Biochimica et Biophysica Acta 1865 (2017) 370–379

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Modifying effects of carboxyl group on the interaction of recombinant S100A8/A9 complex with tyrosinase

Fatemeh NematiNiko [a], Koorosh Goodarzvand Chegini [b], Hamideh Asghari [c], Abbas Amini [d,*], Nematollah Gheibi [e,*]

[a] Department of Nanotechnology, Islamic Azad University of Pharmaceutical Sciences Branch, Tehran, Iran
[b] Department of clinical Biochemistry and Genetics, School of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran
[c] Department of Biotechnology, School of Paramedicine, Qazvin University of Medical Sciences, Qazvin, Iran
[d] Centre for Infrastructure Engineering, Western Sydney University, Bld Y, Locked Bag 1797, NSW 2751, Australia
[e] Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, P.O. Box: 34199-15315, Iran

ARTICLE INFO

Article history:
Received 4 June 2016
Received in revised form 19 December 2016
Accepted 20 December 2016
Available online 23 December 2016

Keywords:
Skin cancer
Mushroom tyrosinase
S100A8/A9
Protein modification
Enzyme activity

ABSTRACT

Tyrosinase is a determinant enzyme for modulating melanin production as its abnormal activity can result in an increased amount of melanin. Reduction of tyrosinase activity has been targeted for preventing and healing hyperpigmentation of skin, such as melanoma and age related spots. The aim of this systematic study is to investigate whether recombinant S100A8/A9 and its modified form reduce the activity of mushroom tyrosinase (MT) through changing its structure. Recombinant His-Tagged S100A8 and S100A9 are expressed in *Escherichia coli BL21* (*DE3*) and modified using Woodward's reagent K which is a carboxyl group modifier. The structures of S100A8/A9 and its modified form are studied using fluorescence and circular dichroism spectroscopy, and the activity of MT is measured using UV-visible spectrophotometry in the presence of its substrate, L-3,4-dihydroxyphenylalanine (L-DOPA). The results show a lower stability of the modified protein when compared with its unmodified form. The interaction of S100A8/A9 with MT changes the structure and successfully reduces the activity of mushroom tyrosinase. Recombinant S100A8/A9 complex decreases MT activity which can control malignant melanoma, the most dangerous type of skin cancer.

1. Introduction

Tyrosinase (EC: 1.14.18.1) is an enzyme essential in the first step of producing melanin. It converts tyrosine into 3,4-dihydroxyphenyl alanine (DOPA), and catalyzes DOPA into dopaquinone. A series of additional chemical reactions convert dopaquinone into melanin in melanosomes of epidermal melanocytes [1–3]. The high activity of tyrosinase has been reported in cancer invasion and metastasis melanoma [4,5]. The tyrosinase expression has been utilized as a molecular marker for evaluating the presence of circulating tumor cells in melanoma patients [6,7]. Melanoma is a source of malignancy that is rapidly spreading in Caucasians and is the second most common cause of death after lung cancer in Western countries [8].

Tyrosinase contains two copper ions associated with the histidine residues in active sites, which are critical for its catalytic activities [9]. To decrease melanocyte's metabolism, tyrosinase inhibitors are used to reduce the melanin synthesis [10]. One approach to curing hyperpigmentation is to inhibit the tyrosinase activity by introducing natural or chemical copper chelators [11]. Many tyrosinase inhibitors such as hydroquinone, kojic acid, azelaic acid, electron-rich phenols and arbutin have been examined for their capacity to prevent an overproduction of melanin [12]. Natural products have also been extensively researched by the pharmaceutical and cosmetic industries as they are deemed safer with fewer side effects and inflammatory responses [13].

Recent achievements in molecular biology have had a pronounced impact on drug discovery. Monoclonal antibodies and recombinant proteins (r-proteins) have shown with a great potential to advance traditional therapeutic methodologies [14]. Small-molecule inhibitors have been pursued as a new cancer therapeutic treatment to block the target protein via protein-protein interaction (PPI) [15]. The PPI can be characterized by changes in some parameters, such as fluorescence intensity,

Abbreviations: MT, mushroom tyrosinase; L-DOPA, L-3,4-dihydroxyphenylalanine; DOPA, 3,4-dihydroxyphenyl alanine; r, recombinant; PPI, protein-protein interaction;; CD, circular dichroism spectroscopy; NMR, nuclear magnetic resonance; WRK, Woodward's reagent K; UV-CD, far ultraviolet circular dichroism spectrum; IPTG, isopropyl β-D-1-thiogalactopyranoside; ANS, anilinonaphthalene-8-sulfonic acid; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; PBS, phosphate buffered solutions..

* Corresponding authors.
E-mail addresses: a.amini@westernsydney.edu.au (A. Amini), ngheibi@qums.ac.ir (N. Gheibi).

http://dx.doi.org/10.1016/j.bbapap.2016.12.013
1570-9639/

circular dichroism spectroscopy (CD), and nuclear magnetic resonance (NMR) [16].

Calprotectin is a heterodimer protein which comprises of a heavy chain (S100A9, 14 kDa) and light chain (S100A8, 8 kDa). These subunits bind with each other in a non-covalent way in the presence of calcium [17,18]. The high expression of calprotectin in neutrophils, monocytes and macrophages has been reported by a number of researchers [19]. Calprotectin is produced in the early inflammatory stages and large amounts are found in many common types of cancers in humans. The similarity of the amino acid sequence between mushroom tyrosinase (MT) and human tyrosinase is only 23% (Gene Bank Accession No. 042713); nevertheless, due to their many similarities (such as reaction pathways, substrate and products), MT has been used as a commonly accepted model for tyrosinase studies [20].

S100A8 and S100A9 have two calcium-binding domains, known as EF-Hand (helix-loop-helix), which are connected by a hinge region [18,21–23]. The intracellular S100A8/A9 might be involved in the activation of phagocyte NADPH oxidase whereas the secreted type has antimicrobial properties and induces apoptosis [24,25]. Ghavami et al. reported that S100A8/A9 induces cell death with mitochondrial damage and lysosomal activation [26]. An effective S100A8/A9 dose for tumor cell apoptosis is 20–25 μg/ml, whereas lower concentrations of S100A8/A9 can cause proliferation of tumor cells [17,18]. After binding with calcium or zinc, the changes in the secondary and tertiary structures of human calprotectin can probably influence the biological functions of this protein [27]. In fact, protein modification can play an important role in the alteration of the S100A8/A9 function [28,29].

In this study, Woodward's reagent K (WRK) is introduced as a carboxyl group modifier for S100A8/A9, as a specific reagent to modify the aspartate and glutamate residues in this protein [30]. MT activity and structural changes are evaluated and analyzed via interaction with r-S100A8/A9 and modified r-S100A8/A9. The results confirm that the modification of r-S100A8/A9 complex leads to changes in its structure and function.

2. Materials and methods

2.1. Materials

Competent *Escherichia coli* (*E. coli*) *BL21* (*DE3*) and pET 15b vector containing N-terminal His-Tag S100A8 and S100A9 gene sequences were obtained from Novagen (Canada). The amino acid sequence of S100A8 and the amino acid residues of S100A9 were respectively: MLTELEKALNSIIDVYHKYSLIKGNFHAVYRDDLKKLLETECPQYIRKKG ADVWFKE LDINTDGAVNFQEFLILVIKMGVAAHKKSHEESHKE and MTCKMSQLERNIETIINTF HQYSVKLGHPDTLNQGEFKELVRKDLQNFLKKE NKNEKVIEHIMEDLDTNADKQLSFEEFIMLMARLTWASHEKMHEGDEGPG HHHKPGLGEGTP. Ampicillin was obtained from Roch (Germany), and sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), sodium chloride (NaCl), calcium chloride ($CaCl_2$), and other reagents were purchased from Merck (Germany). Isopropyl β-D-1-thiogalactopyranoside (IPTG), Ni-NTA resin, Tris, Acrylamide, Imidazole, bacterial media (LB broth), ANS, WRK, MT with a specific activity of 3400 unit/mg (MT; EC: 1.14.18.1) and L-DOPA were purchased from Sigma Aldrich (Germany). Distilled water and ethanol were used as the solution base and reagent, respectively.

2.2. Expression and purification of r-S100A8/A9 subunits

pET 15b vectors containing S100A8 and S100A9 gene sequences were separately transferred to *E. coli BL21* competent cells. The bacteria were incubated for 12 hours at 37 °C in a solid bacterial growth medium ampicillin. Single colonies of transformed bacteria were grown while being vigorously shaken using an incubator (ND_{250} model) until their 600 nm optical density (OD_{600}) reached 0.6. The expression was induced by adding 1 mM IPTG and the resultant cultures were incubated for 4 h at 37 °C while being vigorously shaken. The cells were then harvested by centrifugation at 4000 × *g* for 20 min [31]. The cell lysate was prepared in phosphate buffered solutions (PBS) lysis buffer containing 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, and pH 7.2. The product was sonicated and centrifuged at 12000 × *g* for 30 min to precipitate the cell debris from soluble proteins. The supernatant fractions, which contained soluble proteins, were loaded onto a pre-equilibrated Ni-NTA column. Purification was performed by a buffer containing different concentrations of 10–200 mM imidazole gradient. To remove imidazole, the purified fractions were dialyzed in the PBS buffer containing 100 mM NaCl, 25 mM NaH_2PO_4, at pH 6.5 for 8 h at 4 °C. Finally, the soluble proteins were aliquoted and stored at −80 °C for further studies. The concentrations of purified proteins were obtained according to the Bradford method with bovine serum albumin [32], and corresponding measurements were accomplished using a NanoDrop spectrophotometer.

2.3. Modification of aspartate and glutamate carboxyl group

Aspartate and glutamate sequences are conserved in calprotectin and may play an important role in its performance. As demonstrated by the authors, r-S100A8/A9 have six aspartate residues in total, while S100A8 has nine and S100A9 has 14 glutamate residues [33]. R-S100A8/A9 was modified by incubating with 50 μM WRK in a PBS dialysis buffer at 25 °C for 10 min. In order to prepare a r-S100A8/A9 complex, r-S100A8 and r-S100A9 with a similar concentration were incubated in 10 μM calcium chloride in a PBS dialysis buffer for at least 1 h at 25 °C [33–36]. Next, the secondary and tertiary structures of r-S100A8/A9 and the modified protein were analyzed using the fluorescence and CD methods. The amino acid residues of S100A8/A9, with polar and non-polar interactions with WRK, were evaluated using molecular docking software.

2.4. Molecular docking study

Water molecules, ions and ligands, co-crystallized with the protein, were removed, and hydrogen atoms were added to the functionalizing groups within the protein. By using AutoDockTools version 1.5.6, the variation in Kollman united atom partial parameter was assigned to the S100A8/A9 complex and then non-polar hydrogens of this complex were merged. S100A8/A9 was held rigid and all the torsional bonds of WRK were assumed as free in the molecular docking study. In the docking calculations, the protein was set to be rigid by ignoring the effect of solvent molecules on docking. In order to characterize the binding sites in calprotectin, blind docking was carried out where the grid size was set to 126 × 126 × 126 points with 0.575 Å grid spacing, and the center of the grid was set as 23.96, 39.32, 36.69 Å. The GA population size was considered as 150, along with the maximum number of 2.5 × 107 for energy evolution. A maximum number of 30 conformers were taken into consideration, and the root-mean-square (RMS) cluster tolerance was set to 2 Å.

2.5. Intrinsic fluorescence spectroscopy

The tryptophan and tyrosine residues in the prepared r-S100A8/A9 complex and MT provided us with an opportunity to analyze the tertiary structure of protein using the fluorescence spectroscopy method [37,38]. The prepared r-S100A8/A9 had two tryptophan (Trp) and five tyrosine (Tyr) residues, while MT had twelve Trp and twenty-two Tyr. A Cary eclipse 100 bio Spectro-fluorometer (Varian, Salt Lake, Australia), equipped with a 150 W Xenon lamp and a DR-3 data recorder, was used for the intrinsic fluorescence analysis. The excitation wavelength was 280 nm in the PBS buffer at pH 6.5 and 25 °C, and the emission spectra were recorded at 300–450 nm. To obtain the emission wavelength of the substance, the spectrophotometer was zeroed with a cuvette containing a carrier solution without the protein sample. The structures of