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Effect of His_6-tagging of anterior gradient 2 protein on its electro-oxidation

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ABSTRACT

His-tagged and non-tagged forms of Anterior Gradient 2 (AGR2), oncoprotein and potential cancer biomarker, were studied for the first time using voltammetry at carbon electrodes. In addition to oxidation peak of Tyr and Trp, N-terminal His-tagged AGR2 form yielded characteristic electro-oxidation peak of histidine. Qualitatively similar results were obtained with other N-terminal His-tagged proteins, such as, glutathione-S-transferase, α-synuclein and cytochrome b5. Our results suggested that His-tag modification of proteins (commonly used in recombinant protein preparation) may change adsorption and orientation of the proteins at electrode surfaces. In absence of the His-tag in His-containing proteins, appearance of His peak was influenced by accessibility of His residues, and depended on the carbon electrode type. Oxidation His peak, in combination with Tyr and Trp oxidation responses may find use in label-free analysis of numerous proteins, including those important in biomedicine.

1. Introduction

Progress in genomics, proteomics and bioinformatics over the last decades resulted in dramatic increase of proteins produced by recombinant techniques [1–3]. Introduction of an affinity tag contributed to improvement in the biochemical properties of the target recombinant protein, such as improved protein yield [4], prevention of proteolysis [5], increased solubility [5], etc. On the other hand, adding tag can change protein conformation [6], inhibit enzyme activity [7], alter biological activity [8] etc., but to our knowledge, no information about the effect of His-tagging on the protein interactions with electrically charged surfaces was reported. In recent years, numerous fusion tags have been developed for recombinant protein production [1–3]. PolyHis-tags (containing 3 to 10 His residues [3]) are the most commonly used affinity tags for purifying recombinant proteins [9] used in biophysical and structural studies [2]. Advantages of the polyHis tag include its low immunogenicity and small size, positioning at either the N- or C-terminus. PolyHis-tagged proteins have been studied by different methods [10] but not by methods of electrochemical analysis.

Label-free direct electrochemical methods have been successfully applied for the study of proteins important in biomedicine including α-synuclein [11–13], amyloid β-peptides [14–16], tumor suppressor p53 [17,18], etc. Besides analysis of non-protein part in conjugated proteins [19,20], predominantly, voltammetric oxidation peaks [15,21–25] and/or chronopotentiometric catalytic peak H of proteins [13,17,18,26] were used for direct electrochemistry.

More than 30 years ago it was shown that free monomeric Cys, His, Met, Tyr and Trp are oxidized at carbon electrodes [27–30]. Moreover, it was found that only Tyr and Trp residues are oxidized in proteins [28–30] and peptides [31,32] at positive potentials far from zero. Cai et al. [31] studied biological important peptides, such as neurotensin, bombesin and LH-RH, containing Tyr and/or Trp residues and they showed that these peptides produced well-resolved Tyr and/or Trp peaks in dependence on the Tyr, Trp peptide content. In proteins with more Tyr, Trp residues peaks Tyr and Trp can merge into one Tyr, Trp peak [26]. Further studies of different proteins were in agreement with this work. Recently, oxidation of Met [22,33] and His [21,22] residues in proteins at carbon electrodes was reported but unambiguous experimental evidence of the origin of this oxidation is still needed. Very recently, His oxidation peak was not observed in His-containing angiotensin peptides at a basal plane pyrolytic graphite [32].

AGR2 is a human protein originally identified as a protein expressed in *Xenopus laevis* embryos [34]. It is endoplasmic

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reticulum resident protein required for mucins production as was confirmed by clinical studies showing the key role of AGR2 in inflammatory bowel disease [35] and asthma [36]. Apart from these functions, many studies showed a role for AGR2 in a range of cancer associated processes including cell migration and cellular transformation [37], metastasis [38], and drug resistance [39]. AGR2 is highly expressed in various human cancers and its utility as a cancer biomarker is under intense investigation [40,41].

In this paper, we studied electro-oxidation of recombinant AGR2 oncoprotein in its N-terminal His_6-tagged and no-tagged variants at carbon electrodes with emphasis on elucidation of (i) His oxidation in proteins and (ii) His_6-tag effect at AGR2 and other protein signals. Our results suggest that (a) the potential biomarker, oncoprotein AGR2 and its mutant produce useful electro-oxidation signals, (b) His-containing proteins without His-tag yield His oxidation peak but its appearance and intensity depends on His accessibility and also on the carbon electrode material, (c) His-tagging of AGR2 and all other studied proteins, such as α-synuclein, glutathion-S-transferase, cytochrom b5 and histone H4 resulted in histidine oxidation peak at +1.1 V.

2. Experimental

2.1. Purification of mutant AGR2-E60A protein

$AGR2^{21-175}$ (lacking signal peptide) cloned into pEHISTEV was kindly provided by prof. Hupp [42]. AGR2-E60A mutant was prepared using QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer instructions. $AGR2^{21-175}$-E60A-pEHISTEV was transformed into BL21 (DE3) RIPL chemically competent *E. coli* cells. Bacteria were grown to OD_{600} of ~0.5 at 37 °C. Induction of gene expression was achieved by adding isopropyl β-D-thiogalactopyranoside (IPTG) to the culture (final concentration 1 mM). The bacterial culture was grown at 30 °C for another 3 hrs. Cells were harvested by centrifugation for 10 min at 4600 g, the pellet resuspended in lysis buffer (20 mM Tris, pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 20 mM Imidazole, 1 mM PMSF, 1 mg/ml lysozyme) and disrupted by sonication. Bacterial lysates were obtained by centrifugation for 30 min at 12,000 g. The supernatant was applied to Ni-charged HisTrap FF 5 ml column (GE Healthcare). After washing the column using lysis buffer without PMSF and lysozyme, the immobilized protein was specifically eluted with 250 mM Imidazole. Eluted fraction was loaded on 7 K MWCO Zeba Spin Desalting column (Thermo Scientific) to remove Imidazole. Purified fusion His_6-AGR2 protein was subsequently cleaved with His_6-TEV protease to remove His_6-tag. N-terminal His_6-tag of AGR2 with His_6-TEV protease was then captured using HisTrap FF 5 ml column (GE Healthcare), whereas purified recombinant protein was present in the flow-through fractions. The purified AGR2 was further processed by preparative gel filtration using HiPrep 16/60 Sephacryl S-100HR column (GE Healthcare). The purity of isolated AGR2 protein was confirmed by SDS-PAGE/Coomassie staining (data not shown). Tobacco etch virus protease His_6-TEV(S219V)-Arg_5 was prepared in-house following the modified method of Tropea et al. [43]. Lysozyme was purchased from SERVA Electrophoresis GmbH.

2.2. Material

N-terminal His tagged α-synuclein, histone H4, and cytochrom b5 were purchase from Sigma-Aldrich. His_6-tagged glutathione-S-transferase was obtained as a by-product after TEV cleavage of unspecified His_6-GST tagged protein and further purified using HisTrap FF 5 ml column (GE Healthcare). The proteins concentration was determined spectrophotometrically using the molar extinction coefficient obtained from the ProtParam software on the EXPASY server. His_6 was purchased from Bachem and other chemicals of analytical grade were from Sigma-Aldrich. Solutions were prepared from triple distilled water. Denatured proteins for analysis were prepared by treatment in 8 M urea at 4 °C overnight.

2.3. Apparatus

The experiments were performed with a three electrode system connected to a μAutolab III potentiostat (Metrohm-Autolab). The working electrode was a glassy carbon electrode (GCE, area 3.14 mm^2), a basal plane pyrolytic graphite electrode (BPGE; with geometric area 7.5 mm^2) and a carbon paste electrode (CPE, 7.65 mm^2) controlled by a VA-stand 663 (Metrohm). Ag|AgCl|3 M KCl was used as the reference electrode and platinum wire as the counter electrode. All experiments were carried out at temperature of 18 °C open to air.

2.4. Procedures

The cleaning procedure of the GCE included polishing with alumina 0.3 μm on soft lapping pads for 5 min followed by sonication by Fisherbrand FB 11,020 in deionized water for 1 min. BPGE was pre-treated before each measurement by applying potential +1.7 V for 60 s in background electrolyte. After the electrochemical pre-treatment, the electrode surface was freshly cleaved using cello tape. The carbon paste of 70% graphite powder (Aldrich) and 30% mineral oil (Sigma; free of DNase, RNase, and protease) was housed in a teflon body [44]. Prior to measurements the electrode was polished on weighing paper (Macherey-Nagel).

2.5. Adsorptive Transfer (AdT, ex situ) stripping [26,45]

15 μl of the 5 μM AGR2 aqueous solution were placed onto the electrode surface for accumulation time, t_A of 300 s. Then the AGR2 -modified electrodes were washed with a 50 mM phosphate buffer solution, pH 7.0, and immersed into blank background in the cell, where square wave voltammogram (SWV) was recorded with frequency of 25 Hz from initial potential of +0.3 V to end potential of +1.3 V in blank 50 mM phosphate, pH 7.

3. Results and discussion

3.1. Tyrosine and tryptophan oxidation in AGR2

Very recent paper [46] shows that the protein AGR2 exists in monomer-dimer equilibrium, where the domain containing amino acid residues E60 and K64 is responsible for dimer stabilization [46]. Any electrochemical study of these proteins has been so far missing. Here we attempted to make first investigation of oxidation of these proteins at carbon electrode. Monomeric AGR2-E60A mutant was chosen for our experiments.

5 μM AGR2-E60A protein (non His_6-tagged) was accumulated at open circuit potential for accumulation time, t_A of 5 min from 15 μL drop at GCE surface. Protein-modified electrode was washed and transferred to the electroanalytical cell, where SWV was recorded. Protein AGR2-E60A yielded the oxidation peak of Tyr and Trp (Y,W) at potential of +0.7 V (Fig. 1A,B) in agreement with the results obtained with other proteins [15,23–25,30]. Also His_6-tagged variant of AGR2-E60A (His-AGR2-E60A) produced peak (Y, W) at +0.7, which was about 50% higher than that of AGR2-E60A (Fig. 1), suggesting that His_6-tag presence may influence the protein orientation and adsorption at the electrode surface. Ac voltammograms of both forms showed that adsorption AGR2-E60A differed from His-AGR2-E60A at potentials less positive than +1 V (not shown). This might be related to the reported changes of conformation in some proteins after their His_6-tagging detected by