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The consequences of deglycosylation of recombinant intra-melanosomal domain of human tyrosinase

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Abstract

Tyrosinase, a melanosomal glycoenzyme, catalyzes initial steps of the melanin biosynthesis. While glycosylation was previously studied *in vivo*, we present three recombinant mutant variants of human tyrosinase, which were obtained using multiple site-directed mutagenesis, expressed in insect larvae, purified and characterized biochemically. The mutagenesis demonstrated the reduced protein expression and enzymatic activity due to possible loss of protein stability and protein degradation. However, the complete deglycosylation of asparagine residues *in vitro*, including the residue in position 371, interrupts tyrosinase function, which is consistent with a melanin loss in oculocutaneous albinism type 1 (OCA1) patients.

Keywords

albinism; genetic mutations; N-glycosylation; post-translational modification; protein activity; protein purification

Tyrosinase is an enzyme attached to the melanosomal membrane by a single trans-membrane helix, which catalyzes the initial steps of melanin pigment production through hydroxylation of L-tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA; monophenolase activity) and oxidation of L-DOPA to dopaquinone (diphenol oxidase activity) (Hearing, 1987; Olivares and Solano, 2009). In humans, the tyrosinase enzyme is encoded by the *TYR* gene, where mutations can cause oculocutaneous albinism type 1 (OCA1), an inherited disease linked with decreased pigment in the skin, hair and eyes and with ocular changes, i.e. nystagmus, iris transillumination, foveal hypoplasia (Tripathi et al., 1992; Simeonov et al., 2013). Before arriving at the melanosomal membrane as an active enzyme, tyrosinase goes through posttranslational modifications like N-linked glycosylation or formation of disulfide bonds, then moves from the endoplasmic reticulum (ER) to the Golgi (Wang and Hebert, 2006). Mature human tyrosinase (Tyr, OMIM # 606933) is a type 1 membrane-bound glycoprotein containing two copper ions, CuA and CuB, surrounded by six histidines in the

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active site. N-linked glycosylation is a process, in which carbohydrates are attached to asparagine residues (N) residing within the sequon, a conserved sequence pattern with asparagine-X-threonine/serine (N-X-T/S), where X is any amino acid except proline (Hart, 1992). Human tyrosinase has seven sequons. Genetic changes affecting the conserved patterns near asparagine residues are associated with OCA1 albinism as shown in Table S1. In the cell, N-glycosylation plays an essential role in the transfer of tyrosinase from ER to the cytoplasm and in maintaining its folding, stability, and enzymatic activity (Imokawa and Mishima, 1984; Branza-Nichita et al., 2000; Wang and Hebert, 2006). The glycosylation of tyrosinase might regulate the melanin production in different types of cultured melanoma cells (Mikami et al., 2013). It has been proven in mouse melanoma cells, that inhibition of the early steps of the N-glycosylation process strongly affects tyrosinase activity, thereby changing melanin synthesis. Moreover, the abnormal N-glycosylation process is related to the depigmented phenotype of human melanomas (Negroiu et al., 1999; Branza-Nichita et al., 2000; Olivares et al., 2003; Wang and Hebert, 2006).

We have previously shown that the recombinant intramelanosomal domain of human tyrosinase, hTyrC_{tr}, is a soluble monomeric protein with both, monophenolase and diphenol oxidase enzymatic activities (Dolinska et al., 2014). Additionally, mass-spectroscopy analysis demonstrated, that hTyrC_{tr} contains at least five N-glycosylation sites, which were completely (N86, N337) or partially (N111, N230, N290) modified. Unfortunately, mass-spectroscopy analysis was limited and we were unable to resolve other N-glycosylation sites by this method. To understand the significance of other sites, we performed multiple site-directed mutagenesis. In this regard, at the start, 5 asparagines, N86, N111, N230, N290, and N337, were replaced with aspartic acid to remove glycans (5D mutant) (Figure S1B). However, enzymatic deglycosylation using N-Glycosidase F (PNGase) and Endoglycosidase F1 (F1) showed that the 5D mutant protein is not fully deglycosylated (Figure S2A,B). Therefore, we used additional mutagenesis to remove sugars at N161 (6D mutant) or at both, N161 and N371 (7D mutant) sites (Figure S1C,D).

Recombinant human tyrosinase (residues 19–469 of the native protein) and three partially or fully deglycosylated mutants were prepared and expressed in baculovirus and produced in whole insect *Trichoplusia ni* larvae, as previously described (Dolinska et al., 2014). Proteins were purified by immobilized metal affinity (IMAC) (Figure 1A) and size exclusion chromatography (SEC1 and SEC2) (Figures S3 and 1B). The presence of tyrosinase was confirmed by Western blot using anti-tyrosinase T311 antibody (Figure 1C,D, top panels). Fractions containing tyrosinase were additionally identified using a color reaction with L-DOPA (Figure 1C,D, bottom panels and Figure S3, top panels). All presented proteins showed low expression in crude lysates (protein yield below 10%); however, with the removal of 5, 6, and 7 N-glycosylation sites from the hTyrC_{tr}, protein expression was even lower (Table 1). Total amount of protein after two chromatographies, for hTyrC_{tr}, 5D, 6D, and 7D mutants, steadily reduced probably due to protein degradation.

The final protein purity confirmed by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was significantly decreased for 5D, 6D, and 7D mutants, ~74, 46, and 13%, respectively, compared to that of the hTyrC_{tr}, ~98% (Table 1, Figure S4). Moreover, with increasing protein deglycosylation, their bands were gradually sharper in

comparison to the broad, heterogeneous band of fully glycosylated hTyrC_{tr} (Figure 1C, top panels). Specific L-DOPA activity only shows a modest decrease of enzymatic activity for 5D and 6D mutants (~70 and 58% of hTyrC_{tr}, respectively) and dramatic change for the fully deglycosylated 7D mutant protein (2% of hTyrC_{tr} activity) (Table 1). The 7D mutant activity was significantly decreased compared to that of the enzymatically deglycosylated tyrosinase using F1, which showed ~36% of the residual activity compared to its native form. This suggests that the seven asparagine replacements produced an inactive protein in contrast to the F1 deglycosylation, which does not cause full glycan removal and shows the residual activity of protein. However, the F1 deglycosylation of 5D mutant shows specific activity decreased to ~2% compared to that of the intact protein (Figure S2C). In addition, protein yield decreases with a gradual loss of protein activity due to the consecutive replacement of glycosylated residues in mutant variants as illustrated in Figure 2.

These observations are consistent with our molecular modeling of human tyrosinase, which suggests that the six sites are located at the protein surface and have less influence on the 4-helix bundle structure maintaining the copper-binding sites (Dolinska et al., 2014, 2017). However, the conformation of the 4-helix bundle in a fully deglycosylated tyrosinase (7D) could be affected by N371D mutation due to an additional negative charge introduced in the protein cavity surrounding this residue. Conclusions about the 7D mutant agree with previous data demonstrated that fully deglycosylated tyrosinase, possibly misfolded, was retained in the ER, and degraded but not delivered to the melanosomes (Svedine et al., 2004). Additionally, it is known that not all tyrosinase glycosylation sites have the same effect on proper folding and/or enzymatic activity. The glycosylation on a specific site is crucial for folding; therefore, its absence will result in protein misfolding and followed consequences (Brodsky, 2012). Occasionally, such proteins can reach the melanosomes, which suggests that the partial glycosylation is more critical for enzymatic function, not for the folding properties.

Currently, 26 known missense mutations from OCA1 could be associated to the glycosylation sites (Table S1). The mutations causing a loss of glycans are related to the positions 0 (substitution of N) and +2 (substitution of S or T). In addition, the replacement of amino acids in sequon positions from -2 up to +5 could affect the glycosylation as well (Mazola et al., 2011). The glycosylation site at position 371 appears to be critical for normal tyrosinase enzymatic activity. In the T373K mutation, which is very common in Northern Europeans, the threonine residue is replaced with positively charged lysine, which disrupts N-X-S/T sequence motif. Results from genetic studies agree with our *in vitro* data suggesting that T373K mutation causes the proteolytic degradation of expressed protein, destabilization of the tyrosinase structure, and abolishes protein activity (Dolinska et al., 2017). Previously shown *in vivo* these mutations could prevent tyrosinase from leaving the ER and initiate a severe disease phenotype in OCA1 albinism causing the loss of pigment (Branza-Nichita et al., 2000).

One of the important questions is how the N-glycosylation affects protein conformation and energetics. Unfortunately, we were not able to answer this question by performing the study experimentally due to difficulties in getting high quality deglycosylated mutant variants for the protein stability analysis (Table 1). Therefore, we did a rough relative estimation of

protein stability changes for deglycosylated mutants using our measurements for OCA1B missense mutations (Figure S5). This estimation shows that Gibbs free energies are expected to be 2.29, 2.43, and 3.09 kcal/mol for 5D, 6D, and 7D, respectively. These data demonstrate the changes like that determined for OCA1B missense mutations (Dolinska et al., 2014, 2017). The removal of all glycans for the 7D mutant disturbs the protein stability and potentially could affect protein folding. Stability of 5D and 6D mutant proteins is affected in less degree and the proteins still have a residual catalytic activity.

In summary, we performed the first protein expression, purification, and *in vitro* analysis of deglycosylated human tyrosinases using multiple site-directed mutagenesis to understand a significance of glycosylated asparagines, which were not accessible to mass-spectroscopy analysis. In the 6D mutant the residue N371 was intact and tyrosinase kept a residual catalytic activity. The additional glycosylation of residue N371 seems to be the critical for a loss of tyrosinase function. In general, a total amount of pure protein and enzymatic activity for hTyrC_{tr} and deglycosylated mutants were steadily reduced due to a loss of protein stability followed by protein degradation. In addition, we demonstrated that the replacement of N-glycosylation residues decreases tyrosinase enzymatic activity more effectively compared to the effect of Endonuclease F1. A complete deglycosylation of tyrosinase *in vitro* causes a decrease of functionally active enzyme and agrees with a loss of melanin in OCA1 patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Purification and enzymatic activities for the recombinant hTyrC_{tr} and deglycosylated mutant variants.

Recombinant tyrosinases (hTyrC_{tr}, blue; 5D, purple; 6D, gray; 7D, cyan) were purified from infected larvae by IMAC and SEC using a Bio-Logic Duo-Flow Maximizer workstation (Bio-Rad, Hercules, CA, USA). (A) Soluble extracts were loaded on 5 ml His-Trap FF crude column (GE Healthcare, Piscataway, NJ, USA) equilibrated with Buffer A (20 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole, pH 7.4) and eluted at flow rate of 1 ml/min. A gradient of 0–500 mM imidazole was applied and 2.5 ml fractions were collected. After dialysis against 4 l of Buffer B (50 mM Tris-HCl, pH 7.2, 1 mM EDTA, and 150 mM NaCl, 50 μ M TCEP), concentrated fractions were loaded on 120 ml Superdex 75 16/60 (Figure S3), and then on 30 ml Superdex-75 HR 10/30 columns (GE Healthcare, Piscataway, NJ, USA) at a flow rate of 0.5 ml/min (B); 2.5 and 0.5 ml fractions were collected, respectively. Brown color (intensity proportional) in tubes indicates diphenol oxidase activity, measured for each fraction after 30 min of incubation at 37°C with 3 mM L-DOPA in 50 mM sodium phosphate buffer, pH 7.5 (C and D, bottom panels). Corresponding Western blots, confirmed identity of proteins, were detected using anti-tyrosinase T311 antibody (1:2000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (C and D, top panels).

Figure 2: The consequences of deglycosylation of intra-melanosomal domain of human tyrosinase.

Protein amount in milligrams plotted as a function of the log10 of L-DOPA activity of the recombinant hTyrC_{tr} and 5, 6, and 7-deglycosylated mutant variants (5D, 6D, and 7D, respectively) shown in black. Percent of proteins yield loss, plotted as a function of the log10 of L-DOPA activity of the recombinant hTyrC_{tr} and 5, 6, and 7-deglycosylated mutant variants (5D, 6D, and 7D, respectively) shown in red.

Table 1:

Purification and enzymatic activity of recombinant hTyrC_{tr} and N-deglycosylated mutant variants from 10 g of larval biomass.

	hTyrC _{tr}	5D	6D	7D
Protein purity (%)				
Lysate	9.19 ± 0.76	7.88 ± 1.20	5.74 ± 1.51	6.58 ± 2.34
IMAC	39.92 ± 1.30	29.87 ± 3.08	7.35 ± 0.98	6.30 ± 0.37
SEC1	73.16 ± 2.81	49.99 ± 12.69	19.44 ± 6.24	8.48 ± 1.88
SEC2	97.71 ± 1.41	73.54 ± 7.58	46.06 ± 1.86	13.47 ± 1.22
Total protein amount (mg)				
Lysate	202.35 ± 34.17	183.14 ± 39.28	153.75 ± 69.56	154.15 ± 31.84
IMAC	5.35 ± 0.50	3.52 ± 0.19	0.76 ± 0.14	0.61 ± 0.03
SEC1	2.00 ± 0.29	0.94 ± 0.18	0.28 ± 0.08	0.11 ± 0.03
SEC2	1.18 ± 0.14	0.40 ± 0.14	0.14 ± 0.02	0.02 ± 0.01
Specific activity (U/mg)				
SEC2	78389 ± 6212	54629 ± 3856	45202 ± 5772	1938 ± 756
% of hTyrC _{tr}	–	70	58	2

Protein purity of hTyrC_{tr} and deglycosylated variants (5D, 6D, and 7D) was obtained from the SDS-PAGE gels using UN-SCAN-IT gel™ analysis software (Silk Scientific, Inc., Orem, UT, USA) (see also Figure S4).

The protein concentration was measured in crude lysates, after IMAC (HisTrap FF crude 5 ml column), and after two SEC chromatographies, SEC1 and SEC2 (Superdex 75 16/60 and Superdex 75 10/30 columns, respectively) (GE Healthcare, Piscataway, NJ, USA). The total protein estimated by the Warburg-Christian method using absorbance at 260/280 nm was normalized by its purity measured after each step of purification. The specific activities were obtained as the L-DOPA enzyme activity multiplied by the sample total volume and divided by total protein. The reaction mixture containing 3 mM L-DOPA in 10 mM sodium phosphate buffer, pH 7.4, was incubated for 30 min at 37°C and monitored by measuring the dopachrome formation at 490 nm ($\epsilon_{\text{dopachrome}} = 3520 \text{ m}^{-1} \text{ cm}^{-1}$) using a Varian Cary 300 Bio UV-vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The specific activities were normalized by protein yields.