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Research paper

Chronopotentiometric sensing of anterior gradient 2 protein

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ABSTRACT

The study of new proteins and particularly those involved in cancer development is still a focal point of interest. We studied the anterior gradient 2 (AGR2) oncoprotein, aberrantly expressed in a number of human cancers. For the first time the electrochemical behaviour of various variants of the AGR2 was described using constant current chronopotentiometric stripping (CPS) analysis at mercury electrodes. In the first part, we show that mutation of AGR2 protein at its sole cysteine significantly changed its CPS response compared to wild type AGR2, probably due to their different adsorption and some deviations in their structures, which were obtained by analysis of hydrogen – deuterium (H/D) exchange connected with high resolution mass spectrometry (MS). In the second part we studied the influence of His-tag modification, widely used in the purification of recombinant proteins, on CPS response and H/D exchange. Addition of a His-tag, containing positively charged and electroactive residues, affected the CPS peak H of His_6-tagged AGR2 compared to non-tagged AGR2 protein due to different adsorption as well as variation in the structure at the negatively charged interface. H/D exchange MS analysis confirmed differences in the structure of these two variants even in solution. The uncovering of AGR2 behaviour at charged surfaces gives us the opportunity to study its interactions with other biomedically important proteins.

1. Introduction

In the Nextprot platform, there are about 20 000 records concerning proteins encoded by the human genome [1]. A wide variety of old and new methods in proteomics aim to acquire as much information as possible about proteins. Techniques focused on revealing the structure and conformation of proteins, which determine their functions, are sought after. Electrochemical methods have been utilized in protein structural analysis only rarely [2–4]. A simple label-free chronopotentiometric stripping (CPS) method in combination with an electrocatalytic process and mercury electrodes allows the determination of practically any protein at low concentration, as well as recognition of changes in the protein structures [5]. CPS appeared particularly useful in the analysis of proteins important in biomedicine, including (i) tumor suppressor p53 protein [6] and its sequence-specific interaction with DNA [7], (ii) α-synuclein with its aggregation and oligomerization [8,9], (iii) lectin concanavalin A and its interactions with glycoproteins [10]. These results obtained using the CPS method were in good agreement with results generated by other methods such as fluorescence, dynamic light scattering, gel electrophoresis etc. [5,6,8–10].

To take full advantage of the application of CPS peak H in protein analysis, it is essential to understand the different parameters influencing the catalytic hydrogen evolution reaction (CHER) in a protein. Using model peptides [11] and polyamino acids [12,13] it was shown that only amino acid residues bearing functional groups with exchangeable protons, such as cysteine (Cys), lysine (Lys), arginine (Arg) or histidine (His), can play the active role in the CHER [5]. These amino acid residues transfer the protons from the acid constituent of the buffer (proton donor) onto the highly negatively charged surface [14].

Anterior Gradient-2 (AGR2) is an endoplasmic reticulum resident protein mainly expressed in human epithelial cells. Enhanced AGR2 expression was observed in many human cancers [15]. Apart from its function as a p53 inhibitor [16,17], subsequent studies have further reinforced a role for AGR2 in a range of cancer associated pathways demonstrating that its overexpression enhances the rate of adhesion, resulting in a greater propensity to form metastases [18,19], and supports anchorage-independent

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growth [20]. Recently, AGR2 has been found in human biological fluids such as plasma and urine [21], thereby uncovering new ways for its potential use as a cancer biomarker. The ability to catalyse the formation of disulphide bonds in proteins represents the parameter most important for assigning this protein to a protein disulphide isomerase (PDI) family. This protein, as well as other members of the PDI family, has specific structural features such as thioredoxin (CxxC) or thioredoxin-like domains (CxxS), special C-terminal motifs characteristic of proteins localized in the endoplasmic reticulum, etc. that are necessary for their assignment to a PDI family [22].

In this work, we describe for the first time the electrochemical behaviour of the AGR2 protein at mercury electrodes. We elucidate the influence of Cys and His residues on the electrochemical response of the AGR2 protein. Using a combination of hydrogen/deuterium (H/D) exchange connected with high resolution mass spectrometry (MS) [23] and CPS analysis we show that AGR2 mutation of the sole Cys to alanine (Ala) partially changed the protein structure, and simultaneously significantly changed the electrochemical response caused by the high affinity of Cys to mercury. Also, His_6-tag modification of the AGR2 protein at the N-terminus altered its structure, as well as, its electrochemical response. In both cases, the electrochemical responses of the selected AGR2 variants were influenced by different protein adsorption.

2. Materials and Methods

2.1. Reagents and Solutions

$AGR2^{21-175}$ (lacking signal peptide) cloned into pEHISTEV was kindly provided by prof. T. Hupp [24]. AGR2 mutants C81A, C81S, K95A, K95S were prepared using a QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Purification of AGR2 proteins was described earlier [25]. Purified fusion His_6-AGR2 protein was subsequently cleaved with His_6-TEV protease to remove the His_6-tag. The N-terminal His_6-tag of AGR2 with His_6-TEV protease was then captured using a HisTrap FF 5 ml column (GE Healthcare), whereas the purified recombinant protein was present in the flow-through fractions. After purification, the protein concentration was determined spectrophotometrically using the molar extinction coefficient obtained from the ProtParam software on the EXPASY server. Solutions were prepared from triple distilled water. All chemicals were of analytical grade.

2.2. Procedures

2.2.1. Electrochemical analysis

Protein was adsorbed on the electrode surface from the stirred solution for an accumulation time, t_A of 120 s (if not stated otherwise) at an accumulation potential, E_A of +0.1 V (if not stated otherwise) in the background electrolyte. After accumulation, chronopotentiograms were then recorded in the same solution. An AUTOLAB Analyzer PGSTAT 30 potentiostat (Metrohm-Autolab) in combination with a VA-stand 663 (Metrohm) and three electrode system was used to perform the experiments. The working electrode was a hanging mercury drop electrode (area 0.4 mm^2), Ag|AgCl|3 M KCl was used as the reference electrode and platinum wire as the counter electrode. All experiments were carried out at a temperature of 18 °C (if not stated otherwise) open to air.

2.2.2. Hydrogen/deuterium exchange mass spectrometry

This analysis contained from two different experiments, the first is the peptide identification followed structural analysis. In the first step each AGR2 protein was proteolyticaly digested and the created peptides were identified with high confidence using LC-MS/MS measurement before hydrogen/deuterium exchange cultivation. Aim of this step was determination of the amino acid composition of peptides covered primary structure of the protein. The second step was deuteration of either wt AGR2, or AGR2 mutant C81A and wt His-AGR2 proteins (their sequences are introduced in, Figs. 2 B and 5 B) was initiated by a sequential dilution into deuterated water with 1% dimethyl sulfoxide (v/v) with a 3 μM final protein concentration. The deuterium exchange of wt AGR2 and wt His-AGR2 was carried out at room temperature and was quenched by the addition of 0.875 M HCl in 1 M glycine at 10 min and 30 min followed by rapid freezing in liquid nitrogen. Moreover the following experiment with wt AGR2 or wt His-AGR2 was held at four different temperatures (4 °C, 16 °C, 25 °C (RT), 37 °C) and was quenched by the addition of 0.875 M HCl in 1 M glycine at 60 min followed by rapid freezing in liquid nitrogen. Each sample was thawed and injected onto an immobilized pepsin column (15 μl bed volume, flow rate 100 μl/min, 98% HPLC grade water, 2% acetonitrile, and 0.05% trifluoroacetic acid, v/v/v). Peptides were trapped and desalted on-line on a peptide microtrap (Michrom Bioresources, Auburn, CA) for 3 min at flow rate 50 μl/min. Next, the peptides were eluted onto an analytical column (Jupiter C18, 1.0 × 50 mm, 5 μm, 300 Å, Phenomenex, CA) and separated using a linear gradient elution of 10% B in 2 min, followed by 17 min isocratic elution at 40% B. Solvents were: A – 0.1% formic acid in water (v/v), B – 0.08% formic acid in 80% acetonitrile, and 20% water (v/v/v). The immobilized pepsin column, trap cartridge and the analytical column were kept at 1 °C. Mass spectrometric analysis was carried out using an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) with ESI ionization on-line connected with a robotic system based on the HTS-XT platform (CTC Analytics Company). The instrument was operated in a data-dependent mode for peptide mapping (HPLC-MS/MS). Each MS scan was followed by MS/MS scans of the top three most intensive ions from both CID and HCD fragmentation spectra. Tandem mass spectra were searched using SequestHT against the cRap protein database (ftp://ftp.thegpm.org/fasta/cRAP) containing sequences of all AGR2 proteins with the following search settings: mass tolerance for precursor ions of 10 ppm, mass tolerance for fragment ions of 0.6 Da, no-enzyme specificity and no-fixed or variable modifications were applied. The false discovery rate (FDR) at peptide identification level was set to 1% for the peptides identified with high confidence, 5% for the peptides identified with moderate and higher than 5% for low confidence, respectively. Sequence coverage was analysed with Proteome Discoverer software version 1.4 (Thermo Fisher Scientific). Analysis of deuterated samples was performed in HPLC-MS mode with ion detection in the orbital ion trap and the data were processed using HDX Examiner (Sierra Analytics). Graphs showing the deuteration kinetics were plotted using Draw H/D Protection Plot [26]. Molecular visualization was performed with the AGR2_Human protein (PDB code: 2LNS, residues 41-175) with the software PyMOL (The PyMOL Molecular Graphics System, version 1.5.0.4; Schrödinger, LLC).

3. Results and discussion

3.1. The role of Cys 81 on AGR2 electrochemical behaviour

Previous work has shown, that CPS analysis in combination with mercury electrodes was sensitive to local and global changes in protein structure [5,6,9,27] including those caused by one amino acid exchange [6]. Following these findings we tested wt AGR2 and its mutant form C81A using CPS and a hanging mercury drop electrode (HMDE) in neutral 50 mM phosphate buffer for the first time. Wt AGR2, adsorbed at the HMDE at an accumulation potential, E_A of +0.1 V from an electrolytic cell at accumulation