Spider Hemocyanin Binds Ecdysone and 20-OH-Ecdysone*

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Elmar Jaenicke, Roman Föll‡, and Heinz Decker§

From the Institute for Molecular Biophysics, University of Mainz, D-55128 Mainz, Germany and the ‡Zoological Institute, University of Munich, D-80333 Munich, Germany

Fluorescence quenching studies and binding experiments with [3H]ecdysone reveal that the respiratory protein, hemocyanin, of the tarantula Eurypelma californicum binds ecdysone. The binding constant for ecdysone ranges between 0.5 and 5 mM, indicating a low affinity binding. However, it is comparable with those found for the ecdysone binding to hexamerins from insects. Based on a comparison of sequences and x-ray structures of arthropod hemocyanins, we propose an evolutionary conserved hydrophobic pocket in domain 1 of the hemocyanin subunit that may bind ecdysone.

RESULTS

The ability of hemocyanins to bind ecdysteroids was shown by two different methods, fluorescence quenching and binding of [3H]ecdysone.

Fluorescence Quenching—Fluorescence quenching was employed to show the binding of ecdysone and 20-OH-ecdysone (Fig. 1). In order to record the influence of ecdysone on tyrosine and tryptophan at the same time, the excitation wavelength was set to 280 and 295 nm, while the fluorescence intensity was monitored at 310 and 355 nm, respectively. Hemocyanin dissolved in buffer containing 0.1 M Tris/HCl, 5 mM CaCl₂, and 5 mM MgCl₂ at pH 7.8 was titrated with a stock solution of the respective ecdysteroid dissolved in pure ethanol. The total ecdysone concentration in the solution was calculated after each titration step (Fig. 1A). Ethanol was needed in order to dissolve the high concentration of ecdysteroids in the stock solution. Native oligomeric hemocyanins, however, are stable even at 35% ethanol as was shown by difference absorbance spectroscopy (data not shown). This limited the maximum ecdysteroid concentration in our experiments to about 700 µM, without disturbing the protein due to the presence of too much ethanol. Thus, a complete saturation of the binding sites obviously could not be achieved (Fig. 1A). Since the quenching of the fluorescence intensities seems to follow $F/F_0 = \exp(-K_D^{-1} \times c)$ with the normalized fluorescence intensities $F/F_0$ and the ecdysteroid concentration ($c$), one can simply calculate values for the dissociation constants ($K_D$) to 500 µM for ecdysone and 800 µM for 20-OH-ecdysone (Fig. 1B). The incubation time at each titration step was 3 min. No further quenching was observed at longer incubation times. The volume of the hemocyanin solution was increased by less than 11% after 10 titration steps. A control experiment with equimolar concentrations of tyrosine and tryptophan revealed that titration with ecdysone does not result in any fluorescence quenching. An influence of photoactivation according to Reum et al. (6) during the titration experiments could be excluded, since no deviation was observed when various hemocyanin samples were incubated with different ecdysone concentrations, and their fluorescence intensities were monitored for 15 s.

Binding of [3H]Ecdysone—Another approach also revealed the ability of tarantula hemocyanin to bind ecdysone by bind-

MATERIALS AND METHODS

The animals were purchased from North Carolina Supply Company. The hemocyanin was purified as described previously (10). The protein concentration was determined spectrophotometrically by using a molar extinction coefficient $\epsilon_{280}\text{nm} = 1.1\text{ml} \times (\text{mg} \times \text{cm})^{-1}$ (11). Fluorescence spectroscopy was performed with a SPEX Fluorolog II spectrometer. Tyrosine and tryptophan were purchased from Sigma. In order to avoid any inner filter effect, low hemocyanin concentrations of 0.04–0.06 mg/ml were applied.

Ecdysone and 20-OH-ecdysone were purchased from Sigma. The concentrations of the ecdysteroid stock solution were determined on the basis of the amount of ecdysteroids in a defined volume of ethanol/Tris buffer (35:65, v/v). To avoid light damages, the samples were covered and kept in the dark.

Purified [3H]ecdysone was kindly provided by Dr. Romer (Zoological Institute, University of Mainz, Germany). Before use, it was purified by high performance liquid chromatography in a mixture of water and methanol (1:1, w/w) using an RP 18 column (Lichrosorb, Merck, Germany), which was calibrated by a mixture of ecdysteroids. [3H]Ecdysone activity was measured in a liquid scintillation counter (1900 TR, Packard).

The program Clustal X was used for comparison of the sequences (12). The figures were drawn with WebLabViewer 3.20 (Molecular Simulations Inc., San Diego, CA).
Afterward, the supernatant was fractionated in 500-ml steps. The fluorescence intensity was monitored at 310 or 335 nm, when excited at 280 nm, respectively. A solution of 0.04–0.06 mg/ml hemocyanin, dissolved in buffer, was titrated with a stock solution of ecdysone, which was solved in ethanol. The fluorescence intensities were corrected for the dilution effect, which was calculated to be less than 11% at the maximum. Furthermore, all experiments were controlled for a possible inner filter effect. A fluorescence quenching could be observed for both ecdysone (●) and 20-OH-ecdysone (○) at an excitation wavelength of 280 nm. Minimum values for dissociation constants are estimated to 500 μM for ecdysone and 800 μM for 20-OH-ecdysone, respectively. Titration of the hemocyanin solution with ethanol at an excitation wavelength of 280 nm has no effect on the fluorescence (●). At an excitation wavelength of 295 nm, where only tryptophan is excited, neither for ecdysone ( ■) nor for 20-OH-ecdysone (□) is fluorescence quenching observed. For comparison, fluorescence quenching data at a 280-nm excitation wavelength for the binding of 20-OH-ecdysone (A) and ecdysone (○) to calliphorin, a hexamerin, are also shown (7). B, the fluorescence upon the addition of ecdysone (●) and 20-OH-ecdysone (○) decreases exponentially as shown by this half-logarithmic plot. Therefore, the value for the binding constant $K_D$ was determined by $F/F_0 = \exp(-K_D^{1/2} \times c)$, where $F/F_0$ represent normalized fluorescence intensities and $c$ represents the ecdysteroid concentration.

The amount of $[^{3}H]$ecdysone bound to hemocyanin was calculated in the following way. The total amount of $[^{3}H]$ecdysone of the pellet ($A_{pellet}$) given as activity consists of two parts: bound ecdysone ($A_{bound}$) and ecdysone ($A_{buffer}$) dissolved in the buffer, which is part of the pellet. The mass of this buffer ($M_{buffer}$) can be calculated to be the difference between the mass of the pellet ($M_{pellet}$) and the mass of the hemocyanin ($M_{Hc}$). Although the hemocyanin should be pelletized quantitatively, some hemocyanin can be detected (<20%) spectroscopically in the supernatant. This may be due to the fact that a part of the hemocyanin is accidentally removed from the surface of the pellet while recovering the last aliquot of supernatant above the pellet. Therefore, at least 80% of protein can be recovered in the pellet. This is accounted for by the factor of $x$ in Equation 1, for which we give a value of 0.8, which means 80% recovery. Thus, the amount of $[^{3}H]$ecdysone bound to hemocyanin is given by the following,

$$A_{bound} = A_{pellet} - A_{buffer} - A_{pellet} - (M_{pellet} - M_{Hc} \times x) \times a_{n}^{E}$$

where $a_{n}^{E}$ is the average activity of $[^{3}H]$ecdysone in the supernatant normalized to 1 mg of buffer (Fig. 2). A control experiment was performed by centrifugation of $[^{3}H]$ecdysone dissolved in buffer in absence of hemocyanin (Fig. 2). Thus, a possible $[^{3}H]$ecdysone gradient due to centrifugation can be excluded.

For sake of clarity, the calculations will be demonstrated by one experiment (Fig. 2), which stands for comparable ones. The activity of ecdysone bound to hemocyanin in the pellet was calculated to 9.15 Bq. Assuming that 80% of the hemocyanin was recovered in the pellet, the total amount of ecdysone bound to hemocyanin in this experiment can be calculated as 11.43 Bq. If one subunit can bind one ecdysone, the concentration of $[^{3}H]$ecdysone (in Bq) normalized to 1 mg of solution was determined for each sample and for the pellet. A value of 103.8 mBq/mg was determined for the average activity, $a_{n}^{E}$, of $[^{3}H]$ecdysone in the supernatant normalized to 1 mg of buffer solution. By monitoring the protein-specific absorbance at 278 nm of aliquots 1–22, it was calculated that the pellet contained more than 80% of the hemocyanin used in the experiment. In addition, a control experiment showed that no ecdysone gradient formed due to centrifugation (data not shown). The bars give the S.D.

The abbreviation used is: Hc, hemocyanin.
On the basis of an error propagation, an experimental error of $6 \pm 20\%$ has to be respected for each experiment.

**DISCUSSION**

The two different methods reveal that tarantula hemocyanin binds ecdysone with low affinity. The differences in the binding constants are obviously large comparing the values of 4.4 mM with 0.5 mM, obtained by $[^3H]$ecdysone binding studies and fluorescence quenching.

Several reasons may account for this: First, saturation with bound ecdysone was not reached by the fluorescence technique. Thus, the value for the dissociation constant obtained by fluorescence quenching should be larger than 0.5 mM. Second, similar low binding constants are reported for the binding of steroids to albumin, and two possible error sources are discussed (13), which may also be true for hemocyanin and may explain the difference in dissociation constants by 1 order of magnitude obtained with the two different methods. The presence of ethanol in the fluorescence quenching experiment may alter the steroid-hemocyanin interaction. Additionally, we cannot rule out, that different amounts of fatty acids may have been bound to the hemocyanin used in the fluorescence quenching and the $[^3H]$ecdysone binding experiments, although both hemocyanin batches were prepared in the same manner. Similar effects are reported for binding of steroids by albumin. There, fatty acids are known to interfere with steroid binding.

Although the applied technique contains large errors, we are nevertheless able to demonstrate with this method that...
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Fig. 4. The putative ecdysone binding site in the first domain. a, a top view into the binding pocket in domain 1. The secondary structures of the binding pocket are shown based on the x-ray structure of P. interruptus hemocyanin. The conserved amino acids according to Fig. 3 are colored in red. In order to give an impression about the location of these conserved oily amino acids within the pocket, domain 1 was split in two parts along the yellow line. The interior part of the pocket is presented in two different ways; b and c show the secondary structure and the space-filling representation, while d and e give an impression of the bulky cluster of the conserved oily amino acid chains.

cyanin binds ecdysone, although the binding affinity is low.

We here point out that only tyrosines, and no tryptophans, seemed to be quenched, since tryptophan-specific excitation at 295 nm did not result in any quenching effect (Fig. 1). However, it is not clear whether the quenching of fluorescence is due to a quenching influence of the bound ecdysone on tyrosine or due to a radiationless transmission from an excited amino acid such as tyrosine to the unsaturated 6-keto-group of the ecdysone, as suggested by Enderle et al. (7).

Furthermore, it should be noted that binding of ecdysone does not influence the oxygen binding capacity of tarantula hemocyanin, since no absorbance change is found between 300 and 360 nm, where deoxygenation of hemocyanin can be followed spectroscopically.

Hypothetical Binding Site—Hemocyanins are extracellular proteins in the hemolymph of crustacea and chelicera that are responsible for the oxygen supply of the animals (14). The smallest hemocyanin found in hemolymph consists of six subunits and is structurally related to hexamerins on the basis of their similar quartenary structures in electron microscopical images, a high degree of identity in the sequences, and a common epitope for a monoclonal antibody (14–21).

According to the fluorescence quenching data (7, 19), calliphorin, a hexamerin, binds ecdysteroids in a way comparable with that of the closely related hemocyanin of E. californicum (Fig. 1). Sequences of calliphorin and other hexamerins and hemocyanins of several crustacea and chelicera were compared with respect to the tertiary structure of the hemocyanin subunit from Panulirus interruptus (22) (Fig. 3). Based on this comparison, only one potential ecdysteroid binding site was found where hydrophobic and apolar amino acids are highly conserved. This binding site seems to be a pocket located in the first domain, which is characterized by an evolutionary persistent oily cluster of side chains (Fig. 4). The pocket is built up by four α-helices (α-helices 1.1, 1.4, 1.5, and 1.6), based on the nomenclature of Linzen (15, 22). It is surprising to find a highly conserved pocket within the first domain, since the largest differences between known hemocyanins are found in the first domain due to the variability in homologous amino acids and the omission of the complete 1.2 α-helix in the hemocyanin from chelicera (15, 23). Nevertheless, apparently a reorganization of the α-helices happens in such a way that a highly conserved binding pocket for ecdysteroids or hydrophobic compounds is maintained. Based on our binding experiments, we can only speculate that binding takes place at this particular binding site and not somewhere else in the protein. However, we did not perform a further packing analysis of the void volume for several reasons. First, the x-ray structure of the first domain was obtained in absence of bound steroid hormones. Second, according to the x-ray data, the first domain is characterized by rather high T-factors, indicating a rather high flexibility (22, 23). Third, the first domain is the least stable among the three domains of a subunit, since it unfolds at lower concentrations of denaturing agents than the other two (24).

We cannot yet answer the question of whether the binding pocket exclusively binds ecdysone. Analyzing the binding pocket, one might think that other hydrophobic metabolic compounds can be bound as well. Braun and Wyatt (25) reported that the hydrophobic juvenile hormone is bound by a serum protein belonging to the protein family of hexamerins with an affinity that is more than 5 orders of magnitude higher. The observation of the authors that this hexamerin looses the binding capacity when the N-terminal 53 amino acids are removed supports our hypothesis that the first domain could serve as a binding site for hydrophobic compounds. This hypothesis is supported by an observed protein-lipid interaction in the case of the hemocyanin from Carcinus maenas. Zatta (26) reported that the content of α-helices is decreased from 21 to 15% when the hemocyanin solution is delipidated. We propose that the lost α-helices are located in the first domain, which contains seven α-helices according to the x-ray structures of arthropod hemocyanins (22, 29). The other two domains contain very stable folding motives such as a four-α-helix bundle at the active site (domain 2) and a large seven-stranded β-barrel (domain 3).

One might also consider binding experiments to denatured hemocyanin in order to demonstrate the specificity of the ecdysteroid binding. We did not stress this point, since the binding is obviously of very low affinity, and one should expect that a denatured large protein like hemocyanin provides a lot of hydrophobic cavities and surfaces to bind the more hydrophobic ecdysone, and this most probably with a similar binding constant.

Physiological Considerations—In our study, we report for the first time that native hemocyanin binds ecdysteroids. Several years ago a similar binding behavior was also shown for
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... hemocyanin concentrations range between 0.07 and 1.7 mM based on a molecular mass of 72 kDa per subunit. The weak binding, the variable concentrations of hemocyanin subunits (between 0.002 and 0.15%), and highest values for the binding constants and concentrations of both hemocyanin and ecdysteroids, we calculated that up to 75% of the ecdysteroids will be transported by hemocyanin. However, it should be noted, that almost negligible amounts of hemocyanin subunits (between 0.002 and 0.15%) have ecdysteroids bound at the same time due to the large difference in concentrations of ecdysteroids and binding sites on hemocyanin subunits. This calculation is based on concentrations of ecdysteroids ranging between 100 and 3000 nM as reported for some crustacea and one spider (9, 27). The in vivo hemocyanin subunit concentrations range between 0.07 and 1.7 mM based on a molecular mass of 72 kDa per subunit (28).

Hemocyanin is the major protein component in the hemolymph. This estimation is comparable with the situation of calliphorin, a hexamerin, which shares about 30% sequence identity with hemocyanins from crustacea and chelicerata (7, 19). The weak binding, the variable concentrations of hemocyanin subunits and highest values for the binding constants and concentrations of both hemocyanin and ecdysteroids, we calculated that up to 75% of the ecdysteroids will be transported by hemocyanin. However, it should be noted, that almost negligible amounts of hemocyanin subunits (between 0.002 and 0.15%) have ecdysteroids bound at the same time due to the large difference in concentrations of ecdysteroids and binding sites on hemocyanin subunits. This calculation is based on concentrations of ecdysteroids ranging between 100 and 3000 nM as reported for some crustacea and one spider (9, 27). The in vivo hemocyanin subunit concentrations range between 0.07 and 1.7 mM based on a molecular mass of 72 kDa per subunit (28).

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