Kinetic, Thermodynamic and Structural Studies of Native and N-Bromosuccinimide-Modified Mushroom Tyrosinase

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Abstract

Background: Mushroom tyrosinase (MT) as a metalloenzyme is a good model for mechanistic studies of melanogenesis. To recognize the mechanism of MT action, it is important to investigate its inhibition, activation, mutation, and modification properties.

Objectives: In this study, the chemical modification of MT tryptophan residues was carried out by using N-bromosuccinimide (NBS) and then, the activity, stability, and structure of the native and modified enzymes were compared.

Methods: Chemical modification of MT tryptophan residues was accomplished by enzyme incubation with different concentrations of NBS. The relative activity of native and modified MT was investigated through catecholase enzyme reaction in presence of dihydroxyphenylalanine (L-Dopa) as substrate. Thermodynamic parameters including standard Gibbs free energy change (ΔG25°C) and Melting temperature (Tm) were obtained from thermal denaturation of the native and modified enzymes. The circular dichroism and intrinsic fluorescence techniques were used to study secondary and tertiary structure of MT, respectively. All experiments were conducted in 2015 in biophysical laboratory of Qazvin University of Medical Sciences and Islamic Azad University, Science and Research Branch, Tehran.

Results: The relative activity reduced from 100% for native enzyme to 10%, 7.9%, and 6.4% for modified MT with different NBS concentrations 2, 10, and 20 mM, respectively. Thermal instability of modified enzyme was confirmed by decreased Tm and ΔG25°C values after modification. In accordance with kinetic and thermodynamic results, the lower stability of modified MT was observed from the changes occurred on its secondary and tertiary structures.

Conclusions: Chemical modification of tryptophan residues with NBS reduces the activity and stability of MT simultaneously with its structural change. Thus, this study emphasizes the crucial role of tryptophan residues in the structure-function relationship of MT enzyme.

Keywords: Protein Modification, Tyrosinase, N-Bromosuccinimide, Kinetics, Protein Structure

1. Background

Tyrosinase is a metalloenzyme that exists in different forms of life from the most simple microorganisms to top mammals and plants. It contains a coupled dinuclear copper active site that catalyzes metabolic conversion of monophenoles (tyrosine) to O-diphenoles (L-Dopa) and diphenols to O-quinones (L-Dopa quinone) (1, 2). Mushroom tyrosinase (MT) from Agaricus bisporus, as an intercellular enzyme, with a molecular weight of 120 kDa has been introduced as a heterotetramer protein, which contains four Cu+2 atoms (3). In the sequence of MT, as shown in Figure 1, the residues numbered 61, 85, 94, 259, 263, and 296 are metal binding sites of Cu+2 which among, the amino acids numbered 61, 85, and 94 are binding sites of copper 1 (Cu+2) and amino acids numbered 259, 263, and 296 are binding sites of copper 2 (Cu+2). Also, the amino acids numbered 293-293 are cleavage sites (4, 5). Four genes including Abppo1 (Uniprot protein sequence database entry Q00024) and Abppo2 (O42713) (6), and Abppo3 (C7FF04) and Abppo4 (C7FF05) (7) are responsible for coding of Agaricus bisporus tyrosinase. Chemical modification is a good way to investigate the role of each amino acid residue in physicochemical characteristics to understand the structural-functional relationship of proteins. The polar amino acids including lysine, arginine, glutamate, aspartate, serine, tyrosine, histidine, methionine, and tryptophan can be chemically modified (8). In previous studies, chemical modification of tryptophan residue of D-Amino acid oxidase with Pyridoxal 5'-phosphate (9) and lysine and histidine residues of Bacteriophage T4 deoxynucleotide kinase with Diethylpyrocarbonate has been investigated (10). There are some other modifiers for various residues such as bromoacetic acid for imidazole group of histidine residues (11), methanal for amino group of lysine and histidine residues of mushroom tyrosinase (12), and 2,3-butanedione for guanidyl group of arginine residues (13). It has reported that chemical modification of tyrosinase by two modifiers, diethylpyridinedicar-
mate and bromoacetate, resulted in the reversible inactivation of the enzyme (14). Modification of dextranase with 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide and epoxyalkylα-glucosides caused inactivation of dextranase, indicating that carboxyl groups are essential for the enzyme action (15). High expression of tyrosinase to produce melanin in cancerous melanocytes can lead to an improvement in cancer, the same that can be controlled or treated by reducing or inhibiting the activity of tyrosinase (16). MT is a good model for such studies on structure-function relationship and also, it has a good potential for clinical, industrial, agricultural, and food studies. In line with the previous studies on MT activity, stability, and structure (17, 18).

2. Objectives

This study aimed to investigate chemical modification of MT by NBS. In this work, we tried to find out the significance of tryptophan residues role in activity, structure, and stability of the enzyme by chemical modification of MT with NBS.

3. Methods

3.1. Materials and Buffer Preparation

Mushroom tyrosinase (MT; EC 1.14.18.1) with specific activity 3400 units/mg and N-Bromosuccinimide were purchased from Merck (Germany). L-Dopa was purchased from Sigma (Sigma-Aldrich Co). All experiments were performed from 10 to 90°C. The determination of standard Gibbs free energy of denaturation (ΔG°), as a criterion of conformational stability of a globular proteins, is based on two state theories as follows:

\[ \Delta G^\circ = 2.303 \times 10^{-3} \cdot \Delta Y \cdot (\Delta Y + Y_0) \]

where ΔG° is Gibbs free energy of denaturation, ΔY is change in free energy on denaturation, and Y0 is molar fraction of native protein.

3.2. Modification of Tryptophan Residues

For chemical modification of tryptophan residues, NBS stock solution was 100 mM and the concentrations of NBS incubated with MT were 2, 10 and 20 mM. The concentration of NBS was incubated with MT, as a modifier, for 45 minutes at 25°C in 50 mM PBS (pH 6.8) and dialyzed in a sequential process by 50 mM PBS at 4-5°C for 48 hours. The kinetic assay of catecholase reaction was carried out using Cary spectrophotometer, 100 Bio model, with jacketed cell holders. Freshly prepared enzyme and substrate solutions were used in this work. All enzymatic reactions were run in PBS (50 mM) at pH = 6.8 in a conventional quartz cell thermostated to maintain temperature at 4-5°C. The selected conditions of solvent, buffer, pH, temperature, and enzyme concentration were applied for assaying the oxidase activity of MT according to our previous study (19). In catecholase reactions, depletion of L-Dopa was measured in 475 nm for 3 minutes using 40 unit of MT in each reaction. The catecholase activity of MT was investigated by 2, 10, and 20 mM concentrations of NBS as modifier and 1 mM concentration of L-Dopa as substrate. All the experiments were performed in triplicate.

3.4. Intrinsic Fluorescence Measurement

Cary-Eclipse Spectrofluorimeter at an excitation wavelength of 280 nm was used for recording fluorescence intensities. The maximum emission λ max for the MT was 320 nm. A series of modified MT solutions of 0.2 mg/mL with different concentrations of 2, 10, and 20 mM NBS as quencher were prepared in the 50 mM PBS. Quenching data were analyzed by using Stern-Volmer equation, as follows (20):

\[ \frac{F_0}{F} = 1 + K_{SV} [Q] \]  

where \( F_0 \) and \( F \) are the relative steady-state fluorescence intensities in the absence and presence of quencher, respectively, Q is the quencher (ligand) concentration. The values of Ksv can be derived from the intercept and slope of a plot based on the equation 1. The enzyme stability in thermal stress situation with solution concentration of 0.2 mg/mL for native and modified enzymes was assessed by thermal denaturation. Regarding to excitation wavelength of 280 nm and MT maximum intensity of 320 nm in the intrinsic fluorescence emission assay, the thermal scan was performed from 10 to 90°C. The determination of standard Gibbs free energy of denaturation (ΔG°), as a criterion of conformational stability of a globular proteins, is based on two state theories as follows:

Native (N) ⇔ Denatured (D)

By assuming two-state mechanism for enzyme thermal denaturation, one can explain the process by monitoring changes in the absorbance or fluorescent thermal intensity, calculate the denatured fraction of protein (\( F_d \)) as well as determine the equilibrium constant (K), as shown in the following:

\[ F_d = \frac{(Y_N - Y_{obs})}{(Y_N - Y_D)} \]  

\[ K = \frac{F_d}{(1 - F_d)} = \frac{(Y_N - Y_{obs})}{(Y_{obs} - Y_D)} \]
Figure 1. Sequence Chain View of MT (PDB ID: 2Y9X)

Where $Y_{obs}$ is the observed variable parameter (e.g. absorbance or thermal intensity) and $Y_N$ and $Y_D$ are the values of $Y$ characteristics of a fully native and denatured conformation, respectively. The standard Gibbs free energy change ($\Delta G^o$) for protein denaturation is given by the following equation:

$$\Delta G^o = -RT\ln K$$

Where $R$ is the universal gas constant and $T$ is the absolute temperature. $\Delta G^o$ varies linearly with temperature ($T$) over a limited region (21).

3.5. Circular Dichroism Measurement

The far UV region (190-260 nm) that corresponds to secondary structure of MT was analyzed by an Aviv model 215 Spectropolarimeter (Lakewood, USA). Far UV spectra of MT were studied at the concentration of 0.2 mg/mL with 1 mm path length quartz cell. Enzyme solutions were prepared in 50 mM PBS at pH 6.8. The ellipticity of the enzyme solutions was obtained at the concentrations of 0, 2, 10, and 20 mM of NBS as modifier, after its incubation for about 45 minutes. All spectra were collected in triplicate from 190 to 260 nm with a background correction.
against buffer blank. The data were smoothed by applying a software-based technique, including the fast Fourier-transform noise reduction, which allows the enhancement of most noisy spectra without distorting their peak shapes.

4. Results

4.1. Kinetic

The relative activity analysis through catecholase reaction in Figure 2 showed a decrease in enzymatic activity from 100% for native MT to 10%, 7.9%, and 6.4% for modified MT by 2, 10, and 20 mM of NBS concentrations, respectively, at presence of 1 mM L-Dopa as substrate.

![Graph showing relative activity of native and modified MT by 2, 10, and 20 mM NBS through catecholase reaction in presence of 1 mM L-Dopa as substrate.](image)

**Figure 2.** Relative activity of native and modified MT by 2, 10, and 20 mM NBS through catecholase reaction in presence of 1 mM L-Dopa as substrate (white: native, grey: 2 mM, dark grey: 10 mM, black: 20 mM). ***P < 0.001.

4.2. Thermal Denaturation

The sigmoid curves (Figure 3A) show thermal denaturation of native and modified MT with different concentrations of modifier (2, 10, and 20 mM). The thermodynamic parameters related to the enzyme stability, i.e. $T_m$ and $\Delta G^{25\degree C}$, were calculated according to the relevant formula and the sigmoid curves derived from thermal denaturation (Figure 3B). These curves are for native and modified enzyme with different concentrations of modifier (2, 10 and 20 mM). $T_m$ and $\Delta G^{25\degree C}$ values for native and modified enzyme are shown in Table 1. Decreasing values of $T_m$ from 61°C for native MT to 38.69°C for modified MT and $\Delta G^{25\degree C}$ from 17.14 to 8.07 kJ/mol indicated instability of the modified enzyme in comparison to its native form.

![Graph showing thermal denaturation profiles of MT obtained from intrinsic fluorescence intensity in 320 nm emission wavelength with temperature scanning for native (♦) and modified enzymes with different concentration of modifier (2 mM (□), 10 mM (△), 20 MM (×)); B, Linear secondary plots for thermodynamic parameters $\Delta G^{25\degree C}$ and $T_m$.](image)

**Figure 3.** A, Thermal denaturation profiles of MT obtained from intrinsic fluorescence intensity in 320 nm emission wavelength with temperature scanning for native (♦) and modified enzymes with different concentration of modifier (2 mM (□), 10 mM (△), 20 MM (×)); B, Linear secondary plots for thermodynamic parameters $\Delta G^{25\degree C}$ and $T_m$.

**Table 1.** Thermodynamic parameters of native and modified MT

<table>
<thead>
<tr>
<th>Thermal Denaturation</th>
<th>$T_m$ (°C)</th>
<th>$\Delta G^{25\degree C}$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>61.00</td>
<td>17.14</td>
</tr>
<tr>
<td>2 mM NBS</td>
<td>44.86</td>
<td>14.14</td>
</tr>
<tr>
<td>10 mM NBS</td>
<td>40.08</td>
<td>13.80</td>
</tr>
<tr>
<td>20 mM NBS</td>
<td>38.69</td>
<td>8.07</td>
</tr>
</tbody>
</table>

4.3. Structural Analysis by Fluorescence and CD

The tertiary structure of native and modified forms of MT was determined using fluorescence technique which is depicted in Figure 4A. The intensities of intrinsic fluorescence show a set of curves with gradual descending trend by increasing NBS concentration as MT modifier. Analysis of fluorescence maximum intensities in different NBS concentrations through Stern-Volmer equation (equation.1) in Figure 4B showed the dynamic form of quenching. The gradual reduction of negative elipticity after incubation of MT with different concentrations of NBS in Figure 5 refers to the lower regular secondary structures in the modified
form of enzyme.

**Figure 4.** A, Intrinsic fluorescence intensity of MT at a concentration of 0.2 mg/mL in the excitation wavelength of 280 nm and emission range of 300 - 450 nm for native (—-) and modified enzymes with different concentrations of modifier 2 mM (..-..-), 10 mM (—-), and 20 mM (••••); B, Stern-Volmer plot for determination of quenching type.

**Figure 5.** Far UV-CD Spectra for Native (—-) and Modified Enzymes with Different Concentrations of Modifier 2 mM (..-..-), 10 mM (—-) and 20 mM (••••)

5. Discussion

The relative activity of enzyme through catecholase reaction, depicted in Figure 2, decreased from 100% for native MT to 10%, 7.9%, and 6.4% for modified MT by different NBS concentrations of 2, 10, and 20 mM, respectively. The thermodynamic parameters obtained from thermal denaturation plots of native and modified enzyme (Figure 3) showed a decrease in $\Delta G_{25^\circ C}$ and $T_m$ for the modified MT (Table 1), indicating lower stability of the modified enzyme. The $T_m$ values were obtained as 61°C for native enzyme, and 44.9, 40.1, and 38.7°C for modified enzyme with NBS concentrations of 2, 10, and 20 mM, respectively. According to Figure 4, spectra of MT initial fluorescence intensity gradually decreased by increasing of NBS concentrations, probably due to the changes in microenvironment of tryptophan residues in MT and thus quenching of these chromophores. Environment of tryptophan residues in proteins can be probed by selective oxidation with NBS. The conversion of indole side chain of tryptophan residue to oxindole is accomplished by oxidation reaction, which causes a loss of absorbance at 280 nm (22). According to MT's sequence, the tryptophan residues numbered 93 and 293 are located near the histidine residues of enzyme active site (4). Modification of these tryptophan residues by NBS causes enzymatic inactivation and partial unfolding of MT's structure that leads to its instability. In agreement with this finding, the CD spectra of modified MT in comparison with the spectra of native MT in Figure 5 demonstrate a decline in regular secondary structure of modified MT.

There are some evidences about enzyme modifications in literatures that confirm the enzyme inactivation and instability. In James L. Sartin et al., modification of tryptophan residues by NBS in DNase led to inactivation of the enzyme; they reported that DNase contains three tryptophan residues and the inactivation kinetics revealed that one of them is crucial for enzymatic activity. They supposed that this key tryptophan residue can have an influence on or is located in the active site of the enzyme (23). In another study examining the structure-function relationship of Xylanase, chemical modification of enzyme with NBS resulted in the loss of enzymatic activity and decrease in fluorescence intensity of modified Xylanase (24). Modification of Streptomyces R6i DD-peptidase and bovine pyruvate dehydrogenase by NBS resulted in the complete inactivation of enzyme and the structural analysis by fluorescence and CD techniques showed a decrease in fluorescence intensity and partial changes of regular secondary structures (25, 26). Chemical modification of tryptophan-241 at the C-terminus of the manganese stabilizing protein (MSP) of plant photosystem II with NBS indicated that tryptophan-241 is critical for maintaining appropriate structure and function of MSP (27). In recent study by Rachadech et al. in 2016, examining the NBS modification of Corynebacterium glutamicum, the essential role of the tryptophan residues was recognized. The modification
caused significant decrease in enzyme activity, indicating that tryptophan residues are very important for catalysis (28). As shown in Figure 4B, the quenching data were analyzed using Stern-Volmer equation I and it was proven the static mode of interaction between NBS and MT. It means that NBS binding with enzyme resulted in the structural changes of the enzyme in a concentration-dependent manner, as illustrated in Figure 4A.

In another study by Gheibi et.al in 2015, the monophenolase and diphenolase activities of MT were affected by benzoic acid and pyridine derivatives (29). Chemical modification of some residues such as tryptophan, lysine, and arginine in sulfite reductase, respectively by NBS, N-acetylsucciimide, and phenylglyoxal revealed the inhibitory effect of these three modifiers and delineated the importance role of these residues in the catalytic mechanism of enzyme (30). In a previous study, Histidine, arginine, tryptophan, and lysine residues of Aspergillus niger lipase were chemically modified by using bromoacetic acid (BrAc), 2, 3-butanedione (BD), NBS, and metanal, respectively, and the results suggested that these residues are essential for enzyme activity and might be involved in the catalytic site of the enzyme (31). In agreement with the above investigations, the results of the current study confirmed the inactivation of modified MT in comparison with native MT. Moreover, the changes of tertiary and secondary structures of the modified enzyme obtained from fluorescence and CD spectra revealed that the tryptophan residues are very essential for structural-function relationship of MT. The aim of this study was to evaluate the role of tryptophan residues in activity, structure, and stability of MT. In conclusion, the kinetic, thermodynamic, and structural investigations of NBS-modified MT showed the enzyme inactivation following the modification by changing its structural and thermodynamic properties. Thus, this study indicated that tryptophan residues are very essential for structure-function relationship of MT.

Acknowledgments

The research council of Qazvin University of Medical Sciences and Islamic Azad University, Science and Research Branch, Tehran, are gratefully acknowledged for the financial support of this work. Also, we specially thank the institute of biochemistry and biophysics, university of Tehran, for providing CD facility of biophysical chemistry laboratory.

Footnote

Declaration of Interest: The authors report no conflicts of interest. The authors are merely responsible for the content and writing of the paper.

References