Juvenile Hormone III as a Natural Ligand for Photoaffinity Labelling of JH-Binding Proteins

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Z. Naturforsch. **39 c**, 1145–1149 (1984); received April 26/July 23, 1984

Insect Haemolymph, Hormone Binding Proteins, Juvenile Hormone, Photoaffinity Labelling, *Manduca sexta*, *Locusta migratoria*

Juvenile hormone III, *i.e.* methyl 10,11-epoxy-3,7-dimethyl-2*E*,6*E*-dodecadienoate, was used for photoaffinity labelling of JH-binding proteins. Irradiation of haemolymph from *Manduca sexta* and *Locusta migratoria* in the presence of racemic [10-^{3}H]JH-III results in covalent incorporation of up to 3.2% of the radioactivity into an acetone insoluble protein fraction. Resolution of the proteins by HPLC and by SDS-PAGE confirms the presence of radiolabelled JH-binding proteins. Proteins not involved in JH-binding in insect haemolymph are labelled photochemically with lower yields only. Thus, JH-III is a suitable natural ligand for photoaffinity labelling of high-affinity binding sites. Use of the natural hormone instead of a hormone analog bears the major advantage that the binding with a JH-binding protein will occur at specific sites.

Photoaffinity labelling of biological receptors and enzymes is emerging as a very useful technique for the characterization of these biopolymers and for studies on their interactions with low molecular weight specific ligands [1]. In the field of insect hormones, applications have recently been described for ecdysteroids [2, 3] and for juvenile hormone analogs [4]. In the latter case, Prestwich *et al.* [4a] have used epoxyfarnesol diazoacetate (EFDA) for photoaffinity labelling of high affinity JH-binding proteins in cockroach haemolymph and ovaries. Photoaffinity labelling of a putative JH-receptor in *Drosophila* K_c-cells has also been reported [4b]. The K_D-value of the haemolymph carrier from the cockroach *Leucophaea maderae* is 40-fold higher for EFDA ($K_D = 8 \times 10^{-7}$ M) than for racemic JH-III ($K_D = 2 \times 10^{-8}$ M) [4], and the extent of irradiation induced covalent attachment was reported to be in the range of 3–4% of racemic EFDA.

The use of a ligand-analog bears some inherent difficulties, since it is necessary to prove that the photoattachment occurs at the "true" binding site in each protein system under investigation [1]. Furthermore, a comparatively high K_D-value of a hormone analog together with a quite low yield of covalent bond formation seems to call for a search into alternative methods. We have therefore investigated possibilities to use racemic [10-^{3}H]JH-III, which contains 50% of the natural ligand, for photoaffinity labelling of high affinity binding proteins in haemolymph from two insect species, *Manduca sexta* and *Locusta migratoria*. The chromophore present in JH-III (systematic name: methyl (2*E*,6*E*)-10,11-epoxy-3,7-dimethyldodecadienoate) is a 3,3-dialkylacrylate which *a priori* appears to be a rather poor substrate for such purposes. We found, however, that JH-III is covalently attached to JH-binding proteins upon irradiation at 254 nm.

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0341-0382/84/1100-1145 $ 01.30/0

Materials and Methods

Racemic JH-III was purchased from Fluka (Buchs) and racemic [10-^{3}H]JH-III (spec. activity 11 Ci · mmol^{-1}; radiochemical purity ≧ 98%, as determined by TLC on silica gel with benzene/5% ethyl acetate) from NEN-Chemicals (Dreieich). All glassware used to handle aqueous solutions of JH-III was coated with polyethyleneglycol according to [5].

Haemolymph of 4th instar *Manduca sexta* larvae was generously provided by Dr. K. Lehmann, Köln, and that of 7 days old *Locusta migratoria* 5th instar larvae by Dr. J. A. Hoffmann, Strasbourg. Batches of haemolymph were diluted 1:1 (v:v) with ice cold 10 mM potassium phosphate buffer pH 7.5, containing a few crystals of phenylthiourea, and centrifuged at 4 °C and 6000 × *g* for 10 min. The

supernatants were stored at −70 °C. Before irradiation, aliquots were diluted further four fold with 10 mM potassium phosphate buffer pH 7.5. Esterases were inhibited with 0.1 mM paraoxon and incubation at 20 °C for 30 min. Samples were prepared for irradiation in the following way: aliquots of JH-III and [10-^{3}H]JH-III stock-solutions were pipetted into borosilicate tubes and the solvent evaporated. The quantities of JH-III were calculated so that in the case of *Manduca* haemolymph the concentration was 2×10^{-7} M and in *Locusta* haemolymph 9×10^{-8} M. The radioactivity concentration was always 2×10^{-6} dpm · ml^{-1}. After mixing the JH-III with haemolymph, all samples were kept at 0 °C for 30 min and then divided into two equal portions (usually 1 ml). The first one was irradiated directly (see below), then unlabelled JH-III, dissolved in 5 µl ethanol, was added to give a concentration of 5×10^{-5} M, and the vial was set to 0 °C for 30 min. The second portion was made 5×10^{-5} M with respect to JH-III prior to irradiation and set to 0 °C for 30 min. This time is sufficient in the case of haemolymph JH-binding proteins to reach equilibrium between labelled and unlabelled JH-III at both, specific and unspecific binding sites. Free and bound hormone were not separated before irradiation. The stability of the hormone-protein complex is low (unpublished observations in this laboratory; *cf.* [7]), and absorption of the free hormone to charcoal would result in a rapid disturbation of the equilibrium.

Irradiation was performed in quartz cuvettes, placed into a Rayonette RPR-100 Minireactor, equipped with circularly oriented lamps (4 RPR-1849/2537 and 12 RPR-2537). Samples were cooled to 0 °C, irradiated while stirred for 2.5 min, cooled again to 0 °C, and finally irradiated for another 2.5 min. After irradiation, aliquots of 200 µl were processed in the following way: addition of a solution of 400 µg γ-globulin in 200 µl 10 mM potassium phosphate buffer pH 7.5 was followed by 4 ml acetone and stirring of the mixture at 0 °C for 10 min. Centrifugation for 5 min at $3000 \times g$ and 4 °C resulted in a pellet which was resuspended in acetone. This extraction was repeated two more times. The last pellet was finally resuspended in 0.4 ml 0.1 M potassium phosphate buffer pH 6.8 and subjected to liquid scintillation counting in 4 ml Aqualuma cocktail (Baker, Groß Gerau) or to HPLC on a 0.7 × 60 cm TSK-G-3000-SW column, eluted with 0.1 M potassium phosphate buffer pH 6.8 at a flow rate of 0.3 ml · min^{-1}. Fractions containing radioactivity were dialyzed overnight against H_2O. The contents of the dialysis tube were lyophylized and the residue subjected to SDS-gel electrophoresis in a 5% polyacrylamide gel as described by Fehrnström and Moberg [6]. Radioactivity in the gels was determined by standard methods after slicing individual tracks into 0.5 cm sections and digestion in 1 ml TS-1 (Koch-Light Laboratories, supplied by Zinsser, Frankfurt).

Analysis of photolysis products of JH-III was performed by extraction of an irradiated aqueous solution of 2×10^{-7} M [10-^{3}H]JH-III with ethyl acetate, followed by TLC on silica gel with benzene/15% ethyl acetate/1% acetic acid.

Binding data for determination of K_D-values by Scatchard analysis were obtained after separation of bound and unbound hormone by means of dextrane coated charcoal, adhering precisely to the protocol of Engelmann [7].

Results

The UV-spectrum of JH-III in methanol shows a maximum at 220 nm ($\varepsilon = 13\,800$) [8]. Irradiation of a 10^{-4} M methanolic solution at 254 nm results in a rapid decline in the absorption maximum. Thus, after 90 s, the molar extinction coefficient has decreased from $\varepsilon = 13\,800$ to 9200 at 220 nm, indicating a corresponding loss of the chromophore. Irradiation of a 2×10^{-7} M solution of [10-^{3}H]JH-III in 10 mM potassium phosphate buffer pH 7.2 results in the formation of polar products, as revealed by TLC analysis. Extraction of the reaction mixture with ethyl acetate shows further that approx. 10% of the radioactivity remain in the aqueous phase in a sample irradiated for 5 min. Thus a small amount of ^{3}H is labilized, most probably in form of a proton or triton, respectively, from position 10 of JH-III.

Irradiation of diluted 4th instar *Manduca sexta* larval haemolymph in the presence of [10-^{3}H]JH-III results in a time dependent incorporation of radioactivity into the acetone insoluble protein fraction as shown in Fig. 1. Up to 3.2% of the total radioactivity present in the incubation mixture are bound covalently after 15 min when the concentration of JH-III is 2×10^{-7} M. When the radioactive ligand is exchanged with a 250 fold excess of unlabelled JH-III before irradiation, the amount of label in-

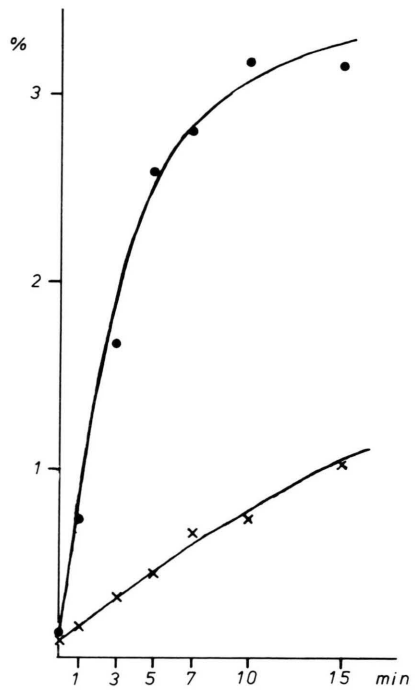

Fig. 1. Kinetics of photochemically induced incorporation of radioactivity from racemic [10-^{3}H]JH-III into *Manduca sexta* 4th larval instar haemolymph proteins. (●) Irradiation in the presence of 2×10^{-7} M [10-^{3}H]JH-III (80000 dpm). (×) Irradiation after equilibration with 2×10^{-7} M [10-^{3}H]JH-III (80000 dpm), followed by a 250-fold excess of unlabeled JH-III prior to irradiation.

corporated is only 1.1%. This result indicates that photoaffinity labelling of high affinity binding sites is more efficient than that of non-saturable nonspecific sites. Similar results were found after irradiation of *Locusta migratoria* 5th larval instar haemolymph. In this case the yield of photoattachment was up to 2.9% at 9×10^{-8} M JH-III, and 1.2% at 5×10^{-5} M. Considering the fact that it is the natural (10 R)-enantiomer which binds to JH-specific haemolymph carrier proteins in *Manduca* [9, 10] and in *Locusta* [11], the actual values of incorporation of the natural ligand may be higher by a factor of up to two.

When the acetone insoluble fraction obtained after irradiation of *Manduca sexta* larval haemolymph in the presence of 2×10^{-7} M JH-III is redissolved in phosphate buffer and separated by HPLC on a TSK-G-3000-SW column, two peaks of radioactivity are detected as shown in Fig. 2a. The first one, corresponding to an apparent M_r of approx. 450000, most likely represents high molecular weight lipoproteins, whereas the second peak corresponds to a M_r of approx. 30000 and represents the JH-specific carrier protein [12]. According to Fig. 2a, the amount of covalent attachment to nonspecific sites appears to be slightly higher than that to the specific ones. It should, however, be kept in mind that specific binding is enantioselective [9, 10], in contrast to nonspecific binding. Therefore, the amount of radiolabelled ligand being present under equilibrium conditions at nonspecific sites may be higher than that at the saturable specific ones. A thorough quantitative evaluation of this question will have to be postponed until optically active JH-III becomes available. Irradiation of *Manduca* larval haemolymph in the presence of

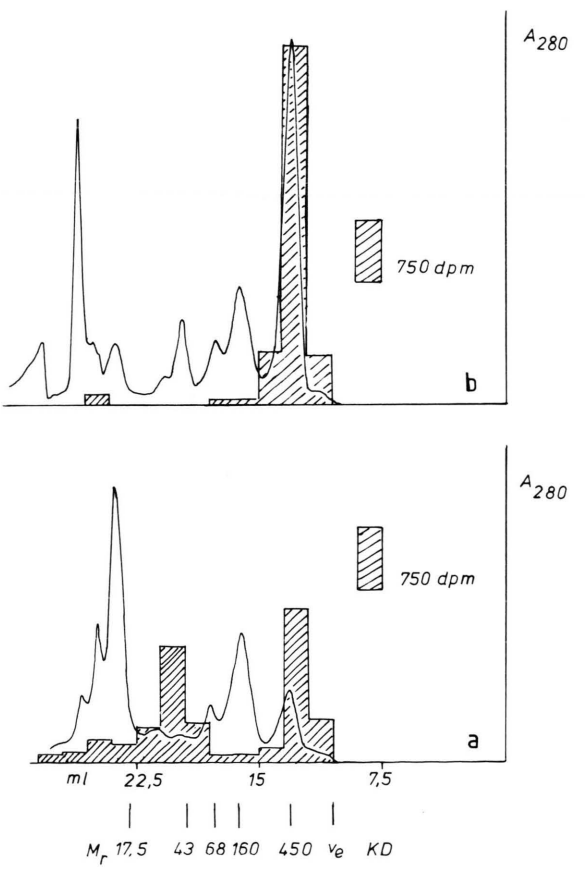

Fig. 2. HPLC-separation of acetone insoluble protein fraction from a) *Manduca sexta* haemolymph, irradiated in the presence of 2×10^{-7} M [10-^{3}H]JH-III; b) *Locusta migratoria* haemolymph, irradiated in the presence of 9×10^{-8} M [10-^{3}H]JH-III. The peak at M_r 160000 arises from γ-globulin which was added prior to acetone precipitation (see materials and methods section).

5×10^{-5} M [10-^{3}H]JH-III leads to incorporation of low amounts of radioactivity into the high M_r protein fraction only. Application of the same analytical procedure to haemolymph from *Locusta* larvae reveals that the radioactivity is associated with a high molecular weight JH-binding protein fraction (M_r approx. 450000) [11], as shown in Fig. 2b.

Covalent attachment of radiolabeled products to the proteins separated by HPLC is further evident from the results of SDS-PAGE, shown in Fig. 3. The high M_r protein fraction obtained from *Manduca* shows an apparent M_r of approx. 90000 (Fig. 3a). The JH-specific binding protein present in *Manduca* larval haemolymph appears as a peak of radioactivity at M_r approx. 28000 (Fig. 3b). Analysis of the radiolabeled fraction obtained after HPLC resolution of irradiated *Locusta* haemolymph shows that the high M_r JH binding protein [11] dissociates into subunits of M_r approx. 90000 in SDS-PAGE (Fig. 3c).

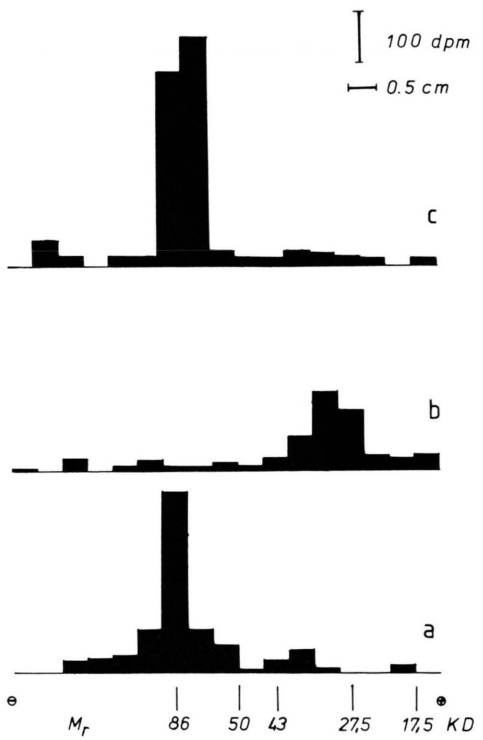

Fig. 3. SDS-PAGE of radiolabeled protein fractions obtained by HPLC separation as shown in Fig. 2. a) *Manduca sexta*, HPLC-fraction at M_r approx. 450000; b) *Manduca sexta*, HPLC fraction at M_r approx. 30000; c) *Locusta migratoria*, HPLC fraction at M_r approx. 450000.

In order to determine the effects of irradiation on the binding properties of the proteins, haemolymph of *Manduca* larvae was exposed to UV light at 254 nm for various time intervals in the absence of JH-III. After 1, 3, 5, and 7 min the amount of hormone bound in a 2×10^{-7} M [10-^{3}H]JH-III solution after equilibration for 30 min at 4 °C was determined by the charcoal assay according to Engelmann [7]. Compared with a non-irradiated haemolymph sample, binding of racemic JH-III was reduced by 7.7% after 1 min irradiation, by 16.3% after 3 min, by 22.7% after 5 min, and by 44.3% after 7 min. The equilibrium dissociation constant showed essentially the same value in irradiated and in non-irradiated haemolymph (found: $K_D = 2.0 \times 10^{-7}$ M after 5 min irradiation; $K_D = 3.8 \times 10^{-7}$ in non-irradiated haemolymph). Binding capacity was essentially unchanged in *Locusta* haemolymph after 5 min exposure to UV light.

When a solution of 2×10^{-7} M [10-^{3}H]JH-III in aqueous buffer was irradiated for 5 min and *Manduca* haemolymph added after turning off the lights, the amount of radioactivity bound to acetone insoluble proteins was essentially *nil*. Likewise, when haemolymph was irradiated for 5 min and radiolabeled JH-III added in the dark, no radioactivity was incorporated into the protein fraction. This demonstrates that the irradiation induced covalent labelling of haemolymph proteins does not occur through eventually present long living reactive intermediates in a dark reaction.

The specificity of labelling was further investigated by irradiating solutions of alcaline phosphatase (1 mg · ml^{-1}) and of BSA (1 mg · ml^{-1}) in the presence of 2×10^{-7} M [10-^{3}H]JH-III for 5 min. The incorporation of radioactivity was in the range of 0.1–0.4%. This result shows that the yield of photoaffinity labelling is low when proteins that are not involved in JH-metabolism and therefore are not expected to bind the hormone with high affinity, are present in the reaction mixture.

Discussion

Acrylic esters, such as the α,β-unsaturated carbomethoxy group present in JH-III, have as far as we are aware not been considered as useful functional groups for photoaffinity labelling of a hormone receptor. The $n - \pi^*$-transition of acrylic esters occurs at a rather low wavelength of 240–250 nm

[13], and reactions of the excited state lead usually to dimerization and polymerization [14]. In the microenvironment where the excited state faces functional groups of the protein in a close geometrical relationship due to high affinity binding, product formation may be different and lead eventually to the desired covalent attachment of reactive intermediates. The present study shows that JH-III may be a useful ligand for photoaffinity labelling of JH-binding proteins. It would, of course, be desirable to obtain informations on the photochemistry involved and this aspect will have to receive proper attention in the next future.

Compared with the hitherto most fruitfully explored application of a JH-analog [4], the natural hormone will bear the major advantage that high affinity binding will necessarily occur between the natural ligand and its specific binding site. This is best demonstrated by the fact that the yield of irradiation induced covalent attachment to high-capacity-low-affinity binding sites is lower than that of low-capacity-high-affinity binders, as revealed by the data shown in Fig. 1, as well as from the experiments with BSA and alcaline phosphatase.

A disadvantage of the present methodology is the apparently limited stability of proteins under the irradiation conditions. In order to optimize the yield of photoactivated binding of the hormone it is necessary to use rather long periods of irradiation at short wavelength. Further studies will therefore be directed towards the question whether the reaction time can be shortened when proteins with K_D values in the range of $10^{-8}-10^{-10}$ M, such as cellular receptors, are used. A great potential of using JH-III for photoaffinity labelling of JH-binding proteins emerges from the possibility to characterize the binding components as shown by SDS-PAGE in this study. It will also be possible to tag individual amino acid residues of the binding region by covalent attachment of the natural ligand.

Acknowledgements

The use of JH as a photoaffinity label was suggested to us by Prof. O. Pongs, Bochum. We would like to express our thanks to him as well as to Prof. J. Koolman, Marburg, and to Dr. H. Heimgartner, Zürich, for discussions. We are indebted to Prof. Wamhoff, Bonn, for providing access to a Rayonette reactor. This work was supported by the Deutsche Forschungsgemeinschaft (Pe 264/2-3).

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