helix 12 adopts a conformation observed in the case of several NRs, such as RXRa/oleic acid (Fig. 2B), RARα/BMS614 (26), and antagonist-bound ER LBDs (24, 25, 27). The groove in which H12 lies is the binding site for the helical NR-box module of nuclear coactivators with a consensus sequence LXXLL, as shown for the LBDs of peroxisome proliferator-activated receptor-γ (28), TRβ (29) and ERs (25). Similarly, in hvUSP, Ile-450, Ala-453, and Leu-454 of H12 lie in approximately the same relative locations as the first, second, and third leucine residues of the LXXLL binding motif (LXXAL instead of LXXLL). As for other antagonist NR conformations, helix 12 packs on a groove formed by residues from helices H3, H4, and the loop L3–4 (Val-261, Arg-265, Met-275, Glu-276, Ile-279, Ile-282, Lys-283). However, for USP, the loop L1–3 also participates in the groove topology, where a few residues (Phe-227, Gln-228, and Phe-229) make van der Waals contacts with H12.

The length of helix H12 is identical to that of antagonist RXRa LBD. However, the structural principle encountered in other antagonist NR LBDs, by which helix H11 unwinds to allow H12 to bind to the coactivator NR box LXXLL motif binding groove, does not fully apply in the case of the USP LBD. Helix H11 is more in the continuity of H10 and superimposes very well to helix H11 of the agonist-bound RXRa LBD, being only two residues shorter than in this case. It follows that the region connecting H11 to H12 is 6 residues long (His-439 to Ile-445, Ser-431, and His-434). The reason for this behavior lies mostly in the different positioning of the ligands in their respective pocket, as readily shown in Figs. 4, B and C, and 5. The 9-cis-RA is buried deep inside the pocket, where its carboxylate group establishes a salt bridge with the conserved arginine residue belonging to helix H5 of RXRa LBD. In contrast, the phospholipid does not penetrate as deep inside the cavity. As an illustration, the tail of the longer fatty acid is located approximately at the position of atom C9 of 9-cis-RA in RXRa LBD, while the tail of the other fatty acid goes as deep as about the β-ionone ring of 9-cis-RA (Fig. 4B). As a consequence, the arginine residue belonging to H5 in hvUSP LBD, Arg-297, does not participate to the anchoring of the ligand, as it is observed for holo RXRa (19) and RARγ (36). Despite the fact that it does not interact with the ligand, this residue adopts a position close to that of the homologous arginine residue in holo-RXRa, which definitely differs from the solvent-exposed position of the apo RXRa conformation (19). Instead of making contacts with the ligand, Arg-297 is hydrogen-bonded to the backbone carbonyl group of Leu-325 (β-sheet) and involved in a water-mediated hydrogen bond network comprising the carbonyl group of Leu-290 (H5) and the side chain of Gln-256 (H3) (Fig. 4C). In particular, two of the structural water molecules, which are involved in these interactions, are positioned about where the two oxygen atoms of the carboxylate group of 9-cis-RA in RXRa LBD would be located, as can be seen in Fig. 4C. The side chain of Gln-256 is positioned inside the ligand-binding pocket with respect to the position of the homologous residue in RXRa, but it could re-orientate as in RXRa to allow a carboxylate group or another functional group to interact with Arg-297.

A superimposition of the cavities of USP and RXRa LBDs is depicted in Fig. 5. The probe-occupied volume of the USP
Thr-444). These amino acids span a 12-Å strand, resulting in an extended conformation for L11–12. The C terminus of H11 contains 3 phenylalanine residues, which are conserved in RXRα. In the case of apo RXRα, the side chains of the first two phenylalanine residues fill the hydrophobic ligand-binding pocket, and the third one is solvent exposed, whereas in the agonist form they exchange their roles (19). The situation in the USP LBD is similar to that of agonist RXRα LBD: Phe-436 and Phe-437 are exposed to the solvent, while Phe-438 contributes to the binding pocket. The side chain of Phe-438 is only slightly rotated with respect to its counterpart in RXRα to face the ligand at the level of its shorter fatty acid. For the antagonist-bound RXRα LBD (26), only the first of these three phenylalanine residues belongs to H11. The other two residues which are part of L11–12 contribute to the ligand-binding pocket and in this antagonist conformation would make a steric clash with the phospholipid ligand.

The Connecting Region L1–3 Precludes the Agonist Conformation—Unlike in all other NR LBDs known up to now, one structural element, L1–3, plays a crucial role in USP. From the superimposition of USP and holo-RXRα LBDs (Fig. 2A), it can be observed that a few residues of hvUSP L1–3 (Asn-237, Ser-236, and Phe-229) lie in approximately the same locations as residues belonging to L11–12 of holo-RXRα (Asp-444, Thr-445, and Phe-450, respectively). This comparison leads to a very intriguing consequence: in its actual conformation, L1–3 precludes the existence of the agonist conformation, since it would sterically interfere with the loop L11–12. The steric hindrance for H112 to adopt the agonist position is therefore due to a constitutive element of the USP LBD structure. The connecting loop L1–3 stabilizes the N terminus of H3 through a hydrogen bond network with Arg-243 and Asn-254 of H3, as depicted in Fig. 3B. The guanidinium group of Arg-243 is anchored by strong hydrogen bonds to the backbone carbonyls of Gly-233, Ser-236, and Val-238 and exhibits a van der Waals contact with the side chain of Val-232. In addition, the backbone amide group of Arg-243 is hydrogen-bonded to the carbonyl group of Pro-239. For Asn-254, its side chain makes direct hydrogen bonds with the backbone carbonyl group of Leu-230, the amide group of Phe-229, and to the side chain of Gln-228. The backbone carbonyl group of Asn-254 is hydrogen-bonded to the side chain of Glu-226.

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The loop L1–3 is also involved in contacts with the N-terminal part of H11 and L11–12 through the residues Gln-228 to Arg-231 and Asp-235 to Asn-237, as shown in Fig. 3B. The backbone carbonyl group of Gln-228 is hydrogen-bonded to the side chain of Ala-442, while that of Phe-229 binds to the NH group of Ala-442. Furthermore, Arg-231 plays a central role by establishing strong stabilizing interactions with L11–12: its backbone amide group binds to the carbonyl group of Leu-440, while its side chain is hydrogen-bonded to the carbonyl group of His-439 and establishes a van der Waals contact with Val-441 and Ala-442. Other interactions involve the backbone carbonyl group of Asp-235 with the side chain of His-439 and a water-mediated interaction with Val-441. The hydroxyl group of Ser-236 also makes a van der Waals contact with the side chain of Leu-440.

It is important to notice the high sequence conservation among all the residues involved in the interactions of L1–3 with H3 and with L11–12. In the case of the interactions of L1–3 with H3, the main interacting residues of H3, Arg-243 and Asn-254, are strictly conserved for all lepidopteran USPs. Similarly, their interacting partners in L1–3 (Glu-226, Phe-227, Gln-228, Phe-229, Leu-230, Val-232, Gly-233, Ser-236, Val-238, Pro-239) are strictly conserved for all lepidopteran USPs, except Phe-227 and Phe-229, which are replaced by leucine and isoleucine residues, respectively, in the case of bmUSP (see Fig. 1). In the case of the interactions of L1–3 with L11–12, apart from Phe-229 and Asp-235, both sets of interacting residues in L1–3 (Gln-228 to Arg-231, Asp-235, and Ser-236) and in L11–12 (His-439 to Ala-442 in L11–12) are strictly conserved among all lepidopteran USPs. These conservations show a strong evolutionary pressure that suggests the functional relevance of the observed conformation for the lepidopteran USPs. The other subgroups of the USP family do not exhibit the same sequence conservation, suggesting the possibility of alternative conformations.

The final conformation of the connecting loop L1–3 adopted by the liganded USP LBD most probably acts as a regulator during the transconformation process of the LBD occurring when the ligand binds. For RXRα, it has been described as a dynamic region acting as a molecular spring to accompany the considerable movement of helix H3 upon ligand binding (19). For USP, L1–3 adopts a totally different conformation from that observed in liganded RXRα, and we cannot exclude this conformation to be different upon binding of a different ligand. However, the observed conformation of the loop L1–3 of USP is likely to reflect a peculiar and specific role played by this structural element inside the family of lepidopteran USPs.

Biological Significance and Concluding Remarks—The ligand captured inside the ligand-binding pocket of the USP LBD is most certainly an endogenous molecule of E. coli used as the protein expression system. The type of ligand found in the ligand-binding pocket of USP is consistent with the nature of the endogenous phospholipids of this bacterial organism, where only three types of phospholipids coexist (phosphatidylethanolamines, 70%; phosphatidylglycerols, 18%; cardiolipins or diphasatidylglycerols, 12%) (39). The phospholipid ligand in the USP LBD cannot be displaced by competition with juvenile hormone ester, nor with methoprene, one of the juvenile hormone mimics, as checked by mass spectrometric studies (data not shown). Although the bound phospholipid is not the natural ligand, it stabilizes the observed conformation, thus favoring the ligand-bound conformation versus the less stable ligand free one.

The large size of the ligand-binding pocket mainly results from the peculiar positioning of the N terminus of H3 and of the loop L1–3. This vast hydrophobic cavity, which accommodates a large-sized phospholipid ligand, is composed of two parts: one containing the ligand and another one near a conserved arginine. The shape and the chemical nature of this empty part of the pocket are similar to the corresponding parts of the RXRα pocket. The guanidinium group of this residue could interact with a ligand functional group, thereby acting as a RXR-like ligand anchoring part. It seems likely that the pocket could hold a different ligand in the region unoccupied by the phospholipid. This would result in a shrinkage of the cavity to a volume closer to that of the RXRα cavity. However, in this case, it is not possible to predict which kind of conformation the LBD would adopt. Identification of JH as the USP ligand is an elusive issue. In the case of the juvenile hormone I (3), docking of the acidic form of the molecule into USP shows that this molecule fits very well inside the USP cavity, being much less constrained than inside a RXR-like cavity. Furthermore, the hydroxyl group of Ser-431 located close to the N terminus of H5 could most likely be involved in stabilizing interactions with the ligand. It is interesting to notice that for the dipteran Drosophila melanogaster and Chironomus tentans, the arginine residue of H5 is not conserved: it is replaced by a cysteine and a methionine residue, respectively (Fig. 1). This suggests a different role for these residues possibly related to a different type of ligand.
On the other hand, the present data suggest that the actual USP ligand could resemble the *E. coli* endogenous phospholipids. One class of molecules found in insects, the diacylglycerides, are worth mentioning. These molecules are the primary circulating lipids in insects (3). In the insect hemolymph, these molecules are bound to carrier proteins (called lipophorins), which enhance the solubility of these molecules. Whether these molecules are relevant, directly or indirectly, to USP cannot be decided from this study.

The functional ecdysone receptor is formed by the heterodimer EcR/USP. It is therefore instructive to substitute, in the crystal structure of the heterodimer RXR/RAR, the RXR LBD by the USP LBD. This is corroborated by the fact that a strong similarity is observed for the homo- and heterodimer interfaces of most NRs (26). This substitution shows that the interface is conserved, as already seen from the sequence alignment (Fig. 1) and suggests that the loop H5-s1, which is only partly resolved in our structure, would embrace the partner interface is conserved, as already seen from the sequence alignment; Dr. J. Fagart for his help with the sequence alignment; Dr. P. Richards for valuable comments and careful reading of the manuscript. 

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