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Electrochemical impedance spectroscopy for the study of juvenile hormones – recombinant protein interactions

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1. ABSTRACT

The interactions of recombinant juvenile hormone binding protein (His_8-rJHBP) with juvenile hormones (JHs), methoprene and farnesol have been studied with electrochemical impedance spectroscopy (EIS). The protein was immobilized on the dodecanethiol (DDT) modified gold electrodes. Each step of electrode modification has been confirmed with cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The conformation changes of His_8-rJHBP upon JHs and methoprene binding have been presented. The EIS determined association constants in the JHs analogs – immobilized His8-rJHBP system indicate that lack of the epoxide moiety in methoprene molecule is not critical for observed high affinity of this compound to the binding region of the His8-rJHBP protein.

2. INTRODUCTION

The juvenile hormones (JHs) are an insect chemical regulators which play a crucial role in the regulation of insect development and reproduction. JHs are structurally unique among animals and constitute a family of esterified sesquiterpenoids (1).

Before 1956, most of the work dealing with JHs was microsurgical. The discovery by Williams (2) that JH can be extracted in relatively large amounts from abdomen of the male Hyalophora cecropia moth has transformed this research fields and attracted chemists to identify natural products. In addition, commercial firms wanting to make JHs and their mimetic substances profitable insect grow regulators.

The mode of molecular action of JH, however, is still obscure. The available evidence suggested actions of JH directly on the genome via nuclear receptors of the steroid superfamily although actions through the cell membrane receptors are also documented (3, 4, 1). In insects, as well as in vertebrates, the circulatory system transports trace amounts of hormones from the site of synthesis to the target cells. In hemolymph, JH secreted from the corpora allata binds to a specific protein called the JH binding proteins (JHBPs) (5, 6). The major functions of JHBPs that have been suggested are to transport JH in the hemolymph, to protect JH from the degradation by hemolymph enzymes and to facilitate JH recognition and uptake by target cells (7, 8). Because interaction of JH-JHBP complex with the target cell membrane could be a way by which JH may reach the cytosol the conformation transition of JHBP upon hormone binding may have physiologically important meaning in JH signal transmission (7).

Diverse techniques have been already applied for the study of interaction JH-JHBP. A quantitative competition assay for this purpose was developed by Goodman *et al.* (9) and Glinka *et al.* (10). This technique is relatively resistant to interference and sufficient sensitive, but required rather complex determination procedure. Krzyżanowska *et al.* applied the spectroscopic methods – circular dichroism (CD) and UV-difference techniques (11) for the study of the JH-JHBP binding process. The another useful assay to be applied for such determination is electrophoresis (12).

Here, we proposed the new electrochemical method for determination of association constants of JHs and their analogs binding by recombinant juvenile hormone binding protein (His_8-rJHBP) of the lepidopteran insect *Galleria mellonella*. This protein was cloned and expressed in the yeast cells of *Pichia pastoris* (13).

The JHBP from *G. mellonella* belongs to class of monomeric, hemolymph JH carrier proteins of low molecular weight and high affinity of binding ($K_a > 10^6$ M^{-1}) (14, 12, 7). This protein has been purified to homogeneity and characterized (15). It is a single chain basic glycoprotein (pI = 8.1), which possess one binding site for JH (14, 15, 16). In SDS-PAGE this protein exhibits a relative molecular mass close to 32 kDa (15, 12).

The His_8-rJHBP expressed in *P. pastoris* was obtained with excellent homogeneity, high yield and with full binding activity. It is stable in the culture medium as well during the purification. This protein exhibited comparable JH-binding activity with the JHBP isolated from the hemolymph of *G. mellonella* (13). Thus, this protein is suitable for study of the mechanism of the interaction with JHs and their analogs.

In the work presented, the His_8-rJHBP was immobilized on the surface of gold electrodes modified with dodecanethiol self-assembled monolayer (DDT–SAM). In this method, proteins are embedded into the hydrophobic layer created by alkyl chains of DDT via hydrophobic and van der Waals forces. In such environment, proteins might be properly oriented and keep sufficient flexibility, which is very crucial for binding of specific stimulants (17-20). Recently, the same methodology has been successfully applied for embedding the artificial receptor, macrocyclic polyamine, sensitive for nucleotides (21).

The properties of surfaces modified with biological molecules could be characterized using not only most popular spectroscopic techniques (22-25), but also electrochemical one such as the cyclic voltammetry (CV) and the electrochemical impedance spectroscopy (EIS) (26-31).

In CV method, redox agents and applied potentials of up to several volts are used in order to force a net current. This is, some time, not fully recommended for non-stable delicate biological systems.

The EIS technique involves measuring of the current that flows in response to a small, sinusoidally modulated voltage that is applied across the interface. This set of conditions represents a very non-intrusive way for characterizing the intrinsic electrical properties of biomolecular interfaces (32). The EIS have been successfully applied for study of protein binding events such as: antigen-antibody (33, 34), avidin and biotin system (35), peptide – drugs interaction (36).

In this report the above specified two electrochemical techniques have been employed for the study of interaction of His_8-rJHBP immobilized on the Au–DDT electrodes with JHs and their analogs (Figure 1).

3. MATERIALS AND METHODS

3.1. Materials

The recombinant juvenile hormone binding protein (His_8-rJHBP) was expressed in the yeast cells (*Pichia pastoris*) according to procedure already published (13). (10*R*,11*S*)-juvenile hormone I, (10*R*,11*S*)-juvenile hormone II and farnesol were purchased from Sigma-Aldrich (Poznan, Poland), methoprene was obtained from Zoecon Corp. (West Schaumburg IL, United States).

3.2. Chemicals

1-Dodecanethiol (DDT, 98%), 3,7-Dimethyl-1-octanol (99%), potassium ferrocyanide (II) ($K_4[Fe(CN)_6]$, 99%), potassium ferricyanide (III) ($K_3[Fe(CN)_6]$, 99%) were purchased from Sigma-Aldrich (Poznan, Poland). Potassium chloride, ethanol, methanol, sodium dihydrogen phosphate anhydrous were obtained from POCH (Gliwice, Poland). Aqueous solutions were prepared with freshly deionized water (18.2 MΩ × cm specific resistivity) obtained with a Simplicity®185 Water System (Millipore, Molsheim, France).

3.3. Modification of gold electrodes

The gold electrodes (Bioanalytical Systems BAS, West Lafayette, IN; diameter 1.6 mm, area 3.2 mm^2) were used as working electrodes for all experiments.

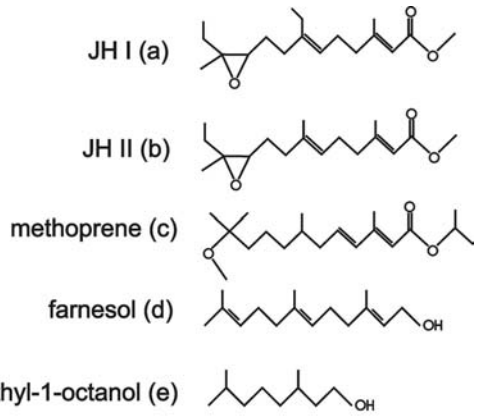

Figure 1. Chemical structure of analytes. JH I, JH II, methoprene, farnesol, 3,7-dimethyl-1-octanol.

The electrodes were polished with wet 0.3 and 0.05 μm alumina slurry (Alpha and Gamma Micropolish; Buehler, Lake Bluff, IL) on a fled pad for 5 min and rinsed repeatedly with deionized water. The polished electrodes were then dipped in the 0.5M KOH solution deoxygenated by purging with argon for 15 min, and the potential was cycled between –0.4 V and –1.2 V with scan rate of
0.1 Vs^{-1} till cyclic voltammograms did not change.

Cleaned gold electrodes were immersed in
1.0×10^{-4} M or 1.0×10^{-6} M ethanol solutions of DDT for 30 min at room temperature. The modification solutions were put into the tubes (8 mm diameter, with no flat bottom). After dipping the electrodes, the tubes were sealing with Teflon tape in order to protect the solvent evaporation. After modification electrodes were rinsed with ethanol and water.

Then 5μl His_8-rJHBP (22μg His_8-rJHBP ml^{-1}) in buffer solution (0.1 M dihydrogen phosphate, 0.1 M KCl, pH 7.2) was dropped on the clean surface of Au–DDT electrode for 20 h at 4^0 C. All Au-DDT-His_8-rJHBP electrodes were stored at 4^0 C in above buffer solution until use.

3.4. Electrochemical measurements

All electrochemical measurements were performed with potentiostat-galvanostat Autolab (Eco Chemie, Utrecht, The Netherlands) with a three electrode configuration. All solutions were deoxygenated by purging with argon 15 min. Potentials were measured versus the Ag/AgCl reference electrode. Ag/AgCl wire was separated from electrolyte by vicor (BAS).

A platinum wire was used as the auxiliary electrode. Au electrodes modified with DDT-His_8-rJHBP were used as a working electrodes. The composition of the supporting solution was as follows: 0.1 M dihydrogen phosphate, 0.1 M KCl, 1.0 mM $K_3[Fe(CN)_6]$ / $K_4[Fe(CN)_6]$, pH 7.2. Cyclic voltammetry (CV) was performed with the potential scanned from 0.6 V to -0.2 V.

In electrochemical impedance spectroscopy (EIS) a sinusoidal ac signal was applied at a frequency from 0.1 to 10000 Hz with 10 mV ac amplitude. The solution composition was as follows the same as for the CV.

The EIS measurements concerning juvenile hormones and their analogs were made in PVC beakers in order to avoid their adsorption to the glass surface.

The aliquots of the stock solution of juvenile hormones and their analogs (in methanol : H_20; 1:1 volume ratio) were added to the supporting electrolyte to obtain the following concentration of analytes: 12.0, 15.0, 19.0 and 21.0 nM. The EIS measurements have been started after 30 min of mixing of particular aliquot of JH stock solution with supporting electrolyte and were carried on until EIS spectra did not change any more. Then, the next aliquot of the JH stock solution was added and measuring procedure was repeated.

4. RESULTS AND DISCUSSION

4.1. Immobilization of His_8-rJHBP on the surface of gold electrode modified with dodecanethiol monolayer

In 1983, Allara and Nuzzo discovered that thiol derivatives could create well ordered monolayers on the surface of gold, silver, cooper or platinum via covalent S-noble metal bonds (37). This discovery open many possibilities for creation of self-assembled monolayers (SAMs) of thiol derivatives possessing additionally different functional groups on the surfaces of metal solid supports.

In the present study, dodecanethiol self-assembled monolayer (DDT–SAM) covalently attached to the gold electrode was selected for His_8-rJHBP immobilization. DDT-SAM provides well ordered hydrophobic environment, which is very suitable for protein immobilization.

Each step of electrode modification was confirmed with using CV and EIS. The base of these measurements was the changes of accessibility of electrons from the redox marker $[Fe(CN)_6]^{-3/-4}$ to the gold electrode surface occurred upon creation of DDT-SAM and His_8-rJHPB immobilization.

The representative cyclic voltammograms for electroactive marker which confirm consecutive steps of the gold electrode modification is showed in Figure 2. The bare Au electrode (Figure 2, curve a) represents reversible redox reactions of $[Fe(CN)_6]^{-4/-3}$ ($\Delta E = 85 \pm 4$ mV). DDT–SAM modification diminished redox current and increased potential shift of redox peaks ($\Delta E = 228 \pm 22$ mV) (Figure 2, curve b). At pH 7.2 His_8-rJHBP molecules are possessing positive charge, therefore, the presence of these molecules on the DDT surface should attracts the negatively charged redox marker and facilitates the redox process. On the other hand, His_8-rJHBP molecules with molecular weight about 32 kDa

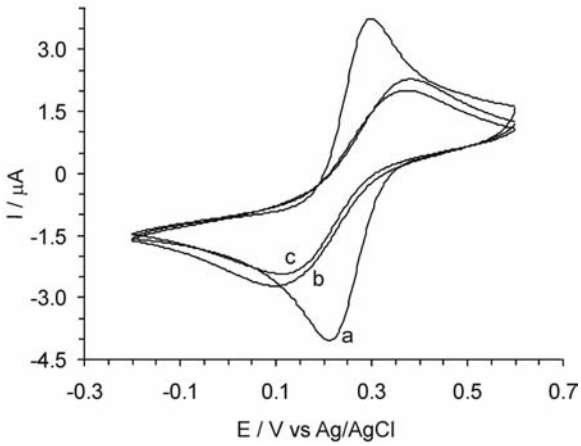

Figure 2. Confirmation of each step of the gold electrode modification by CV. A. bare Au electrode, B. Au-DDT monolayer, C. Au-DDT-His_8-rJHBP. The electrolyte composition: 0.1 M dihydrogen phosphate, 0.1 M KCl, 1.0 mM $K_3[Fe(CN)_6]$ /$K_4[Fe(CN)_6]$, pH 7.2. The measurements conditions: three electrode configuration (see Materials and Methods), scan rate: 100 mVs^{-1}.

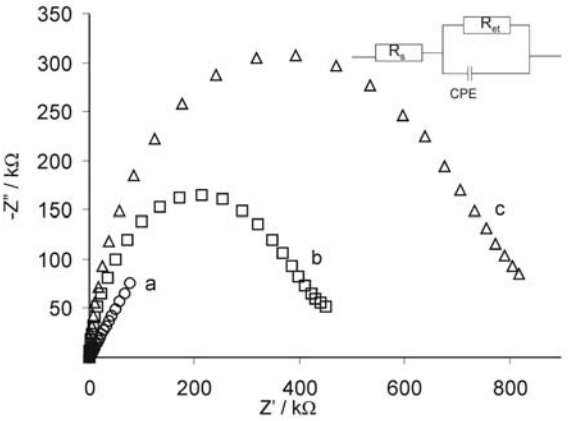

Figure 3. Confirmation of each step of the gold electrode modification by EIS, A. bare Au electrode, B. Au-DDT monolayer, C. Au-DDT-His_8-rJHBP. The electrolyte composition: see Figure 2. The measurements conditions: tree electrode configuration (see Materials and Methods), frequency: 10000 – 0.1 Hz, ac amplitude 10 mV. Inset: equivalent electric circuit diagram of electrochemical interface. R_s – electrolyte resistance, R_{et} – electron transfer resistance, CPE – constant phase element.

(13) increased the packing density of DDT monolayer, which considerably lowered access of $[Fe(CN)_6]^{-4/-3}$ ions to the gold surface. This parameter is dominating, therefore we observed the decrease of reversibility of the system after immobilization of His_8-rJHBP on Au-DDT surface ($\Delta E = 259 \pm 56$ mV) (Figure 2, curve c).

Adsorption of the His_8-rJHBP on Au-DDT electrodes was also studied using redox ion probe and EIS technique (Figure 3). The complex impedance is presented as the sum of the real (Z'_{re}) and imaginary (Z''_{im}) components, that originate from the resistance and capacitance of the cell, respectively. Impedance spectra were recorded after ensuring that the EIS sweeps were stable and reproducible.

The EIS data could be interpreted on the basis of different equivalent circuits. The most suitable one for fitting the values presented consists with R_s- electrolyte resistance, R_{et}- electron transfer resistance, CPE – constant phase element. The CPE is recommended for modeling electrochemical phenomenon occurring on the surfaces which are not ideal homogenous.

From circuit elements obtained by fitting the values, the electron transfer resistance (R_{et}) could be calculated. In this experiment R_{et} could be treated as a good analytical signal. The equivalent electric circuit diagram of the electrochemical interface used for fitting the data presented was illustrated as an inset in Figure 3.

The curve "a" in Figure 3 represents the impedance spectra for a bare gold electrode measured at the formal potential of the redox probe $[Fe(CN)6]^{-4/-3}$ (170 mV). It is almost a straight line that is characteristic for a diffusional limiting step of electrochemical process. The value of R_{et} is very small, about 716 ± 90 Ω. The immobilization of dodecanethiol monolayer on the gold electrode reduced the accessibility of the electrons from the redox agent to the electrode surface, and eventually reduces its response. Because of this, the semicircle shape of EIS spectra was observed at higher frequencies, which corresponds to the electron transfer limited process, followed by a linear part characteristic of the lower frequency, which is attributed to a diffusionally limited electron transfer. The respective semicircle diameter correspond to the electron transfer resistance at the electrode surface. The Au electrode modified with DDT monolayer showed higher R_{et} than bare electrode (490 ± 8 kΩ) (Figure 3, curve b).

The immobilization of His_8-rJHBP on the dodecanethiol SAM creates the insulating protein layer on the surface of Au-DDT. It caused the increase of the electron transfer resistance in the interfacial region (1126 ± 71 kΩ) (Figure 3, curve c). In this case a kinetic-controlled electrode process is predominant.

The results concerning the Au electrode modification with DDT and His_8-rJHBP obtained by using EIS technique (Figure 3) correlate well with those obtained by the CV measurements (Figure 2). Both electrochemical methods confirmed the stable immobilization of His_8-rJHBP molecules on the surface of Au-DDT.

The alkanethiol SAM with COOH functional group was also used for His_8-rJHBP immobilization. But this type of monolayer was less suitable for this purpose (results are not showed). Similar conclusions were reported for the albumin interaction with SAMs of alkanethiols having a different functional headgroups. The albumin adsorption was the most efficient for the dodecanethiol

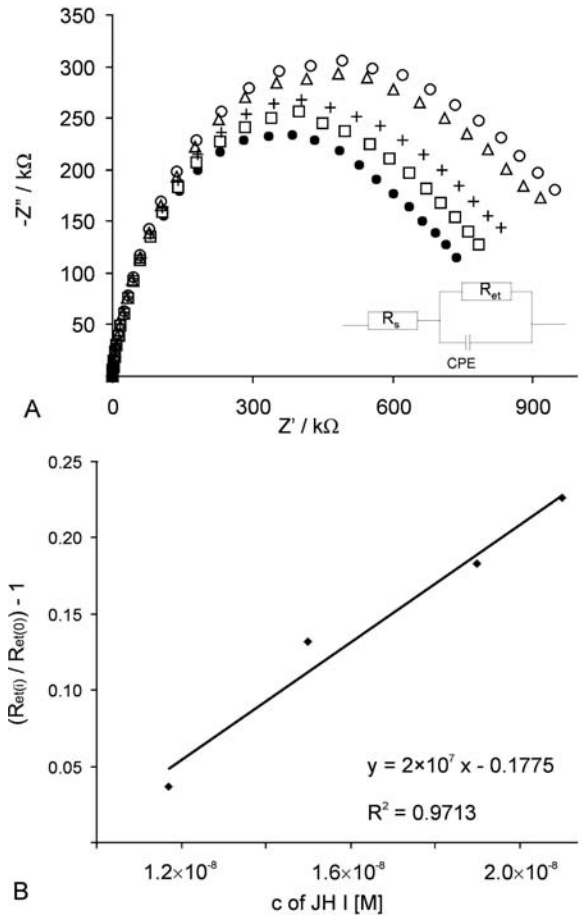

Figure 4. A. Electrochemical impedance spectra of Au-DDT-His_8-rJHBP electrode in buffer solution (○) and after treatment with 12.0 (Δ), 15.0 (+), 19.0 (□), 21.0 (●) nM of the JH I. The electrolyte composition see Figure 2; the measuring conditions see Figure 3. B. The linear relationship of $(R_{et(i)} / R_{et(0)}) - 1$ vs C [M] of the JH I, where $R_{et(i)}$ is the electron transfer resistance of the Au-DDT-His_8-rJHBP electrode after injection aliquot of the JH I and $R_{et(0)}$ is the electron transfer resistance in the absence of hormone.

monolayer in comparison to the OH- and COOH-terminated SAMs (38, 20).

4.2. Interaction between hormones and their analogs with His_8-rJHBP immobilized on the Au-DDT electrodes

The presence of His_8-rJHBP on the surface of Au-DDT electrode was confirmed by CV and EIS methods (Figures 2 and 3). But for the study of interactions between His_8-rJHBP and hormones or their analogs, EIS technique occurred to be more sensitive. This method allows to observe the changes of the interfacial electron transfer resistance (R_{et}) caused by specific adsorption on the electrode surface even very small neutral molecules. This technique was successfully applied for the study of interaction between beta-sheet amyloid and potential drugs (36), antigen – antibody (33, 34), avidin and biotin system (35), just to name a few examples.

Among the juvenile hormones, JH I and JH II have been selected for the present study. It is known that individual JH homologs characterize various levels of biological activity. The JH I and JH II are more potent morphogenetically than JH III in a number of different biological assays using lepidopteran insects (*Manduca sexta, G.mellonella*) (4, 15). It has been also reported that the highest polarity of the JH III out of other JHs causes this compound more prone to degradation.

Apart from the JH I and JH II, methoprene and farnesol have been included to the group of compounds being the objective of the presented study. Methoprene, a chemical analog of JH III with potent biological activity is commercially available as the insect growth regulator (39, 40, 8). Whereas farnesol is considered a precursor of the the JHs (7).

In order to show the selectivity of the analytical system proposed, the non specific interactions of analytes studied with DDT monolayer, without His_8-rJHBP, have been tested. In this case, no change or insignificant increase of the R_{et} were observed (results are not showed). Therefore, it might be concluded that both hormones (JH I and JH II) and methoprene interact specifically with His_8-rJHBP immobilized on DDT monolayer and the analytical system proposed is suitable for research presented.

From the compounds being the objective of our study only farnesol, precursor of JHs, and 3,7-dimethyl-1-octanol (Figure 1) interacted non-specifically with DDT monolayer and decreased its electron transfer resistance. The results obtained from this preliminary experiment allow to concluded that methyl ester and epoxide groups are responsible for the specific interaction with His_8-rJHBP immobilized into DDT monolayer. The presence of π bonds and length of alkyl chain have no influence. Therefore, the analytical system proposed was applied for study of hormones and methoprene interactions with His_8-rJHBP.

The representative EIS spectra obtained for Au-DDT-His_8-rJHBP working electrode upon stimulation with different concentration of the JH I are showed in Figure 4 A. The increasing concentration of the JH I in the supporting solution caused decrease of semicircle diameter, which indicates the reduction of electron transfer resistance ($R_{et(i)}$). These changes were proportional to the JH I concentration. The similar results were obtained in case of the JH II and methoprene (results are not showed).

The decrease of R_{et} occurred upon stimulation of Au-DDT-His_8-rJHBP with hormones and methoprene indicated, that these interactions lead to the His_8-rJHBP conformation changes. This phenomenon modifying the order of DDT monolayer which become more permeable for the $[Fe(CN)_6]^{-4/-3}$ redox marker.

This conclusion is in a good agreement with other scientific reports, which described JHBP conformation changes upon the JH binding observed with range of different techniques such as: the JH-JHBP sediment coefficient and changes of CD and UV spectra, changes in electrophoretic mobility and perturbation of internal chromophores (11).

Table 1. The interaction of Au-DDT-His_8-rJHBP electrode with hormones and methoprene

Compounds	$R_{et(0)}$ / $R_{et(21\ nM)}$ × 100%	Number
JHI	78.6 ± 13.1	5
JHII	77.6 ± 12.2	3
methoprene	71.8 ± 15.1	5

$R_{et(0)}$- the resistance of the Au-DDT-His_8-rJHBP electrode, $R_{et(21nM)}$ - the resistance of the Au-DDT-His_8-rJHBP electrode at maximum concentration of analytes (21 nM)

The JHBP is a medium size protein possessing two disufide bridges between Cys^{10} - Cys^{17} and Cys^{151} - Cys^{195} (41). N- and C-terminal portions of *G. mellonela* JHBP are internally stabilized by S-S bonds. However, this does not exclude the possibility that N- and C- peptides of JHBP participate in the binding pocket formation for the JH binding with the flexible hinge(s) located somewhere between them (41).

To our knowledge, the results presented here are the first report on the His_8-rJHBP conformation changes occurred upon complexation with JHs observed by EIS technique.

It is worthy to underline, that in the measuring system applied, His_8-rJHBP was immobilized on the surface of hydrophobic alkanethiol environment, whereas JHs were dissolved in the aqueous solutions in range close to physiologically observed concentrations (42). Thus, this mimics well the physiological processes occurred in the living organisms.

The strength of His_8-rJHBP interactions with JHs and methoprene were estimated as the relative decrease of R_{et} caused by the highest concentration of analytes studied. The results were collected in Table 1. Both hormones and methoprene caused ca 20% decrease of initial value of $R_{et(0)}$ measured in the absence of any stimulant.

4.3. Determination of association constants in the JHs - immobilized His_8-rJHBP system

The electrochemical impedance spectroscopy EIS cannot provide direct data related with the exact amount of molecules adsorbed on the surface of electrode (43). Therefore, the simplified approach with using a Langmuir isotherm is necessary. This has been successfully applied for determination of association constants between cations and crown ethers (44 - 46), as well as between amyloid and potent drug (36). The Langmuir isotherm assumes equal binding energy for all binding sites.

In the EIS experiment presented, the changes of R_{et} are related to the binding of the JHs or their analogs to the His_8-rJHBP. The relation between the occupied binding sites Θ and the change of R_{et} is as follows (46):

$$\Theta = 1 - \frac{R_{et(0)}}{R_{et(i)}} \qquad (1)$$

$R_{et(0)}$, and $R_{et(i)}$ mean the charge transfer resistance of the monolayer without, and at the presence of particular concentration of hormones studied, respectively. In the case of Langmuir isotherm, Θ can be related to the association constant according to the equation (47):

$$\Theta = \frac{K_a c}{1 + K_a c} \qquad (2)$$

Where K_a denotes association constant and c concentration of molecules in the solution. The linearization of Langmuir isotherm gives:

$$K_a c = \frac{\Theta}{1 - \Theta} \qquad (3)$$

Combination of eqs. (1) and (3) gives:

$$K_a c = \frac{R_{et(i)} - R_{et(0)}}{R_{et(0)}} \qquad (4)$$

The equation (4) has been applied for the calculation of the association constants for the His_8-rJHBP – JH complexes.

The $R_{et(i)}$ varies linearly with the concentration of the JH I in the concentration range from 12.0 to 21 nM (Figure 4B). The similar results were obtained for the JH II and methoprene. Therefore, the association constants could be calculated from the slope of the $R_{et(i)} – R_{et(0)} / R_{et(0)}$ vs C [M]. The results obtained are summarized in Table 2.

The affinity of JH I, JH II and methoprene towards His_8-rJHBP protein were quite similar. It is noteworthy that methoprene, devoid of epoxy group present in JH, interact specifically with JHBP. It has been previously reported that the presence of methyl ester moiety and epoxide group in the structure of JH are critical for binding by JHBPs (11, 14, 48).

However, the weak binding of methoprene to the JH binding site of Manduca JHBP has been reported by Touhara *et al.* [49]. The result of our study indicated also that JHBP can tolerate the lack of the epoxide in effective binding of the ligand. The association constants presented in our study were in range 10^7 M^{-1}. It is in good agreement with those reported previously for binding of the JHs to the low-molecular weight JHBPs which contain the one binding site for this ligand (14, 12).

To our knowledge, the research presented here is the first attempt of assessment of association constants between His_8-rJHBP protein and juvenile hormones or their analogs with using the EIS method.

Thus, it might be concluded that the electrochemical impedance spectroscopy, together with Au electrode modification employing the self-assembled DDT layer is very suitable technique for the above purpose. This measuring system is relatively simple in the comparison to other spectroscopic one and allows to determine not only thermodynamic of the interfacial interactions, but their kinetics as well (50, 43).

Table 2. The association constants for His_8-rJHBP immobilized onto the Au-DDT electrode with hormones and methoprene determined by EIS

Compounds	K_a [M^{-1}]	Number	Mean of values of calculated R^2
JHI	$3.5 \pm 1.7 \times 10^7$	4	0.9635 ± 0.0250
JHII	$2.3 \pm 0.6 \times 10^7$	3	0.9657 ± 0.0340
methoprene	$3.0 \pm 1.7 \times 10^7$	3	0.9279 ± 0.1071

5. CONCLUSIONS AND PROSPECTS

The dodecanethiol (DDT) monolayer deposited on the surface of the gold electrodes through Au-S covalent bonds was proved to be a good hydrophobic environment suitable for immobilization of the His_8-rJHBP protein molecules with keeping their unchanged biological activity. The alternation of the interfacial properties of Au-DDT-His_8-rJHBP electrode upon interactions with JHs or methoprene were successfully traced by electrochemical impedance spectroscopy EIS. The obtained results confirmed the conformation changes of the His_8-rJHBP protein occurred upon JHs or methoprene binding.

The electron transfer resistance of the Au-DDT-His_8-rJHBP electrode varies linearly with the concentration of aqueous solution of hormones and/or related compound studied in the range from 12 to 21 nM. These relationships allow to calculate the association constants.

The measuring system proposed, with His_8-rJHBP immobilized onto hydrophobic environment of DDT monolayer and JHs present in the aqueous solution mimic well the physiological conditions. Thus, it could be recommended for study the interfacial interactions between biomolecules. Also, the analytical system proposed might be applied for the monitoring in the environment of methoprene, which is widely used as an insect growth regulator.

6. ACKNOWLEDGEMENTS

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