Metmyoglobin Promotes Arachidonic Acid Peroxidation at Acid pH*

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The ability of metmyoglobin and other heme proteins to promote peroxidation of arachidonic acid under acidic conditions was investigated. Incubation of metmyoglobin with arachidonic acid resulted in a pH-dependent increase in lipid peroxidation as measured by the formation of thiobarbituric acid reactive products and oxygen consumption. Increased peroxidation was observed at pH levels below 6.0, reaching a plateau between pH 5.5 and 5.0. At comparable heme concentrations, metmyoglobin was more efficient than oxyhemoglobin, methemoglobin, or ferricytochrome c in promoting arachidonic acid peroxidation. Metmyoglobin also promoted peroxidation of 1-palmitoyl-2-arachidonyl phosphatidylethanolamine and methylarachidonate but at significantly lower rates than arachidonic acid. Addition of fatty acid-free albumin inhibited arachidonic acid peroxidation in a molar ratio of 6 to 1 (arachidonic acid:albumin). Both ionic and non-ionic detergents inhibited metmyoglobin-dependent arachidonic acid peroxidation under acidic conditions. The anti-oxidants butylated hydroxytoluene and nordihydroguaiaretic acid and low molecular weight compounds with reduced sulphhydryl groups inhibited the reaction. However, mannitol, benzoic acid, and deferoxamine were without significant effect. Visible absorption spectra of metmyoglobin following reaction with arachidonic acid showed minimal changes consistent with a low level of degradation of the heme protein during the reaction. These observations support the hypothesis that metmyoglobin and other heme proteins can promote significant peroxidation of unsaturated fatty acids under conditions of mildly acidic pH such as may occur at sites of inflammation and during myocardial ischemia and reperfusion. This may be the result of enhanced aggregation of the fatty acid and/or interaction of the fatty acid with heme under acidic conditions.

The mechanisms by which reactive oxygen metabolites mediate tissue injury at sites of inflammation and following ischemia and reperfusion is the subject of intense investigation (1-3). Many studies have focused on examining the role of superoxide anion and hydrogen peroxide in the initiation of tissue injury either directly or indirectly through the generation of additional reactive metabolites including iron-dependent hydroxyl radical (•OH) and myeloperoxidase-dependent hypochlorous acid (HOCl). The source of these oxygen metabolites are multiple and include activated phagocytic cells, mitochondrial electron transport systems, microsomal enzymes, xenobiotics, and xanthine oxidase (1-3). One mechanism by which these reactive oxygen species are thought to mediate tissue injury is through the initiation and propagation of lipid peroxidation reactions in cell membranes (4-6). Several in vivo and in vitro studies have shown increased formation of lipid peroxidation products at sites of acute and chronic tissue injury (7-9), as well as in both plasma and lymph draining sites of ischemia reperfusion injury (10-12).

It has been known for many years that heme proteins will enhance the decomposition of lipid hydroperoxides (13-15) and in the presence of hydrogen peroxide or lipid hydroperoxides will promote peroxidation of unsaturated fatty acids and phospholipids (13-17). This reaction is thought to be mediated by the formation of a ferryl peroxo species (Fe4+-OOH) and has been implicated as a mechanism of red cell and myocyte injury under conditions of oxidative stress (16-19). Additional studies have also demonstrated a quasi-lipoxigenase activity of hemoglobin. This activity is distinct from its ability to promote lipid peroxidation and shows a substrate specificity for dienoic fatty acids (20).

An additional feature of inflammatory reactions and ischemic injury is an associated decrease in tissue pH. Under certain conditions, the pH at sites of inflammation or ischemia may decrease below pH 6.0 and significantly alter cell and tissue function (21). Under these conditions the biochemical mechanisms that may promote tissue injury, especially myocardial ischemia reperfusion injury will be altered. We have undertaken this study to determine the effect of acid pH on heme protein-dependent arachidonic acid peroxidation.

MATERIALS AND METHODS

RESULTS

Incubation of metmyoglobin with arachidonic acid resulted in a pH-dependent increase in the production of MDA as assessed by reaction with thiobarbituric acid (Fig. 2). At pH values above 6.0, minimal MDA was formed over a 30-min incubation period. However, between pH 6.0 and 5.0 there was a pH-dependent increase in MDA formation achieving a maximum at pH 5.5. A comparison of the ability of metmyoglobin to promote arachidonic acid peroxidation with other heme proteins indicated that both, oxidized cytochrome c and methemoglobin, effectively promoted arachidonic acid peroxidation (Fig. 3). However, metmyoglobin was 30 to 50% more effective in promoting the reaction than the other heme proteins.
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The effect of pH on metmyoglobin-mediated arachidonic acid peroxidation. Metmyoglobin (50 μM) was incubated with arachidonic acid (200 μM) in 50 mM sodium phosphate buffer at varying pH for 30 min at 37 °C. The amount of malondialdehyde (micromolar) formed is shown.

Fig. 3. Comparison of the effect of metmyoglobin with ferricytochrome c and methemoglobin on the peroxidation of arachidonic acid. The heme protein (50 μM) was incubated with arachidonic acid (200 μM) at either pH 7.4 or 5.5 at 37 °C for 30 min. The amount of malondialdehyde (micromolar) formed is shown.

The initial rate of reaction between metmyoglobin and arachidonic acid was evaluated by determining the rate of oxygen consumption. Upon addition of arachidonic acid to metmyoglobin (2 μM) there was a marked increase in oxygen consumption (Fig. 4). This increase occurred in the absence of an appreciable lag period following addition of arachidonic acid and the reaction rate was linearly correlated with the amount of arachidonic acid added; approximately 0.06 μmol of oxygen was consumed per min/4 μmol of arachidonic acid present in the reaction. Thus, over a 30-min incubation period 1.8 μmol of oxygen would be potentially consumed per 4 μmol of arachidonic acid. In contrast, the rate of peroxidation was linearly correlated with the square root of the concentration of metmyoglobin (r = 0.998). Incubation of arachidonic acid alone at pH 5.5 resulted in a rate of oxygen consumption equivalent to less than 3% of that observed when metmyoglobin was present. At pH 7.4, the rate of oxygen consumption during reaction of metmyoglobin with arachidonic acid was less than 3% of that observed at pH 5.5. Additional experiments demonstrated that incubation of 1-palmitoyl-2-arachidonyl phosphatidylcholine or methylarachidonate with metmyoglobin at pH 5.5, generated significant amounts of MDA over a 30-min incubation (37 °C) (data not shown). However, studies of the rate of peroxidation quantitated as the rate of O₂ consumed, indicated that whereas metmyoglobin increased the rate of arachidonic acid peroxidation by approximately 30-fold, there was only a 2-fold increase in the rate of peroxidation of methylarachidonate and 1-palmitoyl-2-arachidonyl phosphatidylcholine (Table 1).

The effect of a variety of free-radical scavengers and antioxidants on metmyoglobin-dependent arachidonic acid peroxidation at pH 5.5 was evaluated. The most effective inhibitors of the reaction were butylated hydroxytoluene and nordihydroguaiaretic acid which at concentrations of 2 μM or above inhibited the formation of MDA by greater than 90% (data not shown). The concentration at which both these anti-oxidants are effective inhibitors are much less than either the concentration of metmyoglobin (50 μM) or arachidonic acid (200 μM) present in the reaction. Low molecular weight compounds containing reduced sulfhydryl groups were also effective in inhibiting the reaction although at approximately 500 to 5000-fold higher concentrations than butylated hydroxytoluene or nordihydroguaiaretic acid and approximately 20-fold higher concentrations than metmyoglobin. Neither deferoxamine (20 μM), mannitol (100 mM), nor benzoic acid (50 mM) were effective inhibitors of the reaction. Addition of fatty acid-free bovine serum albumin to arachidonic acid (200 μM) prior to addition of metmyoglobin (50 μM) resulted in a dose-dependent inhibition of the reaction at pH 5.5 (data not shown). Approximately 75% inhibition of MDA formation

| Rate of O₂ consumption μl/min |
|-----------------------------|
| Arachidonic acid alone      | 0.12 |
| + metmyoglobin              | 3.96 |
| Methyl arachidonate         | 0.30 |
| + metmyoglobin              | 0.65 |
| 1-Palmitoyl-2-arachidonoyl phosphatidylcholine + metmyoglobin | 0.30 | 0.62 |
was observed at a concentration of 25 μM BSA. This corresponds to the molar binding ratio of BSA for fatty acids (1 to 6).

**DISCUSSION**

The results of this study indicate that under acidic conditions such as may occur in myocardial tissue during ischemia and at sites of inflammation, metmyoglobin and other heme proteins can promote lipid peroxidation independent of an additional source of oxidant. The observation that the rate of oxygen consumption remained relatively linear even at low concentrations of oxygen suggest that reactions similar to those described in this study may play a role in promoting cell and tissue injury at sites of inflammation and ischemia; conditions where the pH is decreased and low levels of oxygen remain available for peroxidation. Similar conditions may exist in the border regions of ischemic myocardial tissue and during the early period of reperfusion following ischemia. Further studies are required to more clearly define the precise biochemical mechanism of peroxidation and to determine if similar reactions can occur under in vivo conditions.

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Figure 1. The effect of metmyoglobin on the peroxidation of arachidonic acid at pH 5.5. Metmyoglobin at varying concentrations was incubated with arachidonic acid (300 μM) at 37°C in sodium phosphate buffer (10 mM). Incubation of arachidonic acid alone or metmyoglobin (10 μM) alone at 37°C for 60 min resulted in the formation of 0.25 μM and 0.14 μM of MDA, respectively. The amount of malondialdehyde (μM) formed is shown.

Figure 3. Visible absorption spectra of metmyoglobin following incubation with arachidonic acid. The spectra of metmyoglobin alone (- - -) and metmyoglobin + arachidonic acid (---) after 30 minutes incubation are shown.

Table 2. Effect of Detergents on Metmyoglobin/Arachidonic Acid Oxygen Consumption at pH 5.5

| Concentration of Detergent | % Inhibition of O2 Consumption |
|---------------------------|------------------------------|
| Buffer                    | 0.0                          |
| Triton X-100              | 0.1%                         |
| Triton X-100              | 0.3%                         |
| Triton X-100              | 0.6%                         |
| SDS                       | 1.0%                         |
| Tween-20                  | 1.0%                         |
| Nonidet-40                | 1.0%                         |

% Inhibition of O2 Consumption