CRYSTALLIZATION OF QUINONE ADDUCTED TO TURKEY HEMOGLOBIN AND ITS ROLE IN INHIBITING LIPID OXIDATION

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Abstract – Turkey hemoglobin A (HbA) was separated from turkey hemoglobin D (HbD) using ion-exchange chromatography, and then reacted with caffeic acid (CA) for up to 48 hours of iced storage (2 °C). Electrospray ionization mass spectroscopy (ESI-MS) was used to determine covalent adduction(s) by CA to α and β chains of HbA, and 1 molecule of CA was found to covalently bind to HbA α-chains. Crystal growth experiments were conducted with HbA in the presence of CA (576 conditions evaluated at 4 °C and 25 °C) and suitable crystals were examined by x-ray diffraction. A high resolution crystal structure of turkey HbA containing covalently bound CA was obtained. The binding site was the cysteine residue at position 130 in each α-chain. The distal histidine of β-chain in HbA-CA was less stabilized than non-adducted HbA. HbA containing bound CA promoted lipid oxidation less effectively compared to non-adducted HbA based on lipid peroxide and thiobarbituric acid reactive substances (TBARS) values during 2 °C storage. This crystal structure of a turkey hemoglobin-caffeic acid complex provides a template to better understand pro-oxidant-antioxidant interactions in muscle foods.

Key Words – caffeic acid, covalent binding, protein crystallography

I. INTRODUCTION

Lipid oxidation is a major cause of quality deterioration in raw and cooked muscle foods, mainly due to incurring off-flavors, off-odors and discoloration [1]. Hemoglobin (Hb), present in postmortem muscle tissue, has been shown to promote lipid oxidation in model lipid systems [2, 3]. Auto-oxidation and hemin loss from Hb have been reported as crucial roles in promoting lipid oxidation, and hemin loss rate can stimulate lipid oxidation more effectively compared to auto-oxidation rate [4].

Plant polyphenols, as naturally occurring antioxidants, have been seen as an important source of preventing meat and meat products from lipid oxidation [5]. Caffeic acid (CA), 3,4-dihyroxycinnamic acid, has been shown to be a potent antioxidant due to its free radical scavenging and metal chelating properties [6, 7], and previous studies have reported that this phenol can strongly inhibited Hb-mediated lipid oxidation in washed muscles [8, 9]. Phenol-derived quinones can also adduct to thiol groups (e.g. cysteine) of proteins, which can impact functional properties of the modified protein [10, 11].

Therefore, the objective of this study was to investigate the interaction of CA with turkey Hb using protein crystallography and examine the effect of the interaction on lipid oxidation in washed cod muscle (WCM).

II. MATERIALS AND METHODS

Preparation of turkey hemoglobin A

Anticoagulated blood was obtained from market weight turkeys at a commercial farm. The hemolysate was prepared according to the method of Park et al [8], and hemoglobin A (HbA) was separated from hemoglobin D (HbD) according to the method of Cobb et al [12] with modification.

Reaction of hemoglobin with CA

Reduced HbA was oxidized to metHbA as described previously [13]. MetHbA (0.4 mM, heme basis) was reacted with ethanolic caffeic acid (4 mM) as described by Park et al [8], followed by removal of unreacted CA using an FPLC system (Pharmacia Biotech Inc. Uppsala, Sweden) equipped with HiPrep 26/10 desalting columns (GE healthcare Inc. Uppsala, Sweden).

Preparation of washed cod muscle (WCM)
The WCM was prepared according to the method described by Grunwald et al [3] with minor modifications.

Electrospray ionization mass spectrometry (ESI-MS)
The covalent binding between CA and HbA was determined by ESI-MS according to the method of Zarnowski et al [14] with modifications.

Determination of lipid peroxides
The lipid peroxides were determined according to the method of Grunwald et al [3] with slight modifications.

Determination of thiobarbituric acid reactive substances (TBARS)
TBARS were determined according to the method of Grunwald et al [15] with slight modifications.

Protein crystallography
Protein crystallization was operated according to the method of Aranda et al [16] with modifications.

Statistical analysis
Data were analyzed using the SAS (version 9.3). Significance was determined using $p < 0.05$.

III. RESULTS AND DISCUSSION

Hemoglobin is a tetrameric protein comprised of two α chains and two β chains with allosteric oxygen-binding properties, and each chain contains one protoporphyrin IX group. Turkey α-globins consist of α^A- and α^D-globin, which were detected at a ratio of approximately 3:1 in definitive lineage [17]. In this study, the ESI-MS results showed that no covalent binding between CA and the minor component HbD was detected when turkey Hb was incubated with CA. In contrast, the major component HbA was found to form a covalent bond with CA (Table 1). Therefore, HbA was isolated by anion-exchange chromatography and selected for reaction with CA. After incubation with CA on ice for 48 h, very little of parental α-chain (m/z 15,338) was found with the intensity of 6000, compared to the CA-adducted α-chain (m/z 15,516) with the intensity of 135,000. The m/z 178 difference before and after incubation with CA on α-chain corresponds to one molecule of CA covalently bound to the α-chain. ESI-MS results showed that CA was not bound to β-chains after the incubation with CA (Table 1), which could result from less accessibility of CA to reactive amino acid residues in β-chains or insufficient reaction time for β-chains to bind to CA.

Table 1. Mass and relative abundance of the α- and β-chains of HbA and HbA reacted with CA.

	α-chain	Intensity	β-chain	Intensity
HbA	15,338 Da	*330,000*	16,307 Da	*60,000*
HbA +CA	15,338 Da 15,516 Da	*6,000* *135,000*	16,307 Da	*58,000*

After reacting CA with metHbA (2.5 mol CA per mol of metHbA), unreacted CA was removed by FPLC, as CA itself can act as a potent antioxidant due to its ability to scavenge free radicals and chelate metals [9]. Some reduction of metHbA (~30%) occurred during reaction with CA. Thus sodium dithionate was used to prepare fully reduced HbA-CA adduct. Reduced HbA-CA was then added to washed cod muscle (WCM) to investigate its effect on lipid oxidation compared to reduced HbA in the absence of adduct. Reduced HbA and reduced adduct were added separately to WCM at 40 μmol/kg tissue, and the pH of WCM was adjusted to pH 6.3 to be in the range of typical post mortem muscle tissue [3]. As indicators of lipid oxidation, the formation of lipid peroxides as the primary lipid oxidation products and thiobarbituric acid reactive substances (TBARS) as secondary lipid oxidation products were measured. Figure 1 showed that at 0-time reduced HbA and reduced adduct had similar lipid peroxide content, while HbA promoted lipid oxidation more effectively than reduced adduct after that. At each time point including 0.6, 0.8 and 1 day, the amount of lipid peroxides was significantly greater in HbA treated WCM compared to adducted HbA. A similar trend was found when evaluating the formation of the secondary lipid oxidation products. TBARS were significantly less due to HbA-CA adduct compared to HbA at 0.6 and 0.8 day. The different efficacy in propagating lipid oxidation between HbA and HbA-CA adduct may be due to bound CA altering the redox properties of HbA or free radical scavenging and metal chelation by bound CA. Structural assessment of HbA-CA compared

to HbA could also provide insight towards the inhibition of lipid oxidation that was observed.

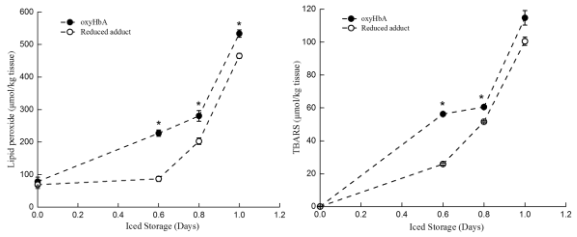

Figure 1. Ability of reduced adduct to inhibit Hb-mediated lipid oxidation in washed cod muscle (WCM) during 2 °C storage was measured by lipid peroxides (left panel) and TBARS method (right panel). * indicates significant difference ($p < 0.05$).

Protein crystallography was used to characterize the location of CA binding to HbA and assess structural attributes of HbA compared to HbA-CA adduct. After initial screenings and further optimizations, suitable crystals of CA reacted with HbA were examined by X-ray diffraction. The crystals belonged to space group $P4_22_12$ and diffracted to 1.9 Å of resolution, at which details of the small molecule interaction with hemoglobin will be unambiguous. A single covalent adduction of CA occurred with the cysteine residue at site H13 in each α-chain. H13 is the 130th amino acid in the primary structure and the 13th residue in the H-helix of α-chain. The sulfhydryl group (-SH) of Cys was the site for interaction with the benzene ring of the catechol unit in CA (Figure 2). Oxidation of the catechol unit to a quinone by tyrosinase was described previously to facilitate covalent binding of catechols to the SH group of bovine serum albumin [18]. It can be noted that there is a distinguishable pocket to allow access of CA to Cys(H13) of α-chain (Figure 3). No covalent binding was observed on β-chain of HbA. That Cys residues in the β-chains are less accessible may explain the lack of a Hb-CA adduct in the β-chains. Glutathione was noted to bind to chicken HbA at Cys(H13) of α-chain and Cys(H4) of β-chain while interior Cys residues in each chain remained unreacted [19]. Initial screening of the HbA-CA structure aligned with HbA indicated small to no structural differences at sites CD3/CE3, E10, E11, and E14 which previously were described to impact autooxidation and hemin loss rates when structural changes were noted upon comparing fish and mammalian Hbs [17]. A water molecule stabilized the distal histidine of HbA but not HbA-CA in β-chains. This suggested CA binding can cause structural perturbations that decrease oxidative stability of the heme protein.

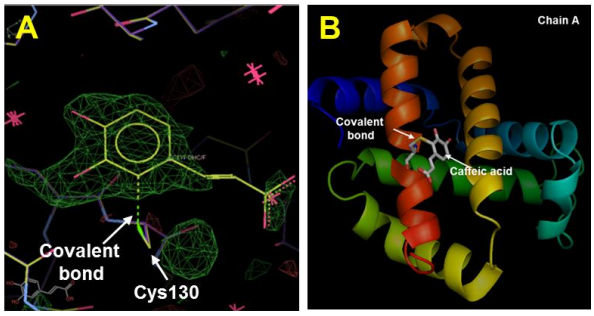

Figure 2. (A): electron density map obtained after molecular replacement, and caffeic acid docked into the difference electron density. (B): PyMol structure indicates that caffeic acid covalently binds to Cys130 in α-chain of turkey HbA.

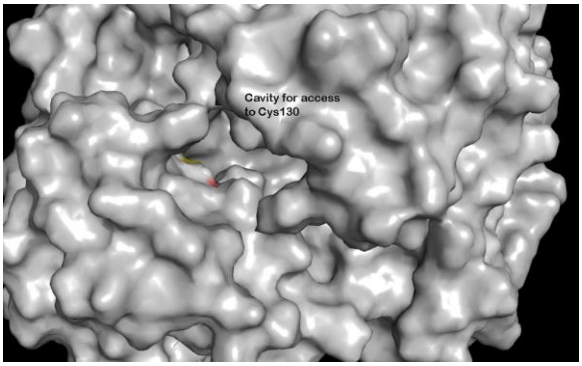

Figure 3. A cavity for small molecules to interact with Cys^{130} in α-chains of turkey HbA is observed. The sulfhydryl and carbonyl group of Cys^{130} in one of the α-chains is shown in yellow and red, respectively.

IV. CONCLUSION

The high resolution crystal structure of turkey HbA reacted with CA demonstrated covalent bonding between the sulfhydryl group of Cys^{130} in α-chains and the catechol moiety of CA. HbA-CA promoted lipid oxidation less effectively than HbA. At the same time, there was structural evidence that CA binding destabilized the distal heme pocket of the β-chains. These studies enhance our understanding of heme protein interactions with phenolic compounds.

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