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Electrochemical sensing of interaction of anterior gradient-2 protein with peptides at a charged interface

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ABSTRACT

Anterior gradient-2 protein (AGR2) is overexpressed in many human cancers, and this protein presents a novel promising cancer biomarker. We show significant progress in understanding of the electric field effects on AGR2-peptides complexes using constant current chronopotentiometric stripping (CPS) analysis. Surface-attached AGR2-peptide complexes can be disintegrated as a result of their exposure to negative potentials. By controlling the exposure time and temperature, peaks of weakly bound non-specific complexes can be discriminated from tightly bound specific complexes. Using CPS analysis we found that mutant E60A-AGR2 forms weaker complex with peptide aptamer in comparison to wild type AGR2. These data highlight the utility of this method for studying real-time dynamics of surface-attached protein-peptide complexes.

1. Introduction

Combining powerful techniques from organic synthesis, biophysical chemistry, molecular biology and genetics allows researchers all over the world to design new molecules, identify key biochemical pathways and develop targeted strategies to combat cancer. Understanding of cancer-causing processes at the molecular level and introducing new methods significantly accelerate progress within this field. Previously we showed that the label-free and reagent-less chronopotentiometric stripping (CPS) analysis in combination with mercury-containing electrodes is suitable for studying dynamic changes in protein nanolayers at electrical interfaces [1]. CPS peak H resulting from the catalytic hydrogen evolution by proteins and peptides [2] allowed to analyze free proteins [1], including membrane proteins [3], and those in complexes with nucleic acids [4,5] and proteins [6,7]. The CPS peak H appear at highly negative potentials, at which a non-homogenous electric field appears in the vicinity of the electrode surface [8,9] and can influence the behavior of molecules attached to the surface. By applying high negative current intensities we are able to reduce the time for which the layer is exposed to such negative potentials to milliseconds [10]. Such short exposure times can eliminate protein denaturation and/or complex disintegration [11]. Thus using various stripping current intensities, the CPS allows discrimination between specific and non-specific protein-DNA binding and provides valuable information on stability of the nucleic acid-protein interactions at the polarized interfaces [4,12]. The obtained data suggest that the proposed method can be used to study both the stability and integrity also of the other protein complexes.

The emergence of aptamers created an attractive alternative to antibodies [13]. In 1990 two revolutionary studies presented the method of *in vitro* generation of high-affinity nucleic acid aptamer molecules against selected targets [14,15]. Although the utilization of the aptamers as affinity, diagnostic, or drug reagents is not sufficiently utilized in the cancer field, the biotechnology sector has exploited their properties to target clinically relevant proteins [16,17]. In 1996, Colas et al. [18] described peptide aptamers that represent other alternative to antibodies. Peptide aptamers usually consist of 5–20 amino acid residues that are selected according to their ability to interact with the protein of interest [19]. By specific binding to target proteins they may contribute to their identification and/or may also block their biochemical functions thus representing an attractive alternative to antibodies [20]. Their binding affinity is comparable with antibodies and can be used in a variety

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of environmental conditions, plus their production is straightforward and robust *in vitro* [19]. Due to these advantages, aptamers have gained interest over the past decade and are widely used [21,22].

Recently, the AGR family of proteins have attracted researchers' attention due to their putative involvement in developmental processes and carcinogenesis [23]. AGR2, the most studied member of the family, plays an important role in many human solid tumors [24]. Thus an in-depth characterization of AGR2 structural, molecular and electrochemical properties may contribute to its use in cancer research. Interestingly, specific peptide aptamers recognizing AGR2 were selected and used to develop a quantitative microarray assay [25]. Within this aptamer-based capture assay the PTTIYY peptide core was identified.

In this paper we tested binding of peptide aptamer and AGR2 oncoprotein using label-free CPS analysis for the first time. Using this analysis we were able to effectively distinguish between specific and non-specific protein-peptide interactions, since specific AGR2-peptide aptamer complexes produced almost no response (CPS peak H), while under the same conditions the non-specific AGR2 binding to arbitrary peptide yielded well-developed CPS peak H as that of AGR2 protein alone. Additionally, we identified that peptide aptamer complexes of with *wild type* AGR2 (wt-AGR2) are more stable that those with mutant E60A-AGR2.

2. Experimental

2.1. Material and apparatus

Recombinant AGR2 protein was purified as described previously [26]. Peptides (PTTIYY, FPTIYYFS, biotin-SGSGPTTIYY, HHHHHH, DRVYIHPF) were purchased from Clonestar (Brno, Czechia) and other chemicals were of analytical grade.

Electrochemical measurements were performed using an AUTOLAB Analyzer (EcoChemie, Utrecht, Netherlands) in combination with VA-Stand 663 (Metrohm, Herisau, Switzerland); a hanging mercury drop electrode (HMDE, 0.4 mm^2) served as the working electrode in a standard cell in a three-electrode system. An Ag|AgCl|3 M KCl electrode was used as the reference and a platinum wire as the auxiliary electrode. Experiments were carried out in open air, at a controlled temperature of 18 °C using a thermostat from Julabo, USA. Data were collected using GPES version 4.9. Experiments were replicated at least 3 times for each measurement.

2.2. Electrochemical sensing

40 μM AGR2 protein was incubated with 400 μM peptide in 3.8 mM Tris-HCl, pH 7.5, 45.6 mM KCl, 4.56 mM NaCl, 0.38 mM $MgCl_2$, 0.38 mM $CaCl_2$ and 2.05 M DMSO in a volume of 20 μL on ice for 2 h. High concentration of DMSO was used for dissolving hydrophobic PTTIYY peptide. 300 nM sample was adsorbed on the working electrode at accumulation potential −0.3 V for an accumulation time of 120 s in background electrolyte 50 mM Na-phosphate, pH 7, followed by chronopotentiogram recording with stripping current, I_{str} −22.5 μA or alternating current voltammogram with amplitude 50 Vrms, scan rate 9.2 mV/s, frequency 223 Hz and phase angle 90°. Accumulation was done under stirring.

PTTIYY modified HMDE: 3 μM peptide was adsorbed at accumulation potential −0.3 V for 120 s from 50 mM Na-phosphate, pH 7, on HMDE followed by washing and transfer PTTIYY-modified electrode to background electrolyte in presence or absence of AGR2.

2.3. Immunoprecipitation

To verify AGR2's interaction with peptide aptamers, HEK-293FT cells were transiently transfected as described previously [27] with expression plasmid pCDNA3 bearing the coding sequence for either wt-AGR2 or mutant E60A-AGR2. 48 hrs post transfection, cells were collected into lysis buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3CO_4, 1% Nonidet P40). Lysates containing the same amount of total protein were incubated overnight with biotinylated peptides conjugated to streptavidin-agarose beads (Milipore, Billenca, MA, USA) at 4 °C. Immunoprecipitates were eluted with 0.1 M glycine-HCl pH 2.5 and neutralized with 1.5 M Tris-HCl pH 8.8. The proteins were analyzed by immunoblotting and detected as described previously using specific anti-AGR2 K31 rabbit polyclonal antibody [28]. Immunoprecipitation with purified AGR2 proteins was performed according to the same protocol as for the cell lysates.

3. Results and discussion

3.1. Specific binding of peptide aptamer

Previously, the electrochemical behavior of AGR2 protein was described at HMDE [29]. Obtained CPS data were in good agreement with complementary analysis for the study of protein structure, namely hydrogen-deuterium exchange connected with high resolution mass spectrometry. Here we used the same arrangement to study CPS responses of AGR2 protein after specific binding to the peptide aptamer PTTIYY. 300 nM AGR2 or AGR2-peptide complex (prepared *in vitro*) were adsorbed at HMDE from background electrolyte, followed by chronopotentiogram recording. Under these conditions, AGR2 protein yielded a well-developed peak H in agreement with a previous study [29], while PTTIYY peptide did not produce any peak H (Fig. 1A). Peak H of AGR2 after incubation with the peptide aptamer almost disappeared (Fig. 1A). This dramatic change in the peak H of the AGR2-PTTIYY complex (as compared to free AGR2) can correspond to the binding of AGR2 with PTTIYY peptide. Large differences between the peak H areas of free AGR2 and AGR2-PTTIYY complex were observed in a wide range of surface concentrations depending on accumulation time (Fig. 1B). Inhibition of the catalytic hydrogen evolution reaction of AGR2-PTTIYY complex can be explained by (i) decreased accessibility of catalytically active aa residues (lysine, arginine, histidine and cysteine) [1,30,31] in the AGR2-peptide complex, as well as (ii) different adsorption of the AGR2-peptide complex compared to free AGR2 protein and/or (iii) altered interfacial properties of AGR2-PTTIYY complex and free AGR2 protein. Decrease of CPS peak H after specific binding of PTTIYY peptide to AGR2 was similar as we observed after specific DNA-protein interaction [4,12]. In both cases neither peptide nor DNA yielded any CPS peak H. On the other hand, specific interaction of lectins with glycoproteins resulted in increase of CPS response comparing to that of free lectin and glycoprotein [6,7]. This phenomenon may be explained by the changes in orientation and/or conformation of lectin and/or glycoprotein after their specific binding (biorecognition process).

To investigate the adsorption behavior of AGR2-peptide complex, we further studied AGR2 protein, PTTIYY peptide and their complex using phase sensitive alternating current (AC) voltammetry out-of-phase (sensitive to non-faradaic, capacitance processes). AGR2 protein was adsorbed in almost the whole range of studied potentials, which was accompanied by a decrease of its capacitive current compared to that of the background electrolyte, as described earlier [29]. Peptide was also adsorbed on the HMDE surface and its adsorption was weaker than that of AGR2 between −0.1 V and −1 V (Fig. 1C). The AC voltammogram of AGR2-