International Journal of Biological Macromolecules 88 (2016) 306–312

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

New insights into xanthine oxidase behavior upon heating using spectroscopy and *in silico* approach

Loredana Dumitraşcu, Nicoleta Stănciuc, Iuliana Aprodu*

Dunarea de Jos University of Galati, Faculty of Food Science and Engineering, Domnească Street 111, 800201 Galati, Romania

ARTICLE INFO

Article history:
Received 11 February 2016
Received in revised form 29 March 2016
Accepted 31 March 2016
Available online 1 April 2016

Keywords:
Xanthine oxidase
Structural changes
Fluorescence
Synchronous spectra
Molecular events

ABSTRACT

Thermal dependent conformational changes of xanthine oxidase (XOD) were studied using sensitive and non-destructive methods like fluorescence spectroscopy and molecular modeling in the temperature range of 25–85 °C. Intrinsic fluorescence studies showed that the microenvironment of tryptophan and tyrosine residues becomes more exposed to solvent as the temperature increased up to 85 °C, whereas in case of flavin cofactor is rather conserved. At higher temperatures, the flavin adenine dinucleotide is displaced from the core of the protein, but is not fully released as shown by the Stern Volmer quenching constant and accessible fraction of the cofactor. Anyway, no significant changes in the structure of XOD monomer were identified after running molecular dynamics simulations at temperatures 25 °C, 65 °C and 85 °C. Therefore, we can conclude that the most important changes in the protein structure at thermal treatment mainly consist on molecular aggregation and dissociation events.

1. Introduction

XOD (EC 1.17.3.2) is a versatile enzymefound from bacteria to humans and in various tissues of mammals, XOD from milk being one of the most studied enzymes. XOD is a homodimer with a molecular mass of 290 kDa. Each subunit of XOD contains an N-terminal domain that includes two iron sulfur centers, a central flavin adenine dinucleotide (FAD) cofactor and a C-terminal molybdopterin-binding domain with the four redox centers aligned in an almost linear fashion [1,2]. Each subunit is acting independently during catalysis, the cofactors being positioned in three domains of approximately 85, 40 and 20 kDa [3].

XOD plays a key role in the metabolism of endogenous compounds such as purines, where it catalyzes the oxidation of hypoxanthine and xanthine to uric acid with concomitant reduction of molecular oxygen [4,5].

XOD is involved in various forms of ischemic and types of tissue, inflammatory diseases and chronic heart failure [2]. The enzyme catalyzes the reaction to produce bactericide superoxide radicals and hydrogen peroxide in the presence of oxygen that can be used, among others, to activate the lactoperoxidase system [6]. The inhibition of XOD prevents lifestyle-related diseases such as gout and hyperuricemia [7].

XOD is a nonspecific enzyme naturally present in milk in the fat globule membrane, the highest activity being recorded for milk of bovine origin. As other important milk proteins such as lactoferrin and lactoperoxidase, XOD has some specific properties that have been found to be beneficial for the shelf life and quality of dairy products [8]. Thus, in dairy industry, XOD is present in different applications out of which we outline the following [9]: index of heat treatment in the temperature range of 80–90 °C, involvement in lipid oxidation process, responsible for the oxidation of purine bases and for reduction of nitrate to nitrite (nitrate being recognized to inhibit the germination of butyric acid bacteria spores in cheese). At pH 5.2 the milk XOD exhibits nitrate reductase activity, when the Mo-containing site becomes crucial. According to Ananiadi et al. [10], the switch from xanthine oxidase to the nitrate reductase activity is possibly only with considerable conformational changes of the enzyme molecule.

Although XOD has been the subject of many studies, being characterized from structural, mechanistic and biological point of view, little information are related with the influence of heat treatment on protein conformational states. The aim of this research was to investigate the influence of heating on XOD structural features using fluorescence spectroscopy approach. Moreover, the *in silico* technique was further used to add some useful details regarding the thermal behavior of XOD. Taking in consideration the importance of XOD for milk and dairy industry, the study is expected to deliver

* Corresponding author at: Dunarea de Jos University of Galati, Faculty of Food Science and Engineering, Domneasca Street 111, Building E, Room 104, 800201 Galati, Romania.
E-mail address: Iuliana.Aprodu@ugal.ro (I. Aprodu).

http://dx.doi.org/10.1016/j.ijbiomac.2016.03.072
0141-8130/

some important insights that can be used for better understanding of heat induced structural behavior of this protein. Therefore, the data may elucidate the XOD behavior in relation with other target inhibitory compounds, adding valuable information for other research fields like life sciences, chemistry, pharmacy or medicine.

2. Materials and methods

2.1. Materials

XOD from bovine milk was purchased from Serva (Serva Electrophoresis GmbH, Heidelberg, De), while acrylamide and potassium iodide from Sigma (Sigma–Aldrich Co., St. Louis, MO). Unless otherwise stated, all other reagents were of analytical grade.

2.2. Heat treatment

Plastic tubes (1 cm diameter) of 1 mL were filled with 0.20 mL XOD solutions containing 2 mg mL^{-1} protein. The samples were heated at different temperatures ranging from 25 to 85 °C for 10 min in a thermostatic water bath (Digibath-2 BAD 4, RaypaTrade, Barcelona, Spain). Then, the tubes were immediately transferred into ice water to avoid further thermal denaturation.

2.3. Intrinsic fluorescence spectroscopy experiments

The samples used for fluorescence measurements were prepared in 0.05 M phosphate buffer solutions at pH 7.5. Thus, 5 μL of protein solution were suspended in 2.5 mL phosphate buffer solution to measure tryptophan (Trp), tyrosine (Tyr) and FAD fluorescence intensity. The fluorescence spectra of XOD were obtained using LS-55 luminescence spectrometer (Perkin Elmer Life Sciences, Shelton, CT, USA) equipped with Perkin Elmer FL Winlab software. Emission spectra were monitored using the excitation wavelengths of 290 nm for Trp, and 278 nm for Tyr residues. In agreement with Przybyt et al. [11] the emission spectra of FAD were recorded using two excitation wavelengths of 370 nm and 450 nm respectively. All fluorescence spectra were monitored with a quartz cell of 10 mm path length, excitation and emission slits being set at 10 nm each. Each presented spectrum represents the average of three scans, and is expressed as arbitrary units. Phase diagram method, synchronous spectra and three dimensional fluorescence spectra were employed as previously described by Dumitraşcu et al. [12].

2.4. Quenching experiments

Fluorescence quenching experiments were performed with acrylamide (8 M), and potassium iodine (5 M). Freshly prepared stock solutions of quenchers were added into cuvettes containing buffer and protein solution. The concentration of acrylamide varied from 0 to 0.36 M, while the concentration of potassium iodine varied from 0 to 0.24 M. Before fluorescence measurements the mixtures were allowed to stand for 5 min to reach equilibrium. Data were analyzed using the Stern Volmer equation Eq. (1):

$$F_o/F = 1 + K_{SV}[Q] \quad (1)$$

where F_o and F are the fluorescence intensities of XOD in the absence and presence of the quencher, K_{SV} is Stern-Volmer quenching constant determined by linear regression by plotting the ratio between F_o and F versus [Q], and [Q] is the concentration of the quencher. Since quenching experiments of FAD in the presence of KI displayed nonlinear correlations by using Eq. (1), experimental data were fitted to the modified Stern-Volmer Eq. (2):

$$F_o/(F_o - F) = 1/(f_a K_{SV}) \times 1/[Q] + 1/f_a \quad (2)$$

where f_a is the accessible fraction of FAD molecule to the quencher, K_{SV} is the modified Stern-Volmer quenching constant for the accessible fluorophore and was calculated using the linear plot of $F_o/(F_o\text{-}F)$ versus 1/[Q].

2.5. Molecular modeling

The high resolution crystal structure of bovine milk XOD (PDB cod 3UNA) [13] was used as basis for the molecular mechanics and molecular dynamics computations. Only one monomer out of two were used in the simulations. After refinement, the XOD structure was energetically optimized with Gromacs 4.6.1 package [14] by using Gromos 43a1force field. The protein system was afterwards inserted into a cubic box (13.28 × 13.28 × 13.28 nm) that was filled with single point charge explicit water molecules. Additional minimization steps were performed using Steepest Descent method to allow the relaxation of the water molecules and to remove any large interaction forces acting between protein atoms and water molecules. The solvated system was then heated up to 25, 65 and 85 °C in 100 ps using the Berendsen thermostat with a time step of integration of 0.5 fs. After reaching the target temperature each system was equilibrated for additional 20,000,000 steps to remove any large oscillation of the temperature or energy values. Finally, details on the energetic and conformational particularities of XOD were collected by means of Gromacs and PDBsum tools [14,15].

2.6. Statistical analysis

The obtained data were analyzed using Microsoft Office Excel version 2007. Values are represented as means ± standard deviation. For multiple comparisons, one way ANOVA test was used, and p value was less than 0.05.

3. Results and discussions

3.1. Phase diagram

The *phase diagram* method gives a detailed description of the protein unfolding pathway, by detecting partially folded species and hidden intermediates [16]. In brief, the essence of this method is to construct the diagram of $I_{\lambda 1}$ versus $I_{\lambda 2}$, the spectral intensity values being measured at wavelengths λ_1 and λ_2 for the different experimental conditions that induce the structural changes of the protein. In this study, temperature was the experimental variable, although this approach can be applied to any extensive parameter generated by other methods as being important for folding/unfolding proteins [17]. When dealing with protein unfolding, the resulted plot is linear if changes in the protein environment lead to an *all-or-none* transition between the two conformations. On the other hand, the non-linear plot will reflect the sequential character of structural transformations [18].

In this study, the method was employed to analyze the kinetics of XOD unfolding/refolding by identifying intermediate states upon heating the enzyme. In Fig. 1 is displayed the phase diagram of heat induced structural changes of XOD solutions resulted by plotting the change of XOD fluorescence intensity at 320 nm as function of that at 365 nm for different temperature values.

A clear linear dependence was obtained (Fig. 1) showing an *all-or none* transition that indicates the presence of two distinct conformational species of the thermal denaturation pathway of XOD, including the native state (N) and the folded state (F). These results suggest that XOD experiences large conformational changes during heating such as aggregates formation. Regardless of the excitation wavelength used in the study, the highest fluorescence intensity value was registered at 25 °C, corresponding to native protein which is present as an active dimer. Heating the XOD solutions