Differences in the Antioxidant Mechanism of Carnosine in the Presence of Copper and Iron[†]

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Carnosine is a β-alanylhistidine dipeptide found in skeletal muscle. Carnosine (1.0–25 mM) is capable of inhibiting copper- and iron-catalyzed oxidation of phosphatidylcholine liposomes as measured by thiobarbituric acid reactive substances (TBARS) and lipid peroxides. The ability of 5 mM carnosine to inhibit the formation of TBARS and lipid peroxides was 2.5- and 8.8-fold higher, respectively, for copper- than iron-catalyzed lipid oxidation. Carnosine (0.05–10.0 mM) is capable of inhibiting copper-catalyzed oxidation of ascorbic acid but was ineffective at preventing iron-catalyzed ascorbate oxidation. Carnosine inhibits iron-dependent microsomal lipid oxidation but does not inhibit the oxidation of NADPH by the enzyme system. ^{1}H NMR spectra of carnosine show peak broadening in the presence of copper but not iron. These data suggest that carnosine forms a complex with copper which decreases its catalytic activity; however, carnosine does not form a complex with iron.

INTRODUCTION

Lipid oxidation is controlled in skeletal muscle by a number of different systems. Iron-catalyzed lipid oxidation is inhibited enzymically by ceruloplasmin (Osaki et al., 1966). Both iron- and copper-catalyzed lipid oxidation are controlled nonenzymically by chelators such as transferrin, ferritin, serum albumin, amino acids, phosphates, and carboxylic acids (Halliwell and Gutteridge, 1986). Lipid oxidation is also inhibited by enzymes that inactivate reactive oxygen species (glutathione peroxidase, catalase, and superoxide dismutase) and by nonenzymic factors that scavenge free radicals (α-tocopherol, uric acid, serum albumin, and ubiquinone; Kanner et al., 1987).

Carnosine is a β-alanylhistidine dipeptide found in skeletal muscle (Crush, 1970). Pork, beef, and chicken white muscle contain approximately 4.0, 8.0, and 15.0 mM carnosine, respectively (Crush, 1970). Carnosine is capable of inhibiting iron-catalyzed lipid oxidation under the pH and temperature conditions expected during the processing and storage of muscle foods (Decker and Faraji, 1990). Addition of carnosine to uncooked (Decker and Crum, 1991a) and cooked (Decker and Crum, 1991b) ground pork results in the inhibition of lipid oxidation during storage. These data suggest that carnosine could contribute to the inherent antioxidant potential of muscle and also suggest that carnosine could have application as a "natural" antioxidant in processed meat products.

In addition to iron (Boldyrev et al., 1988), carnosine has been reported to inhibit a number of different lipid oxidation catalysts including hydrogen peroxide-activated hemoglobin, lipoxidase, photoactivated riboflavin (Decker and Faraji, 1990), peroxyl radicals (Kohen et al., 1988), and hydroxyl radicals (Aruoma et al., 1989). While carnosine is an active antioxidant, Boldyrev et al. (1988) and Kohen et al. (1988) have reported that the individual amino acids, β-alanine and histidine, have little to no antioxidant activity. However, the antioxidant role of histidine

in different lipid oxidation systems is not consistent. Erickson et al. (1990) found that histidine stimulated the oxidation of flounder sarcoplasmic reticulum, while Karel et al. (1966) reported that histidine inhibited lipid oxidation in a freeze-dried model system. The antioxidant mechanism of carnosine is also not well understood but has been suggested to be due to a combination of chelation and free-radical scavenging (Kohen et al., 1988).

The objectives of this research were to determine if the antioxidant mechanism of carnosine was different in the presence of copper or iron. Differences in antioxidant mechanism were determined by the ability of carnosine to chelate iron and copper as determined by changes in the catalytic activity of the metals and by changes in the ^{1}H NMR spectra of carnosine in the presence of iron or copper. Elucidation of the antioxidant mechanism of carnosine will provide a better understanding of the antioxidant role of carnosine in situ and in processed meat products.

EXPERIMENTAL PROCEDURES

Materials. Carnosine, ascorbate, soybean phosphatidylcholine (IV-S), and deuterium oxide were obtained from Sigma Chemical Co. (St. Louis, MO). (Trimethylsilyl)propionic acid and 4,6-dihydroxy-2-mercaptopyrimidine (TBA) were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade or purer.

Methods. Liposomes were prepared from soybean phosphatidylcholine by homogenization and sonication and were quantitated by measuring phosphate (Decker and Hultin, 1990). Lipid oxidation studies were performed in a model system containing 0.02 mg of phosphatidylcholine liposomes/mL of 0.12 M KCl–5 mM histidine buffer (pH 7.0) plus ascorbate (100 μM) and FeCl$_3$ (15 μM) or CuCl$_2$ (30 μM) and various concentrations of carnosine. Ascorbate oxidation was minimized by preparing all solutions immediately prior to use. All reactions were run at 37 °C for 30 min. Lipid oxidation was monitored by measuring thiobarbituric acid reactive substances (TBARS; McDonald and Hultin, 1987) and lipid peroxides (Beuge and Aust, 1978). Percent inhibition was calculated as

$$\left(1 - \frac{\text{activity in the presence of inhibitor}}{\text{activity in the absence of inhibitor}}\right) \times 100$$

Iron- and copper-catalyzed ascorbate oxidation was measured spectrophotometrically at 265 nm (Davies et al., 1986) on a Mil-
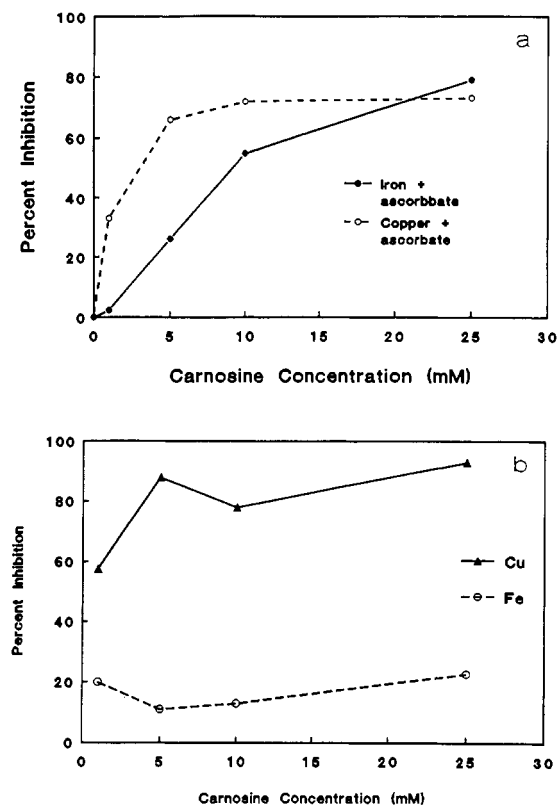
Figure 1. Inhibition of copper- and iron-catalyzed oxidation of phosphatidylcholine liposomes by various concentrations of carnosine as measured by thiobarbituric acid reactive substances (TBARS; a) and lipid peroxides (b).

Figure 2. Effect of various concentrations of carnosine (0–10 mM) on copper- (a) and iron-catalyzed (b) oxidation of ascorbate.
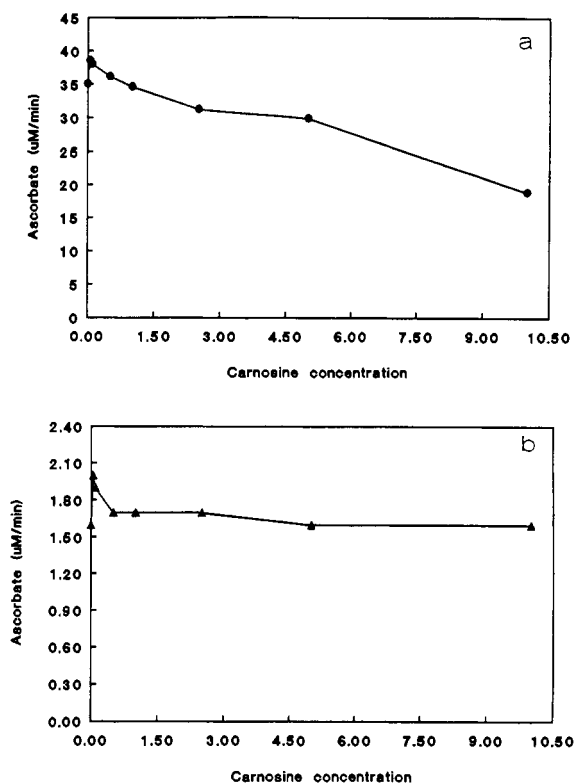
ton Roy 1201 spectrophotometer. Initial rates of ascorbate oxidation were determined every minute for a total of 5 min. Reactions were performed in 100 mM phosphate buffer (pH 7.0). Initial ascorbate concentration was 100 μM, and FeCl$_3$ and CuCl$_2$ concentrations were 15 μM.

Iron-dependent microsomal lipid oxidation was determined in chicken muscle microsomes. Microsomes were isolated from chicken thigh muscle (<4 h post-mortem) as described by Lin and Hultin (1976). Assays contained 0.3 mM ADP, 0.3 mM NADPH, 10 μM FeCl$_3$, 25 mM carnosine, and 0.5 mg of microsomal protein per milliliter of 0.12 M KCl–5 mM histidine buffer (pH 7.0). Controls contained no carnosine. Lipid oxidation was monitored by measuring thiobarbituric acid reactive substances (TBARS; McDonald and Hultin, 1987). NADPH concentration was monitored with a Gilson Response double-beam, double-wavelength spectrophotometer at 450 and 340 nm every 3 min for 30 min. NADPH concentration was calculated as the differences between Abs$_{340}$ and Abs$_{450}$ using 6.20 × 10^3 as the molar extinction coefficient (Dawson et al., 1986).

^{1}H nuclear magnetic resonance spectra of carnosine and carnosine in combination with either copper or iron were recorded on a Varian VXRS-400 spectrometer operating at 400 MHz. Carnosine (10 mM) was dissolved in deuterium oxide containing (trimethylsilyl)propionic acid as the internal standard. FeCl$_3$, FeCl$_2$, CuCl$_2$, and CuCl (10 μM) were added immediately prior to analysis.

RESULTS

Carnosine was capable of inhibiting both iron- and copper-catalyzed oxidation of phosphatidylcholine liposomes. When lipid oxidation was measured by TBARS (Figure 1a), low carnosine concentrations ($\leq$10 mM) more effectively inhibited lipid oxidation catalyzed by copper than by iron. At 1, 5, and 10 mM, carnosine inhibition of TBARS production was 11-, 2.6-, and 1.3-fold higher in the presence of copper than that in iron. The antioxidant activities of 25 mM carnosine were similar in the presence

of either iron or copper. The antioxidant activity of carnosine was also greater in the presence of copper than of iron when the oxidation of phosphatidylcholine liposomes was measured by lipid peroxides (Figure 1b). Inhibition of lipid peroxide formation was 2.9-, 8.0-, 6.6-, and 4.5-fold higher at 1, 5, 10, and 25 mM carnosine, respectively, in the presence of copper. The ability of carnosine to inhibit copper- and iron-catalyzed lipid oxidation was different when lipid oxidation was measured by lipid peroxides as compared to TBARS. Changes in antioxidant activity as determined by lipid peroxides could be due to the ability of metals to decompose lipid peroxides (Labuza, 1971) or to differences in sensitivity between the peroxide and TBA assays. Neither histidine nor β-alanine (25 mM) was found to inhibit iron- or copper-catalyzed formation of TBARS or lipid peroxides (data not shown). This is in agreement with the findings of Boldyrev and co-workers (Boldyrev et al., 1988) and Kohen and co-workers (Kohen et al., 1988), who reported that both histidine and β-alanine have little to no antioxidant activity.

The ability of carnosine to inhibit the catalysis of ascorbate oxidation by copper and iron is shown in Figure 2. Low concentrations of carnosine (0.1 and 0.05 mM) slightly accelerated the catalysis of ascorbate oxidation by both metals. Higher carnosine concentrations (1.0–10.0 mM) had no effect on iron-catalyzed but decreased copper-catalyzed ascorbate oxidation. Oxidation of ascorbate by copper was decreased 46% in the presence of 10 mM carnosine compared to that in controls containing no carnosine. Chelators such as EDTA and uric acid have been reported to inhibit both iron- (Davies et al., 1986) and copper-catalyzed (Uchida and Kawakishi, 1989) ascorbate oxidation by rendering the metals catalytically inactive.

Carnosine was capable of inhibiting the production of TBARS by an iron/NADPH-dependent lipid oxidation system in chicken muscle microsomes (Figure 3). Inhibition of microsomal lipid oxidation by carnosine ranged
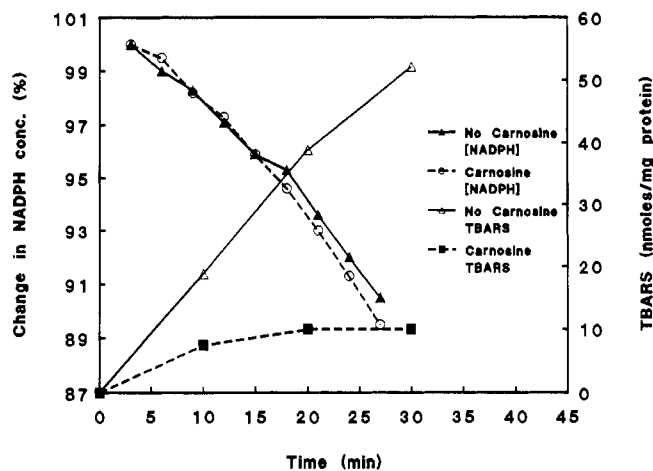
Figure 3. Formation of thiobarbituric acid reactive substances (TBARS) and consumption of NADPH by an iron-dependent chicken microsomal lipid oxidation system in the presence (25 mM) and absence of carnosine.

from 56 to 80% during the 30 min of incubation. Oxidation of NADPH by the enzyme system (as measured spectrophotometrically at 340 nm) did not change in the presence or absence of carnosine. The rates of NADPH oxidation were 5.4 and 5.6 nmol/min in the presence and absence of carnosine, respectively. NADPH concentration did not decrease and TBARS did not increase in the absence of iron (data not shown).

The ^{1}H NMR spectra of carnosine, carnosine plus Fe^{2+}, and carnosine plus Cu^{1+} are shown in parts a, b, and c, respectively, of Figure 4. Peaks at 2.6, 3.0, 3.2, 4.7, 7.7, and 6.9 ppm represent the protons of β-CH_2 of β-Ala, β-CH_2, of His, α-CH_2 of β-Ala, α-CH of His, and C-2 and C-4 of the imidazole ring of carnosine, respectively (Brown and Antholine, 1980). The peak observed at 4.9 ppm represents water. The ^{1}H NMR spectrum of copper–carnosine was different from that of carnosine alone in that peaks at 6.9 and 7.7 ppm were absent and peaks at 2.6, 3.0, 3.2, and 4.7 ppm showed significant broadening. Spectra for carnosine and carnosine plus Fe^{2+} were similar. ^{1}H NMR spectra for carnosine plus the oxidized states of copper or iron were similar to those of the reduced states of the metals (data not shown).

DISCUSSION

Potential antioxidant mechanisms of carnosine against prooxidant metals include inactivation of free radicals and formation of carnosine–metal complexes that exhibit decreased reactivity. Carnosine was found to inhibit both iron- and copper-catalyzed oxidation of phosphatidylcholine liposomes as measured by either TBARS (Figure 1a) or lipid peroxides (Figure 1b). However, the antioxidant activity of carnosine was greater in the presence of copper than iron, especially at low carnosine concentrations. Increasing the concentration of carnosine from 0 to 10 mM decreased the ability of copper to oxidize ascorbate but did not decrease iron-catalyzed ascorbate oxidation (Figure 3).

Iron catalyzes lipid oxidation in a chicken skeletal muscle microsomal system in the presence of ADP and NADPH (Lin and Hultin, 1976). Oxidation has been postulated to occur through the reduction of ferric to ferrous ions which catalyze the decomposition of hydrogen or lipid peroxides to lipid and oxygen radicals (McDonald and Hultin, 1987). If carnosine–iron chelates were formed in a manner that prevents iron from interacting with the enzyme system (either through steric hindrance or prevention of redox
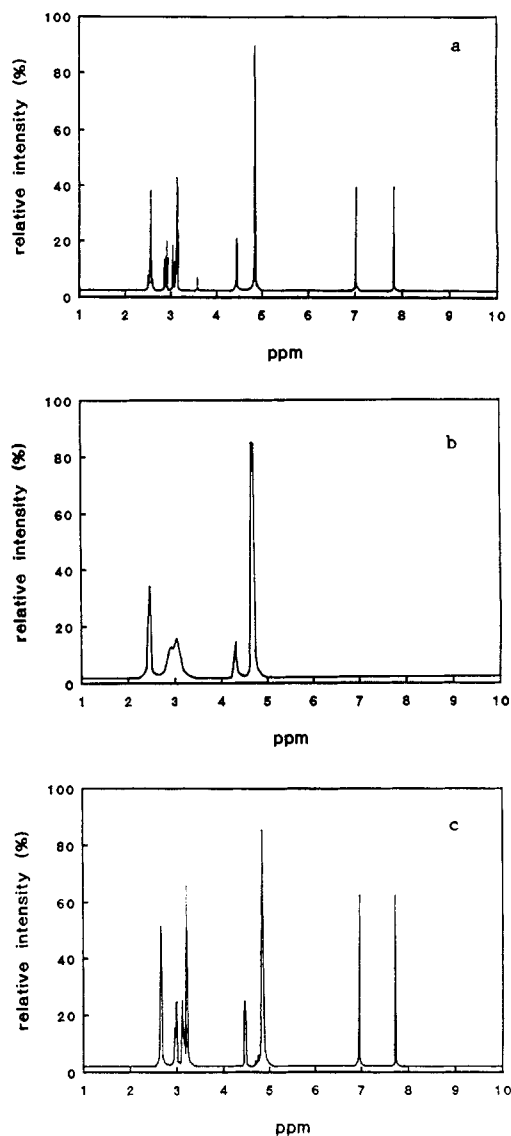
Figure 4. ^{1}H NMR spectra for carnosine (a), carnosine plus Cu^{1+} (b), and carnosine plus Fe^{2+} (c). Carnosine concentration was 10 mM, and copper and iron concentrations were 10 μM. Peaks at 2.6, 3.0, 3.2, 4.7, 7.7, and 6.9 ppm represent the protons of β-CH_2 of β-Ala, β-CH_2 of His, α-CH_2 of β-Ala, α-CH of His, and C-2 and C-4 of the imidazole ring of carnosine, respectively. The peak observed at 4.9 ppm represents water.

cycling), then the rate of NADPH oxidation by the enzyme should decrease along with the catalysis of lipid oxidation. However, carnosine was seen to only decrease TBARS production and have no effect on the consumption of NADPH by the microsomal enzyme system (Figure 3).

The ability of carnosine to decrease copper-catalyzed lipid and ascorbate oxidation suggests that carnosine forms an unreactive complex with copper. However, it is also possible that copper bound to carnosine is catalytically active and able to produce radicals but the close proximity of the carnosine to the site of radical production results in immediate scavenging and decreased lipid or ascorbate oxidation. Histidine has been shown to interact with oxygen radicals in the presence of ascorbate and copper (Uchida and Kawakishi, 1989, 1990a,b). The inability of carnosine to inhibit iron-catalyzed ascorbate oxidation and to inhibit the interaction of iron with chicken muscle microsomes suggests that carnosine does not complex iron in a manner which decreases its reactivity.

Carnosine could form complexes with both iron and copper without affecting the ability of the chelated metal

to catalyze oxidative reactions. ^{1}H NMR spectra of carnosine, carnosine plus Cu^{1+}, and carnosine plus Fe^{2+} indicate that carnosine forms a complex with copper but not with iron (Figure 4). Similar ^{1}H NMR spectra were observed for carnosine plus Cu^{2+} or Fe^{3+}, indicating that the binding of copper or iron was not significantly influenced by the oxidation state of the metal. Brown (1981) reported that carnosine will form a tetramer with Cu^{2+} when the carnosine concentration is 100–1000 times that of copper. The carnosine concentration used in this study was 1000 times that of iron or copper. This tetramer involves the binding of four molecules of carnosine to copper through the N-3 of the imidazole ring. This explains the absence of C-2 (7.7 ppm) and C-4 (6.9 ppm) peaks of the imidazole ring of carnosine in the presence of copper (Figure 4b). Peak broadening at 2.6 (β-CH$_2$ of β-Ala), 3.0 (β-CH$_2$ of His), 3.2 (α-CH$_2$ of β-Ala), and 4.7 (α-CH of His) ppm is due to carnosine–carnosine interactions within the tetramer structure (Brown and Antholine, 1980).

NMR spectra indicate that carnosine did not form a complex with iron; however, carnosine was still capable of inhibiting iron-catalyzed lipid oxidation (as measured by TBARS or lipid peroxides) of phosphatidylcholine liposomes or chicken muscle microsomes. In addition to iron and copper, carnosine can also inhibit nonmetal lipid oxidation catalysts including peroxyl radicals (Koyhen et al., 1988), lipoxidase, and photoactivated riboflavin (Decker and Faraji, 1990). The ability of carnosine to inhibit iron-catalyzed lipid oxidation without chelation and the ability of carnosine to inhibit nonmetal lipid oxidation catalysts suggest that carnosine could be capable of inactivating free radicals. If carnosine is capable of inactivating free radicals in addition to forming catalytically inactive complexes with copper, then it would suggest that the antioxidant mechanism of carnosine in situ could be multifunctional.

ACKNOWLEDGMENT

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