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Effect of Glutathione on Oxymyoglobin Oxidation

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The oxidation of oxymyoglobin (OxyMb) to metmyoglobin (MetMb) is responsible for fresh meat discoloration. Glutathione (GSH) is an important tripeptide reductant that can protect lipid and protein from oxidation. The objective of this research was to investigate the effect of GSH on MetMb formation in vitro and in bovine skeletal muscle cytosol. Equine MetMb formation was greater in the presence of GSH than controls at pH 5.6 or 7.2 and 25 or 37 °C ($p < 0.05$); GSH addition to purified bovine OxyMb solution also resulted in more MetMb formation at pH 7.2 and 25 or 37 °C ($p < 0.05$). This effect on MetMb formation was partly or completely inhibited by EDTA or catalase in the GSH-equine OxyMb system ($p < 0.05$). The addition of GSH to bovine muscle cytosol inhibited MetMb formation at pH 5.6 or 7.2 and 4 or 25 °C ($p < 0.05$); the effect was concentration-dependent. The inhibitory effect was observed in a high molecular weight (HMW) but not low molecular weight fraction of cytosol at pH 7.2 and 25 °C ($p < 0.05$); there was no effect when HMW was heated at 90 °C for 15 min. These results suggest the antioxidant effect of GSH on bovine OxyMb is dependent on heat-sensitive HMW cytosolic component(s).

KEYWORDS: Glutathione; EDTA; cytosol; oxymyoglobin oxidation; sulfhydryl group

INTRODUCTION

Color is an important characteristic of fresh meat. It is dependent on the redox state of heme iron and the extent of oxygenation in myoglobin (Mb). The cherry red color of fresh meat that consumers prefer is attributed to oxymyoglobin (OxyMb). The red to brown discoloration of fresh meat during storage results from oxidation of OxyMb to metmyoglobin (MetMb) in which heme iron is converted from a ferrous to ferric state with loss of bound oxygen.

Glutathione (GSH) is an important intracellular tripeptide reductant that contains cysteine, glycine, and glutamate, and occurs in concentrations that range from about 8.2 mM in rat liver cytosol to 11.0 mM in mitochondria (*1*). Faustman and Cassens (*2*) reported that nonprotein sulfhydryl group content was 1.13 μmol/g wet weight for beef and that GSH accounted for 76% of nonprotein sulfhydryl groups. GSH has been reported to scavenge free radicals such as the hydroxyl radical and to serve as a cofactor in breaking down H_2O_2 by GSH peroxidase (*3*). Both hydroxyl radical and H_2O_2 are potent oxidants of lipid and protein. GSH can also conjugate with lipid oxidation products such as 4-hydroxynonenal (4-HNE) and 4-hydroxydecenal, a reaction that is catalyzed by GSH transferases (*4*−*5*). 4-HNE is a reactive product of lipid oxidation and can bind covalently to OxyMb to favor the formation of MetMb (*6*). GSH can also regenerate the antioxidant vitamins E and C in biological tissues (*7*−*9*). Rifkind (*10*) investigated the effect of GSH on horse hemoglobin (Hb), a tetrameric protein containing four prosthetic groups with subunits structurally similar to Mb. Both GSH and GSH disulfide (GSSG) decreased the apparent autoxidation rate of purified Hb, and the concentration of GSSG necessary to produce the effect was approximately 2 orders of magnitude greater than the concentration required when GSH was used. However, a pro-oxidant effect GSH on human oxyhemoglobin (OxyHb) was also reported by Gorbunov et al. (*11*). The addition of 2.0 mM GSH to commercial human OxyHb resulted in increased ferric Hb formation as manifested by a decreased absorbance at 577 nm and increased absorbance at 501 and 630 nm at pH 7.4 and 37 °C. The basis for the contradictory effect of GSH on Hb is unknown.

Kortz (*12*) investigated the relationship between the thiol-containing compounds and color stability in fresh pork meat and reported a high correlation coefficient between water-soluble −SH groups, or water-soluble protein −SH groups, and meat color stability. The addition of reduced GSH to pork samples increased color saturation, and GSH reduced some MetMb to OxyMb in vitro. However, GSH was not capable of completely reducing MetMb even when added at high concentrations, and there is no study of GSH effects on OxyMb stability. Our objective was to determine the effect of GSH on OxyMb oxidation in vitro and in skeletal muscle cytosol.

MATERIALS AND METHODS

Materials and Chemicals. Bovine *biceps femoris* muscle was purchased locally and stored at −20 °C until required. Equine heart Mb, Sephacryl S-200, sodium hydrosulfite, sodium phosphate, glutathione, Tris[hydroxymethyl]aminomethane hydrochloride (Tris-HCl), ethylenediaminetetraacetic acid (EDTA), 5,5′-dithio-bis(2-nitrobenzoic

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acid) (DTNB), methanol, and catalase (2350 units/mg solid) were obtained from Sigma Chem. Co. (St. Louis, MO). Ammonium sulfate and sodium citrate were purchased from Fisher Scientific (Fair Lawn, NJ). All chemicals were reagent grade.

Bovine Mb Purification. Bovine Mb was purified by ammonium sulfate precipitation and gel filtration chromatography according to Faustman and Phillips (*13*). Bovine *biceps femoris* muscle was homogenized in buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) for 1 min with a Waring blender (New Hartford, CT) and centrifuged (5000*g*) for 10 min with a Sorvall RC-5B centrifuge (Newtown, CT). The supernatant was brought to 70% ammonium sulfate saturation and centrifuged (18 000*g*) for 20 min. The resulting supernatant was saturated with ammonium sulfate and centrifuged (20 000*g*) for 1 h, and the precipitate was resuspended in homogenization buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and dialyzed against the same buffer for 24 h. Mb was separated from other proteins by gel permeation chromatography on Sephacryl S-200 (2.5 × 100 cm). The elution buffer contained 5 mM Tris-HCl and 1 mM EDTA (pH 8.0), and the flow rate was 1 mL/min. All steps were performed at 4 °C to minimize the formation of MetMb.

Equine and Bovine OxyMb Preparation. OxyMb was prepared by hydrosulfite-mediated reduction of commercial equine or purified bovine Mb, and residual hydrosulfite was removed by dialysis (12−14 kDa MWCO, Spectra/Por regenerated cellulose membrane, Rancho Dominguez, CA) with phosphate (pH 7.2) or citrate (pH 5.6) buffer. Mb concentration was determined by its absorbance at 525 nm ($\epsilon_{525\ nm} = 7.6\ mM^{-1}\ cm^{-1}$) and diluted to 0.07 mM in all reaction systems (*13, 14*).

Bovine Muscle Cytosol Preparation. Bovine muscle cytosol was prepared according to Kanner et al. (*15*) with minor modification. Bovine *biceps femoris* muscle (50 g) was trimmed to remove visible fat and ground in a Vitantonio tabletop grinder (4.0 mm; Eastlake, OH). Ground beef was homogenized further in 100 mL of 50 mM sodium citrate (pH 5.6) or sodium phosphate (pH 7.2) buffer with a Waring blender (New Hartford, CT) for 1 min. The homogenate was centrifuged (100 000*g*) at 4 °C for 30 min with a Beckman Optima LE-80 K ultracentrifuge (Fullerton, CA), and the resulting supernatant was filter-sterilized with a Corning filter system (0.22 μm cellulose acetate membrane, Corning, NY) and used as the soluble cytosolic extract. Separation of the cytosol into high (HMW) and low (LMW) molecular weight fractions was accomplished by using an Amicon Centricon filter device (10 kDa MWCO, Bedford, MA) in a Sorvall RT7 centrifuge (1700*g*, Newtown, CT). A heated HMW preparation was obtained by incubating the HMW fraction at 90 °C for 15 min. The preparation was then filtered through Whatman No.1 filter paper to remove any precipitate to yield the heated HMW. It is important to note that endogenous Mb (i.e., from *biceps femoris*) was a component of soluble cytosol and the unheated HMW; purified Mb was added back to LMW and heated HMW fractions to obtain an equivalent concentration of heme protein so that the fractions could be compared.

OxyMb Oxidation. OxyMb oxidation was measured according to Krzywicki's method (*16*) with minor modification. Briefly, an aliquot sample was withdrawn from the reaction system (reaction systems without added GSH, EDTA, or catalase were described as controls, see figure legends for details) and scanned from 650 to 450 nm with a Shimadzu UV-2101PC spectrophotometer. The absorbance values at 525, 545, 565, and 572 nm were used to calculate MetMb formation according to the following expression:

$$\%\text{MetMb} = ((-2.514 \times A_{572}/A_{525}) + (0.777 \times A_{565}/A_{525}) + (0.8 \times A_{545}/A_{525}) + 1.098) \times 100$$

Total Sulfhydryl Content Measurement. Total sulfhydryl content was determined by Ellman's assay according to Sedlak and Lindsay (*17*). Aliquots of a 0.3 mL sample were mixed in 15 mL test tubes with 1.7 mL of 0.2 M Tris buffer, pH 8.2, and 0.1 mL of 0.01 M DTNB. The mixture was brought to 10.0 mL with 7.9 mL of absolute methanol. A DTNB reagent blank (without sample) and a sample blank (without DTNB) were prepared, and the absorbance values were subtracted from the absorbance of the actual sample reaction analysis. Color in blanks and sample mixtures were developed for 20 min at room temperature, and absorbance was measured at 412 nm ($\epsilon_{412\ nm} = 13\ 600\ M^{-1}\ cm^{-1}$) with a Shimadzu UV-2101PC spectrophotometer.

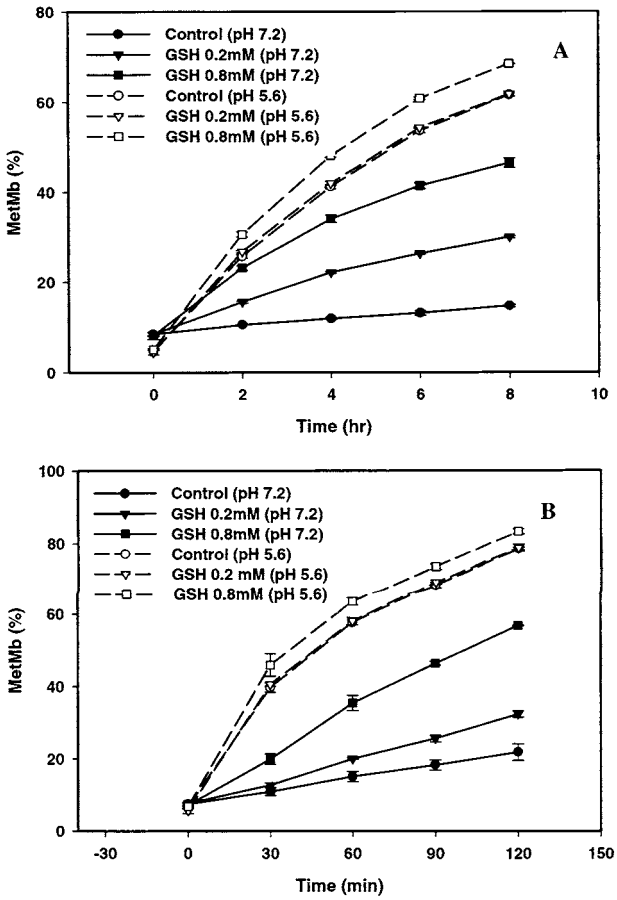

Figure 1. Equine MetMb formation in the presence of GSH at pH 5.6 or 7.2 and 25 °C (A) or 37 °C (B). Controls lacked added GSH. Standard deviations are indicated.

Statistical Analysis. Results are expressed as mean (standard deviation) of three independent studies. Data were analyzed using the MIXED procedure of SAS, and differences among means were detected at the 5% level using LSD with appropriate correction for multiple comparison (*18, 19*).

RESULTS AND DISCUSSION

Equine MetMb formation was greater in the presence of 0.2 and 0.8 mM GSH than controls (no GSH addition) at pH 7.2 and 25 °C ($p < 0.05$); at pH 5.6, only 0.8 mM GSH had a pro-oxidant effect at 25 °C ($p < 0.05$; **Figure 1A**). Similar results were observed at 37 °C ($p < 0.05$; **Figure 1B**). Equine OxyMb oxidation increased with temperature and was greater at pH 5.6 than pH 7.2 ($p < 0.05$; **Figure 1**), results consistent with previous reports (*20, 21*). The increased MetMb formation at higher temperature may be caused by increased rate of various pro-oxidant reactions (*22*); while increased MetMb formation at low pH is likely because of pH-related conformational changes in globin, which exposes the heme group to its surrounding environment and predisposes it to oxidative stress (*23*). When GSH was added to purified bovine OxyMb solution, more MetMb was formed in the presence of GSH at pH 7.2 ($p < 0.05$; **Table 1**); no effect was observed at pH 5.6 at the concentration used (results not shown).

The basis for the pro-oxidant effect of GSH on OxyMb in the present study is not known. One mM EDTA decreased approximately 83% of GSH-induced equine MetMb formation after 2 h at 37 °C ($p < 0.05$; **Figure 2**). This suggested that

Table 1. Bovine MetMb Formation (%) in the Presence of GSH at pH 7.2 and at 25 or 37 °C

	25 °C			37 °C		
time (hr)	control	GSH (0.2 mM)	GSH (0.8 mM)	control	GSH (0.2 mM)	GSH (0.8 mM)
0	6.2a[a]	5.9a	5.5a	11.2a	10.8a	10.1a
2	7.3a	12.4b	22.6c	24.2a	39.6b	59.2c
4	9.3a	18.2b	29.2c	36.2a	53.0b	69.2c

[a] Values in rows with different letter are different within a temperature condition ($p < 0.05$).

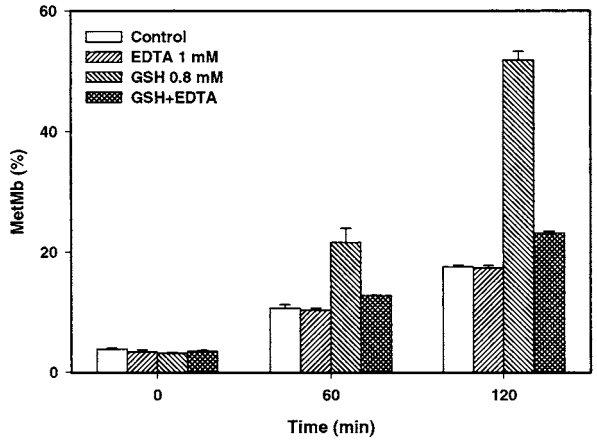

Figure 2. Equine MetMb formation in the presence of EDTA (1 mM) or GSH (0.8 mM) at pH 7.2 and 37 °C. Controls lacked added GSH and EDTA. Standard deviations are indicated.

metal ion-mediated reactions contributed to the pro-oxidant effect of GSH on OxyMb oxidation. Pro-oxidant effects of GSH on lipid in liposome−ADP−Fe^{3+} and human OxyHb in EDTA−Hb systems have been reported previously (*11*, *24*, *25*). In a liposome−ADP−Fe^{3+} system, GSH appeared to increase lipid oxidation by direct reduction of Fe^{3+} to Fe^{2+} in a concentration-dependent manner with subsequent autoxidation of Fe^{2+} to produce H_2O_2 (*24*). In a human Hb−EDTA system, hydrogen peroxide was implicated as an essential intermediate and was hypothesized to be formed by the reaction of GSH with O_2 (*25*). Since hydrogen peroxide was suggested as an intermediate product in those processes, catalase was added to the equine OxyMb−GSH reaction system and GSH-induced OxyMb oxidation was completely inhibited ($p < 0.05$; **Figure 3**) by the addition. Therefore, it is likely that hydrogen peroxide was also a possible active intermediate in this process.

The effect of GSH on bovine OxyMb oxidation was very different in bovine skeletal muscle cytosol. There was less MetMb formation in the presence of GSH relative to controls ($p < 0.05$), and the effect was concentration-dependent (**Figures 4** and **5**). The addition of 0.8 mM GSH decreased MetMb formation by 35% (**Figure 4A**) and 40% (**Figure 4B**) at pH 7.2 and 5.6, respectively, after 8 h incubation at 25 °C. Following 48 h storage at 4 °C, the inhibitory effect was more pronounced at pH 5.6 than 7.2 ($p < 0.05$; **Figure 5**). MetMb formation was decreased by 72% in the presence of 0.8 mM GSH as compared to controls at pH 5.6, while at pH 7.2 the decrease was 29%. The effect of pH on inhibition of OxyMb oxidation was less at 25 °C than at 4 °C, a result consistent with the known effects of pH and temperature on redox stability of heme protein (*21*).

Direct reduction of ferric to ferrous heme iron was proposed to explain the protective effect of GSH on purified Hb (*10*) and

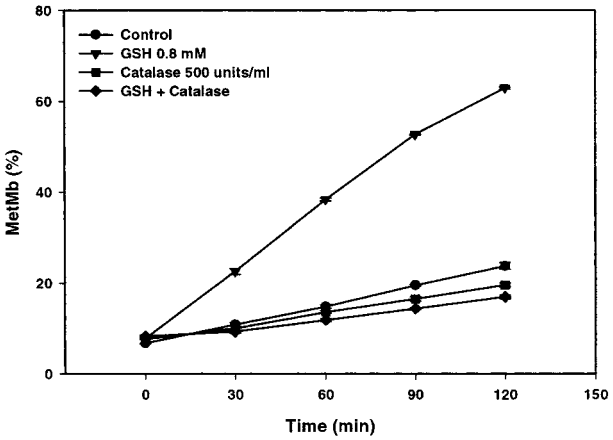

Figure 3. Equine MetMb formation in the presence of GSH (0.8 mM) or catalase (500 units/mL solution) at pH 7.2 and 37 °C. Controls lacked added GSH and catalase. Standard deviations are indicated.

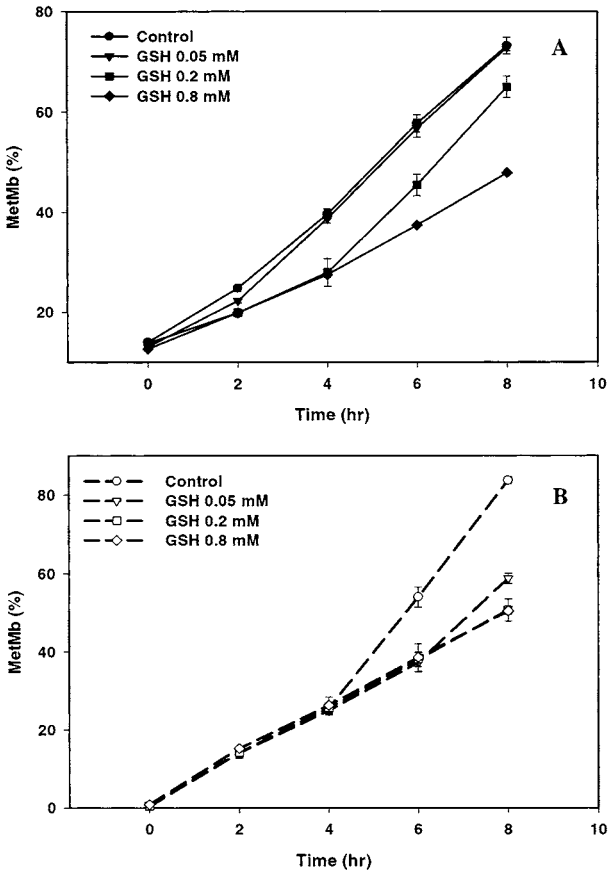

Figure 4. Bovine MetMb formation in the presence of GSH in skeletal muscle cytosol at pH 7.2 (A) or 5.6 (B) and 25 °C. Controls lacked added GSH. Standard deviations are indicated.

Mb (*12*). This cannot explain any observation in our study because results demonstrated that GSH exhibited a protective effect on bovine OxyMb redox stability only when combined with muscle cytosol.

In an attempt to further characterize the potential cytosolic components contributing to the antioxidant effect, soluble cytosol was separated into HMW and LMW fractions. The addition of exogenous GSH to HMW decreased bovine MetMb formation relative to controls at pH 7.2 and 25 °C ($p < 0.05$; **Figure 6**); however, no antioxidant effect was observed in LMW ($p > 0.05$; results not shown). When HMW was heated, there was no difference in MetMb formation between added GSH

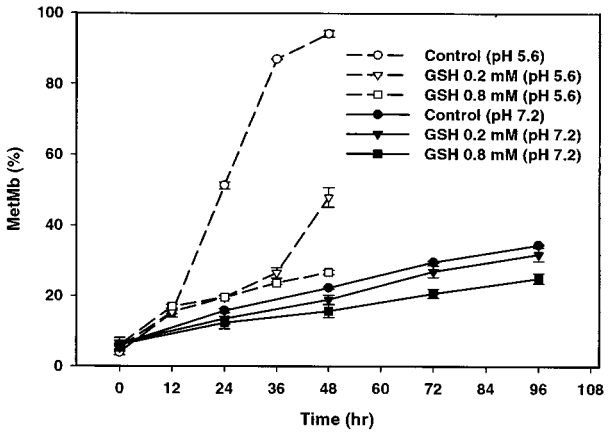

Figure 5. Bovine MetMb formation in the presence of GSH in skeletal muscle cytosol at pH 5.6 and 7.2 and 4 °C. Controls lacked added GSH. Standard deviations are indicated.

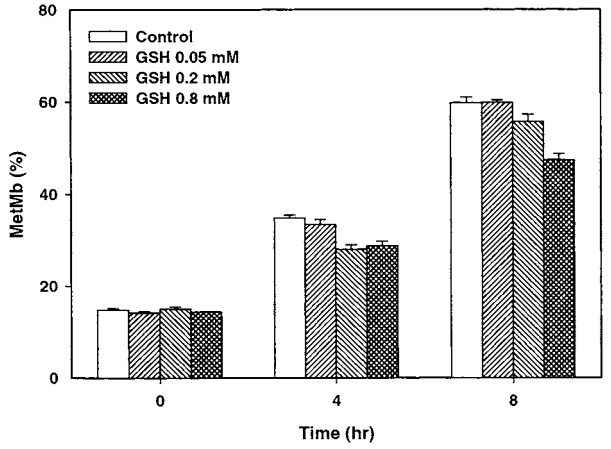

Figure 6. Bovine MetMb formation in the presence of GSH in unheated high molecular weight fraction at pH 7.2 and 25 °C. Controls lacked added GSH. Standard deviations are indicated.

and control treatments ($p > 0.05$; results not shown). These results suggested that the GSH antioxidant effect in cytosol was dependent on heat-labile HMW component(s). The cytosolic fraction of muscle tissues contains pro-oxidant and antioxidant enzymes, myoglobin, ferritin, H_2O_2, metal ions, and reducing components (*15*, *26*, *27*). When it is separated into LMW and HMW fractions, enzymes and Fe-containing proteins are retained in the HMW. Antioxidant enzymes expected to be contained in the HMW fraction include superoxide dismutase, catalase, GSH peroxidase, and GSH *S*-transferase, all of which are capable of inhibiting oxidative processes (*26*, *28*).

Similarly, a rat liver cytosol-specific antioxidant effect of GSH was reported for microsome lipid oxidation (*29*); GSH alone inhibited lipid oxidation only slightly, while cytosol had a pro-oxidant effect. However, the combination of cytosol plus GSH demonstrated a marked protective effect against lipid oxidation, and GSH *S*-transferase was suggested to be partly responsible for this effect. Further study is needed to identify the responsible factors in our system.

We analyzed total sulfhydryl concentration and MetMb formation in cytosolic reaction systems (**Figure 7**). Added GSH inhibited bovine MetMb formation as compared to controls ($p < 0.05$), and it contributed to a measured increase in total sulfhydryl content in treatments initially. However, total sulfhydryl concentrations decreased rapidly in GSH treatments while they were stable in controls during 8 h at pH 7.2 and 25 °C. It

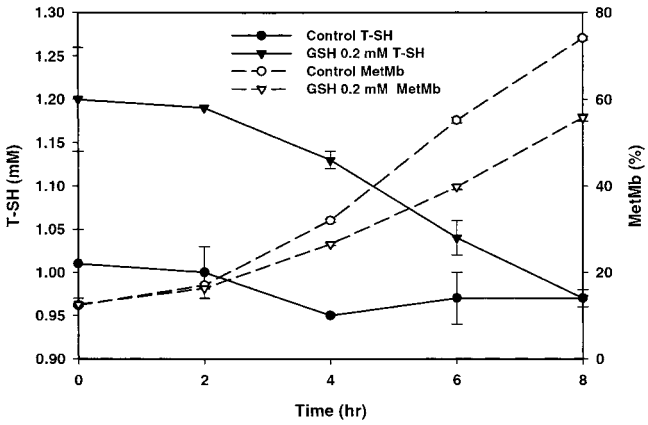

Figure 7. Comparison of total sulfhydryl (T-SH) group content and bovine MetMb formation between GSH (0.2 mM) treatments and controls at pH 7.2 and 25 °C. Controls lacked added GSH. Standard deviations are indicated.

is likely that the sulfhydryl groups from added GSH were responsible for the inhibitory effect on MetMb formation and that they were oxidized during the process. Rapid disappearance of GSH and concomitant increase in GSSG concentrations were reported in skipjack tuna and Japanese jack mackerel muscle during storage at 4 °C (*30*). The authors attributed the loss in GSH to decreased activity of GSH reductase, which acted to regenerate GSH (*30*). Denaturation, hydrolysis by proteases, or loss of cofactors could all decrease enzyme activity during storage.

In conclusion, the effect of GSH on MetMb formation depended on the reaction environment (i.e., buffer vs muscle cytosol). GSH enhanced OxyMb oxidation in vitro; however, it significantly inhibited bovine OxyMb oxidation in muscle cytosol, and this inhibitory effect was related to heat-sensitive HMW compound(s). Both EDTA and catalase addition decreased GSH-induced equine OxyMb oxidation in vitro.

ABBREVIATIONS USED

GSH, glutathione; Mb, myoglobin; OxyMb, oxymyoglobin; MetMb, metmyoglobin; Hb, hemoglobin; OxyHb, oxyhemoglobin; MWCO, molecular weight cutoff; GSSG, glutathione disulfide; HMW, high molecular weight; LMW, low molecular weight; T-SH, total sulfhydryl group; 4-HNE, 4-hydroxynonenal; Tris-HCl, tris[hydroxymethyl]aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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