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Electrochemical behavior of sarco/endoplasmic reticulum Ca-ATPase in response to carbonylation processes

David Novak[a], Jana Viskupicova[b,c], Martina Zatloukalova[a,d], Vladimir Heger[b,e], Silvia Michalikova[b], Magdalena Majekova[b], Jan Vacek[a,*]

[a] Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Republic
[b] Center of Experimental Medicine, Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Dubravska cesta 9, 841 04 Bratislava, Slovak Republic
[c] Department of Biotechnologies, Faculty of Natural Sciences, University of Ss. Cyril and Methodius, Nam. J. Herdu 2, 917 01 Trnava, Slovak Republic
[d] Faculty of Chemistry, University of Warsaw, Pasteura 1, 02-093 Warsaw, Poland
[e] Department of Biochemistry, Faculty of Natural Sciences, Comenius University in Bratislava, Mlynska dolina, Ilkovicova 6, 842 15 Bratislava 4, Slovak Republic

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ABSTRACT

Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is a transmembrane protein that transfers calcium ions from the cytosol of the cell to the lumen of the sarco/endoplasmic reticulum (SR) at the expense of ATP hydrolysis during muscle relaxation. Here we report the electrochemical monitoring of a SERCA1 isoform isolated from rabbit skeletal muscle. The constant-current chronopotentiometric stripping (CPS) analysis was applied for the analysis of solubilized SERCA1 based on a catalytic hydrogen evolution reaction (CHER). Consequently we applied an optimized CPS electrochemical protocol for monitoring the interaction of SERCA1 with the dicarbonyl compounds methylglyoxal, glyoxal and 3-deoxyglucosone. We observed that SERCA1 readily interacts with methylglyoxal at pH 7.4. Electrochemical analysis showed that 1 mg/ml (9.1 μmol/l) SERCA1 was fully modified by methylglyoxal (3 mmol/l) after 24 h. The dicarbonyl binding decreased the enzyme activity of purified SERCA1 in the following order: methylglyoxal ≫ glyoxal > 3-deoxyglucosone. The methodology presented here could be used in further studies of the structural integrity and intermolecular interactions of membrane transporters and the study of their oxidative modifications, carbonylation or glycoxidation.

1. Introduction

The sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is a pump belonging to the family of P-type ATPases. SERCA is a ubiquitous transporter which is able to translocate calcium ions from the cytoplasm into the sarco/endoplasmic reticulum (SR) [1]. At present, more than ten SERCA isoforms have been identified, these isoforms exhibit both tissue and developmental specificity [2]. The SERCA1 isoform consists of 994 amino acid residues and accounts for 96% of adult transcripts in rabbit fast-twitch skeletal muscle. The SERCAs are integral membrane proteins, in which much of the polypeptide chain lies in the cytoplasm containing the phosphorylation site and nucleotide binding domain. The polypeptide chain has 10 hydrophobic sequences (M1-M10 helices), which provide membrane traversing segments that hold the protein in the lipid bilayer and the cytoplasmic region is connected to the membrane-bound portion by a stalk-like structure [3]. The function of the SERCA pump is modulated by the broad spectrum of endogenous and exogenous low-molecular substrates and proteins, *e.g.* phospholamban and sarcolipin. Despite the fact that the SERCA plays an important role in muscle contraction, cell signaling and also in other cellular processes, a number of investigations have focused on understanding its role in cardiac and skeletal muscle function and pathophysiology [1,2,4]. For this reason, novel methodologies that will aid in understanding the mechanism of SERCA's function, regulation and intermolecular interactions are being sought. Spectroscopic methods and computational tools are the most popular of the well-established approaches [5,6]. To the best of our knowledge, electrochemical methods have not been utilized for the investigation of SERCA transporters.

There is a battery of electrochemical methods which could be used for the sensitive analysis of proteins and for the study of their functional and structural changes [7]. Generally, the methods that are useful for protein label-free electrochemical sensing can be divided into two classes. The first focuses on the electroactivity of the prosthetic group in conjugated proteins, which is usually based on the principle of direct electron transfer [8]. A wider application range is attributed to the electrochemistry of non-conjugated proteins or the protein parts of

* Corresponding author.
E-mail address: jan.vacek@upol.cz (J. Vacek).

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conjugated proteins, where redox or electrocatalytically active amino acids residues are involved in the sensing principle [7,9]. This opens up the possibility to not only detect the protein at low concentration ranges, but also to observe protein structural changes (folding), chemical (oxidative) modifications and intermolecular interactions, for example protein-ligand, protein-protein or protein-DNA binding [7,10,11]. Non-direct electrochemical approaches based on labeling or immunochemical reactions of proteins have also been reported [12]. In contrast to the labeling of other biomacromolecules (*e.g.* DNA [13]), protein labeling with redox active probes could be connected to undesired protein structural changes and modulation of protein functional properties. This results in labeling approaches being limited to analytical work with a low level of applicability in mechanistic structural and functional studies.

In contrast to the electrochemistry of water-soluble proteins, the electrochemical investigation of water-poorly soluble (membrane associated) proteins is a more complex issue. The reason for this is the presence of solubilization agents (detergents), stabilizing lipidic components and other additives in the investigated protein samples. In light of this, the optimization of sample composition has to be based on additives that do not interfere with the electrochemical detection endpoint [14–16]. To resolve this, membrane proteins can be analyzed after the reconstitution step, which could stabilize the protein structure and keep the protein adsorbed layers more under control [17,18].

Finally, the importance of electrochemical methods for the study of proteins is based on not only finding their redox properties but also evaluating their interfacial behavior. This could contribute to determining protein functioning in the adsorbed state, which is important for biomedicine (implants adhesion and biocompatibility), technological fields (surface passivation phenomena) and the construction of novel analytical devices (nanoparticle interactions) as well [19–21].

Here we focused on the study of SERCA1 isoform using chronopotentiometric stripping (CPS) at a mercury electrode. The electrochemical analysis was supplemented by protein functionality evaluation, electrophoretic characterization and PyMol molecular visualization using X-ray crystallography data [22]. The CPS approach was consequently applied for the study of interactions of purified SERCA1 protein with the highly-reactive dicarbonyl compounds methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3-DG). The elevated level of the listed dicarbonyls is associated with pathological states, such as diabetes mellitus, atherosclerosis, cancer, progression of neurodegenerative diseases and aging in general [23,24]. These dicarbonyls react with the amino acid residues of proteins, altering their function, which subsequently impairs cellular function. Recently, increased dicarbonyl stress has been linked to multi-organ failure in critical illness [25]. Even though certain conditions, such as obesity and diabetes, could be associated with SR stress and calcium level imbalance [26], the glycoxidation or carbonylation processes of SERCA1 have not yet been fully described.

2. Materials and methods

2.1. Chemicals and pH measurement

All chemicals of analytical grade were obtained from Sigma-Aldrich or Merck (Darmstadt, GE) only 3-deoxyglucosone was purchased from Cayman Chemical Comp. (Michigan, USA). The pH measurements were carried out with a HI 2211 pH/ORP Meter (HANNA instruments, IT).

2.2. Isolation of SR vesicles and SERCA1 protein

SR vesicles containing 70–80% of Ca^{2+}-ATPase protein were isolated from the fast-twitch skeletal muscle of a New Zealand female rabbit (about 2.5 kg) according to previously reported protocols [27,28]. Firstly, skeletal muscle (100 g) was mixed thoroughly with 150 ml of buffer A (see below) at a temperature of 4 °C. The homogenate was centrifuged for 15 min at 8000 ×*g*, at 4 °C. The supernatant was filtered and centrifuged for 90 min at 37000 ×*g*, at 4 °C. The sediment was homogenized in 30 ml of buffer B using a piston homogenizer and centrifuged for 2 h at 37000 ×*g*, at 4 °C. The sediment containing SR vesicles was resuspended in buffer C and dialyzed overnight at 4 °C. SR vesicles were solubilized using potassium cholate in buffer C (0.224 mol/l) in the ratio of 0.4 mg cholate to 1 mg protein under vigorous shaking. The protein suspension was transferred to a discontinuous sucrose density gradient (15–35% in buffer D) and centrifuged in a swinging bucket for 18 h at 95000 ×*g*, at 4 °C. A fraction of purified enzyme was collected, resuspended in buffer D and centrifuged for 1 h at 95000 ×*g*, at 4 °C. The pellet was finally resuspended in buffer C at 4 °C. Detergent was removed by dialysis (24 h) against buffer C containing Amberlite XAD–4. The fractions containing soluble SERCA1 protein at purity ≥80% were stored at −80 °C.

The compositions of the buffers (pH 8 for all) used for individual isolation and purification steps were as follows. Buffer A: 0.3 mol/l sucrose, 20 mmol/l L-histidine, 1 mmol/l dithiotreitol (DTT), and 5 μmol/l phenylmethylsulfonyl fluoride (PMSF) in 96% ethanol. Buffer B: 0.3 mol/l sucrose, 10 mmol/l L-histidine, 0.6 mol/l KCl, 1 mmol/l DTT, and 5 μmol/l PMSF in 96% ethanol. Buffer C: 0.25 mol/l sucrose, 1 mol/l KCl, 50 mmol/l K_2HPO_4, and 50 mmol/l KH_2PO_4. Buffer D: 1 mol/l KCl, 50 mmol/l K_2HPO_4, and 50 mmol/l KH_2PO_4.

2.3. Determination of SERCA1 activity

The enzyme activity of SERCA1 was measured by NADH-coupled enzyme assay as previously described in [28]. The protein samples (final concentration 12.5 μg protein/cuvette) were added to the assay mixture (40 mmol/l HEPES, pH 7.2, 0.1 M KCl, 5.1 mmol/l $MgSO_4$, 2.1 mmol/l ATP, 0.52 mmol/l phosphoenolpyruvate, 1 mmol/l EGTA, 0.15 mmol/l NADH, 7.5 IU of pyruvate kinase, 18 IU of lactate dehydrogenase), preincubated for 10 min at 37 °C. The reaction was started by the addition of $CaCl_2$ (final concentration 1 mmol/l). The reaction rate was determined by measuring the decrease in NADH absorbance at 340 nm at 37 °C and pH 7.2.

The specific activity of SERCA (IU/mg = μmol substrate/min/mg of protein) was calculated using the following equation:

$$\frac{IU}{mg} = \frac{\Delta A_{340\ nm} \cdot V \cdot d}{6.22 \cdot m},$$

where ΔA_{340nm} is the change in absorbance at 340 nm in a 1-min interval, V is the total volume of reaction mixture in ml, d is the thickness of the cuvette, 6.22 is millimolar extinction coefficient for NADH at 340 nm, m represents the total amount of protein in the reacting mixture in mg.

2.4. SDS gel electrophoresis

The samples (20 μg protein per lane) were separated by denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [29], using a mini-PROTEAN II electrophoresis cell (Bio-Rad, Germany). Buffer (25 mmol/l Tris, 1% SDS, 0.192 mol/l glycine, 1% bromphenol blue, pH 6.8) containing freshly added 5% β-mercaptoethanol was used. The samples were incubated for 30 min at room temperature and loaded onto an SDS-polyacrylamide gel (7.5% separating, 4% stacking gel). The separation was performed for 0.5 h at 50 V and then 1.5 h at 150 V. Visualization was performed by Coomassie blue staining.

2.5. Protein determination

Protein content was measured and adjusted by two independent detection approaches using BSA as a standard (Bio-Rad Laboratories, Richmond, CA). For more details, see refs. [30,31].