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THE ROLE OF H_2O_2-GENERATED MYOGLOBIN RADICAL IN CROSSLINKING OF MYOSIN

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The mechanism of myoglobin/H_2O_2 derived peroxidation of myosin was studied by comparing the catalytic activity of myoglobin and horseradish peroxidase using O-dianisidine, N-acetyl tyrosine and myosin as substrates. It was found that both hemoproteins induced myosin crosslinking and concomitant tyrosines oxidation to bityrosines, suggesting inter-molecular coupling of tyrosines in the crosslinking. The enzymatic activity of both hemoproteins on myosin was weak compared to small substrates. While horseradish peroxidase was much more active than myoglobin on small substrates, the reverse was true for myosin peroxidation. Since the suicidal interaction of myoglobin with H_2O_2 forms unstable tyrosine radicals, we suggest that the increased activity of myoglobin on myosin results from an efficient electron transfer between surface tyrosines of myosin and myoglobin but not horseradish peroxidase. These conclusions were supported by evidence that sperm whale myoglobin, which contains two active tyrosines - the heme-adjacent (tyrosine-103) and the surface (tyrosine-151), is more active as a mediator of myosin peroxidation than horse heart myoglobin which is devoid of the surface tyrosine.

KEY WORDS: Myosin, myoglobin, bityrosine, horseradish peroxidase, crosslinking.

ABBREVIATIONS: HA, high aggregates; HMb, horse myoglobin; HRP, horseradish peroxidase; Mb, myoglobin; MHC, myosin heavy chains; MLC, myosin light chains; My, myosin; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; VHA, very high aggregates; WMb, sperm whale myoglobin.

INTRODUCTION

Heme-containing molecules which are ubiquitous in all aerobic cells, can be divided into two major categories. The first includes those that stabilize oxygen molecules for transport, like hemoglobin and myoglobin, and the second are the electron transport proteins such as the mitochondrial hemoproteins or the plant enzyme horseradish peroxidase. Both myoglobin and hemoglobin were elucidated to function also, although with much lower efficiency, as "real" peroxidases like HRP.[1,2] Myoglobin ferryl compound was initially proposed as a model for HRP compound II, but was later shown to be structurally different from both compound I and II of horseradish peroxidase.[3,4] The myoglobin ferryl complex retains only one of the two H_2O_2 oxidation equivalents and the second is centered on aromatic residues of the globins.[5,6]

Despite the low efficiency of myoglobin and hemoglobin as peroxidases, a large amount of information on their peroxidative activities was collected during the last

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three decades because they are accessible and exist in high concentrations *in vivo*. Both proteins were documented to trigger lipid peroxidation in the presence of H_2O_2.[7] In addition, recent studies have implicated induction of self crosslinking of neighboring proteins by several hemoproteins. Undecapeptide from cytochrome c was shown to rapidly cause crosslinking of crystallins.[8] Red cell membrane structural proteins such as the cytoskeletal proteins, spectrin and protein 4.1, were shown to undergo hemoglobin and hemin induced crosslinking in the presence of H_2O_2.[9] We recently reported that myoglobin triggers crosslinking of the main skeletal protein, myosin.[10] Along with crosslinking reactivity of the oxygen binding hemoproteins, HRP was also reported to be involved in peroxidative crosslinking of proteins. Pancreatic ribonuclease and galactose oxidase are examples of proteins that were shown to crosslink by HRP in the presence of H_2O_2.[11,12] Peroxidative activity of HRP on low density lipoproteins similar to that of hemoglobin and myoglobin was reported as well.[13,14]

A survey of this literature leaves one with the impression that despite their low reactivity as peroxidases, hemoglobin and myoglobin are especially active in peroxidations that lead to crosslinking of proteins. It was felt that a systematic comparison of the reactivity of a classic peroxidase and an oxygen binding hemoprotein on the same substrates might explain this unclear phenomenon. We examine here the peroxidative activity of HRP and Mb on nonprotein substrates compared to their activity on a large protein like myosin. This work and the extensive information available on these hemoproteins, allowed us to postulate a mechanism by which the low activity of myoglobin as a "classic" peroxidase is increased when a protein like myosin is used as a substrate.

MATERIALS AND METHODS

Materials

Polyacrylamide gel electrophoresis (PAGE) chemicals were from Bio-rad and all other chemicals were purchased from Sigma. WMb was a Fluka product bought from Sigma, cat No. 70020. HRP was type X, cat No. 6140, with a specific activity of 250 units/mg. HMb was cat No. 0630, O-dianisidine was cat No. 3252, and the L form of N-acetyl tyrosine was No. 2513. All other chemicals were of analytical grade.

Methods

Preparative procedures Myosin was prepared from rabbit leg muscles according to Tonomura[15] and Spudich and Watt.[16] Its concentration was determined spectrophotometrically, using $\varepsilon(1\%) = 5.5$ at 280 nm for myosin (M.W = 500 kD).

Commercial sperm whale and horse muscle myoglobin forms were dissolved in the suitable buffer. After centrifugation for 5 min at 12,000 g, the precipitate was discarded and the supernatant was filtered to eliminate undissolved material. The concentration of this Mb solution was determined spectrophotometrically using a molar extinction coefficient of $\varepsilon(mM) = 9.47$ at 505 nm.[17]

Electrophoretic evaluation of myosin crosslinking Myosin was mixed with myoglobin, and H_2O_2 was added to start the peroxidation reaction. In com-

parative reactions all concentrations and conditions were matched. The pH of the reactions was neutral or increased to high pH values to yield higher amounts of products. The reactions were terminated by SDS containing sample buffer, incubated in a boiling water bath for 2 min and applied to Laemmli SDS-PAGE for analysis.[18] Electrophoresis was carried out on slab gels with either 8% homogeneous or 6% and 12% double layer polyacrylamide. The latter gel composition assisted resolution of proteins in mixtures that contained combination of very high ($\geq$200 kD) and small ($\leq$20 kD) MW polypeptides. Electrophoresis was carried out using an electric field of 40 mA per gel for approximately 90 min. All gels were stained with Coomassie blue stain.

Conversion of spectral data to concentration units Dianisidine: The difference of the mM extinction coefficients ($\Delta\varepsilon$ mM) at 460 nm for oxidized and reduced dianisidine was determined experimentally by measurement of ΔOD (460) of a fully oxidized (by excess HRP) dianisidine solution of a known molar concentration. Tyrosines: N-acetyl tyrosine with a known molar concentration was fully oxidized (by excess HRP) and the fluorescence intensity was determined at the maximum of the emission, 403 nm, under specific spectrofluorimeter conditions which were kept constant throughout the experiments. The ratio of $f = F_{403}/[\text{tyrosine}]$ was used as the factor to convert fluorescence intensity into molarity of oxidized tyrosines. This value was used for measurements of free, as well as protein tyrosines oxidation.

Instruments A Shimadzu UV-160 spectrophotometer and a Hitachi-Perkinelmer Model 44B spectrofluorimeter were used.

RESULTS

HRP and Mb as Mediators of Myosin Crosslinking

To determine the link between Mb-induced crosslinking and peroxidation of myosin, the ability of Mb to crosslink myosin was compared to that of the classical peroxidase, HRP. Myosin solutions were incubated in the presence of H_2O_2 and identical concentrations of myoglobin or HRP. At the concentrations of enzymatic activity expression, nM range, neither hemoprotein affected the SDS-PAGE pattern of myosin. In the μM range of concentrations, HRP, like Mb, induced covalent aggregation of myosin, but the activity of HRP was always lower than that of Mb. Figure 1 illustrates two examples of the protein pattern: one at a high concentration of H_2O_2 and short incubation time and the other at a lower H_2O_2 concentration and prolonged incubation time (see experimental conditions in the legend). As seen by comparing lanes 4 & 5, at 200 μM H_2O_2, most of the myosin monomers were crosslinked by Mb within 10 min. In the presence of HRP, myosin remained practically monomeric and only a minor fraction of the protein aggregated. Note that the propagation of the reaction is expressed by the fraction of aggregated myosin as well as the increased size of the aggregates. Prolonged incubation resulted in increased aggregation of myosin, but HRP was still less efficient than Mb (not shown). At lower H_2O_2 concentration, myoglobin was also more active and its efficiency in promoting myosin aggregation even more pronounced (compare lanes 6 & 7).

Considering that crosslinking of myosin is a result of the hemoproteins' perox-

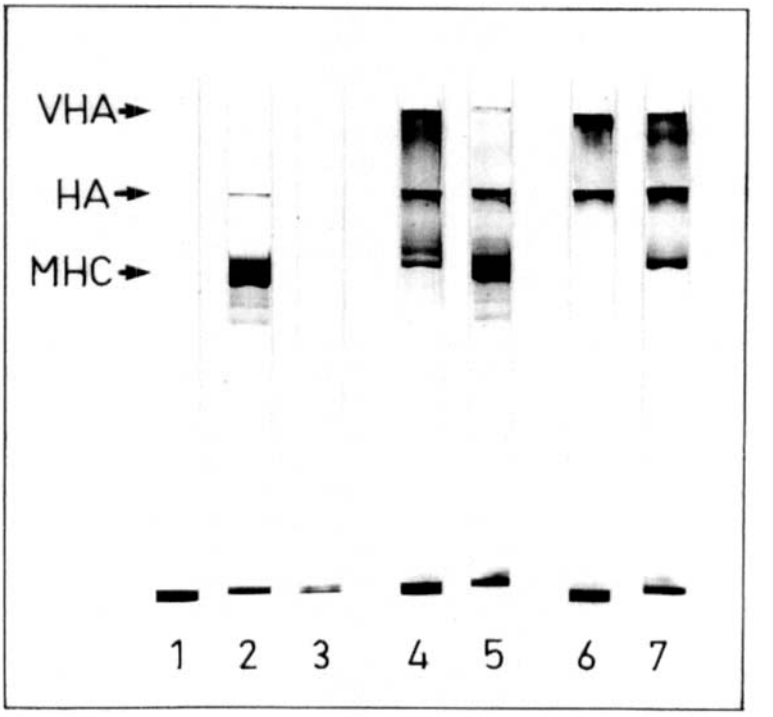

FIGURE 1 Peroxidative crosslinking of Myosin: relative activity of Mb and HRP as inducers. All reactions were carried out in 0.1 M pyrophosphate buffer, at pH 7.3. Concentration of proteins: My-2 μM; Mb & HRP-30 μM. The gel contains 6% acrylamide. Bottom bands Mb and HRP have the same mobilities under the experimental conditions. Lane 1: Mb Lane 2: My Lane 3: HRP Lane 4: Mb in presence of My and 200 μM H_2O_2 after 10 min. Lane 5: HRP in presence of My and 200 μM H_2O_2 after 10 min. Lane 6: Mb in presence of My and 20 μM H_2O_2 after 1 hr. Lane 7: HRP in presence of My and 20 μM H_2O_2 after 1 hr. Note that MLC did not participate in the crosslinking reaction.

idative activity, one could attribute the non-enzymatic dose of μM HRP required for crosslinking of myosin to inhibition of the heme reactivity by the steric hindrance of the large protein substrate. This rationale would lead one to expect a decreased rather than an increased myoglobin peroxidative activity compared to HRP. It was thought that a comparison of the relative sensitivity of other substrates for Mb and HRP would explain these results.

Peroxidative Activity of HRP and Mb on Various Substrates

Initial peroxidation rates of the different substrates by H_2O_2 under identical conditions were measured using HRP or Mb as mediators. The first substrate compared was dianisidine, a widely used HRP substrate. At pH 9.5, 25°C, in the presence of 10^{-11} M HRP, the rate of 31 μM dianisidine peroxidation was 4.3 μM/min (data not shown). Replacement of HRP by the same Mb concentration had no effect on the dianisidine oxidation; and an increase of five orders of magnitude in the Mb concentration, namely 10^{-6} M, was required to reach the rate of dianisidine oxida-

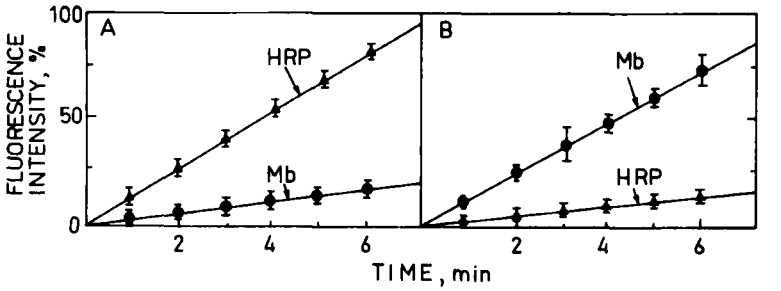

FIGURE 2 Tyrosines as substrates for HRP and Mb induced peroxidation. Buffer: *Pyrophosphate-0.1 mM; pH-9.5; Temp-25°C; Concentrations:* H_2O_2-200 μM *Inducers:* Mb & HRP (note the different concentrations in A & B). Excitation wavelength-327 nm; Emission wavelength-403 nm. *Substrates:* N-acetyl tyrosine-50 μM; Myosin-0.2 μM; A - N-acetyl tyrosine as a substrate. HRP-0.05 μM; Mb-0.5 μM Rates: Mb-1 $\times$ 10^{-8} M/min; HRP-5 $\times$ 10^{-8} M/min. Note that a higher concentration of Mb had to be used to demonstrate both enzymes on the same scale. B-Myosin as a substrate. HRP & Mb-0.5 μM Rates:Mb-3.9 $\times$ 10^{-10} M/min.; HRP-0.67 $\times$ 10^{-10} M/min.

tion induced by HRP. This poor activity corresponds with the well documented low potential of Mb as a peroxidase.[1,2]

Oxidation of tyrosines in various structural and globular proteins was shown in previous literature to be induced by peroxidases.[19] The red cell structural proteins spectrin and protein 4.1 were shown to form bityrosines when crosslinked by hemoglobin.[9] Recent studies in our laboratory indicated that under conditions of myosin crosslinking,[10] bityrosines are formed as well (unpublished results). Hence, in further experiments the initial rates of myosin peroxidation with Mb or HRP was followed by the appearance of a fluorescent signal typical to bityrosines. Under all conditions employed, the action of Mb and HRP on this protein was similar and in the μM range. Figure 2B illustrates an example of such a reaction (see legend for details of experimental conditions). Under identical concentrations of the two hemoproteins, the rate of peroxidation of tyrosines in myosin was higher with Mb than with HRP. When we compared peroxidation of tyrosine monomers in the form of N-acetyl tyrosine (to block the oxidation of the free amino group), as mediated by Mb or HRP, the HRP induced peroxidation far exceeded that of Mb. As shown in Figure 2A, HRP is less active in N-acetyl peroxidation than dianisidine, but still more active than myoglobin (see legend for experimental details).

Since heme comprises the active site of both HRP and Mb peroxidative activity, we further compared its stability in the two hemoproteins under the conditions employed in the above experiments. H_2O_2 (200 μM) was added to identical concentrations (10 μM) of each protein (pH 7.3), and time dependent changes in the spectra of the Soret band region were followed. Similar to other studies, the Mb Soret peak decreased irreversibly with time, while that of the HRP remained stable.[20] Under the experimental conditions of the current study, 75% of the heme Soret absorption of myoglobin was lost within 30 min (not shown). The relative activity of HRP and Mb in catalyzing peroxidation of various substrates by H_2O_2 is summarized in Table 1. The numbers represent concentrations of the peroxidase required to yield an identical initial peroxidation rate of 4.3 μM/min, for all substrates. This rate of HRP-catalyzed dianisidine peroxidation, which was the highest recorded, was

TABLE I
Concentrations of HRP & Mb required to achieve the same peroxidation rates[1] of all substrates employed

Enzyme	Substrate		
	Anisidine	N-Acetyl-Tyr	Myosin
HRP	$1.0(\pm 0.12) \times 10^{-11}$	$4.3(\pm 0.30) \times 10^{-6}$	$3.2(\pm 0.12) \times 10^{-2}$ [2]
Mb	$1.0(\pm 0.12) \times 10^{-6}$	$2.1(\pm 0.15) \times 10^{-4}$ [2]	$5.5(\pm 0.38) \times 10^{-3}$ [3]

(1) 4.3 μM/min
(2) Due to myosin limited solubility this value was unmeasurable and was therefore drawn by linear extrapolation from experimental data collected in the current study at lower substrate concentrations.
(3) Due to trivial fluorescence quenching (filter effect) by Mb, this value was linearly extrapolated from lower Mb concentrations.

chosen as the standard rate. Note that some numbers in the table are experimental, while others were extrapolated from measurable rates when technical difficulties prevented measurement of the required rates.

Relative Activity of Sperm-Whale and Horse Myoglobins in Myosin Peroxidation

In order to understand the specific protein crosslinking activity of myoglobin on myosin, the structural details available for different myoglobins and their interactions with H_2O_2 were used. Sperm-whale myoglobin, employed up to this stage as

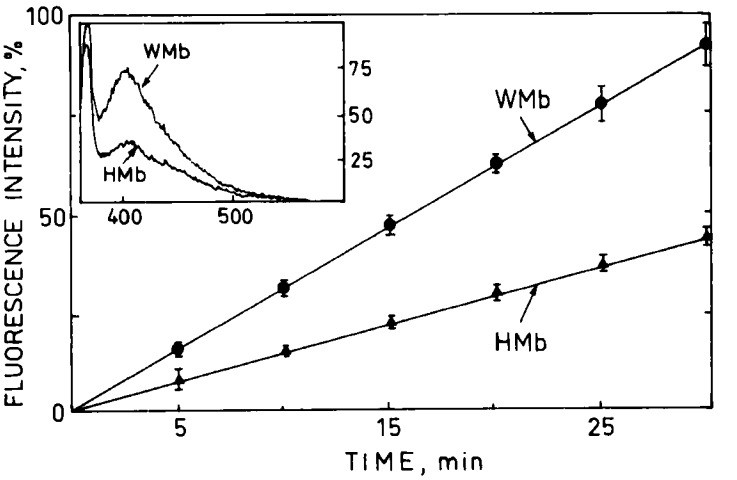

FIGURE 3 Bityrosine formation in Myosin: relative activity of various myglobins. Buffer: Pyrophosphate-0.1 mM; pH-9.5; Temp-37°C; Concentrations: H_2O_2-200 μM; Myoglobins-0.2 μM; Myosin-0.2 μM, equals to 40 μM in tyrosine residues. Excitation wavelength-327 nm. *Main figure*: Relative activity of bityrosines formation as reflected by reaction initial rates. Emission wavelength-403 nm. Rates: Sperm-Whale Mb-13 nm/min.; Horse heart Mb-6.5 nm/min. *Insert*: Emission spectra of reaction mixtures excited at 403 nm after 25 min of incubation.

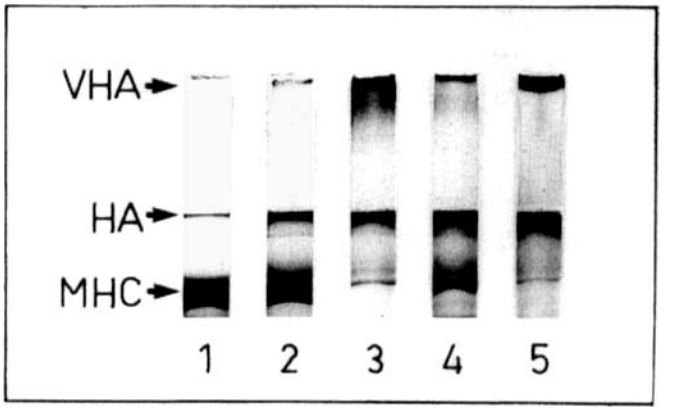

FIGURE 4 Peroxidative crosslinking of myosin: relative activity: of various mloglobins*. Buffer: Pyrophosphate-0.1 M; pH-7.3; Temp 25°C; concentrations: Myoglobins-30 μM; Myosin-2 μM; H_2O_2-200 μM 1. My alone 2. My with horse muscle Mb and H_2O_2; incubated for 30 min 3. My with sperm whale muscle Mb and H_2O_2; incubated for 30 min 4. My with horse muscle Mb and H_2O_2; incubated for 90 min 5. My with sperm whale muscle Mb and H_2O_2; incubated for 90 min. *This figure demonstrates the upper part of the gels containing myosin heavy chains and higher molecular weight area, since no differences appeared in low M.W. areas between the various lanes.

a myoglobin representative, contains 3 tyrosine residues: an inactive Tyr-146, an active Tyr-103 near the heme active site, and a surface tyrosine in position 151 which is missing in horse myoglobin.[21,22] Hence, the activity of sperm-whale and horse myoglobins as catalysts of myosin peroxidation by H_2O_2 was compared. Figure 3 compares the initial rates of the formation of myosin-bityrosines induced by the two myoglobins as measured by the intensity of bityrosines fluorescence at 403 nm. Under the experimental conditions chosen (see legend), the rate of oxidation catalyzed by sperm-whale Mb was double that catalyzed by horse Mb (13 nM/min and 6.5 nM/min respectively). The activity of both myoglobins as catalyzers of myosin crosslinking was than compared. Figure 4 shows the protein pattern at two time points of the reaction mixtures containing myosin, H_2O_2 and one of the myoglobins (details in the legend). After 30 min incubation under the experimental conditions chosen, most myosin heavy chains were still monomeric in the reaction driven by HMb, while a substantial crosslinking occurred in the WMb-induced one (lanes 2 & 3). Within 90 min most myosin underwent crosslinking in the WMb-catalyzed reaction, but a significant fraction of myosin was still monomeric in the HMb-induced reaction (lanes 4 & 5).

Does Ferryl Stability Correlate with the Actively of Mb in Myosin Crosslinking?

In this experiment we compared the level of peroxidised heme, namely ferryl state, and myosin crosslinking. Myoglobin was activated by moderate excesses of H_2O_2 at pH 9.5 to increase the ferryl level. Formation of ferryl was followed spectrophotometrically at 540 nm until maximum absorption was reached (within 10 min at 37°C). At this stage, catalase was added to stop peroxidation, and the decay of the ferryl intermediate was followed as described previously.[23,24] The time course

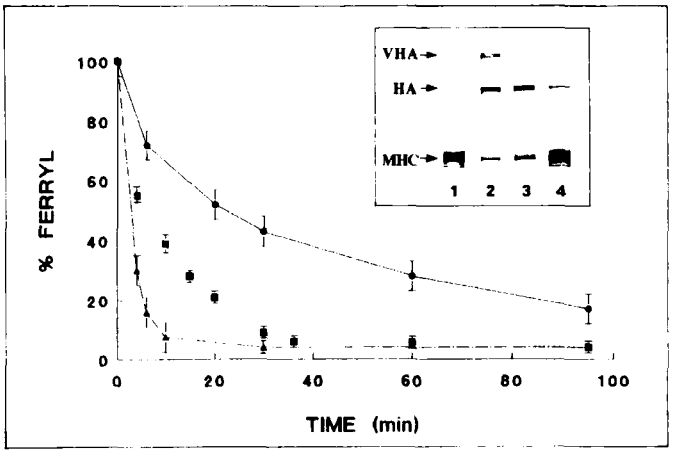

FIGURE 5 Effect of uric acid and tyrosine on myoglobin activity. All reactions were carried out at pH 9.5 and the temperature was 37°C. *Main figure*: Time course of myoglobin ferryl decay. Concentration of reactants: Mb-30 μM; H_2O_2-200 μM; Tyr & uric acid-50 μM; My-1-20 μM; (●—●—●) ferryl alone; (■···■···■) ferryl + tyrosine; (▲—·▲—·▲) ferryl + uric acid *Insert*: Myoglobin mediated crosslinking of myosin* Concentration of reactants: Mb-30 μM; H_2O_2-200 μM; Tyr & uric acid-50 μM; My-5 μM; incubation time was 30 nim. *This figure demonstrates the upper part of the gels containing myosin heavy chains and higher molecular weight area, since no differences appeared in low M.W. areas between the various lanes.

of the ferryl decay under the experimental conditions specified in the legend is demonstrated in Figure 5. Substrates of Mb catalyzed peroxidation were expected to act as scavengers of the activated heme. Uric acid, an active antioxidant known to scavenge Mb ferryl,[25,26] was chosen as reference, and its effect on the Mb ferryl level was compared with that of N-acetyl tyrosine and myosin. All potential ferryl scavengers were added to the Mb/H_2O_2 reaction mixtures immediately after termination of oxidation by catalase. Myosin at increasing concentrations (1–20 μM) and at various pH conditions had no effect on the time course of the ferryl decay. On the other hand, both uric acid and N-acetyl tyrosine accelerated the rate of Mb ferryl decay, with uric acid being more effective (Figure 5). The presence of the above three effectors in the reaction mixtures prior to H_2O_2 addition affected the results as expected: myosin had no effect on the level and rate of ferryl state formation whereas the level of Mb ferryl decreased in the presence of N-acetyl tyrosine, and more so with uric acid (data not shown). The results in Figure 5 demonstrate reactions carried out with WMb, but similar effects were obtained when ferryl state of HMb was studied (not shown). We then followed the protein pattern of the above reaction mixtures, in the presence and absence of tyrosine or uric acid, on SDS-PAGE. The WMb induced peroxidative crosslinking of myosin was obstructed by the presence of uric acid and N-acetyl tyrosine, albeit to a lesser degree (insert to Figure 5).

Previous studies illustrated that in the absence of any other protein, the H_2O_2/Mb mixture yields some globin dimerization via tyrosines in WMb, but not in HMb.[22] We therefore followed the effect of myosin on the dimerization of WMb. The latter was incubated with H_2O_2 in the presence and absence of myosin, and the patterns of the protein mixtures were analyzed by SDS-PAGE (see legend of

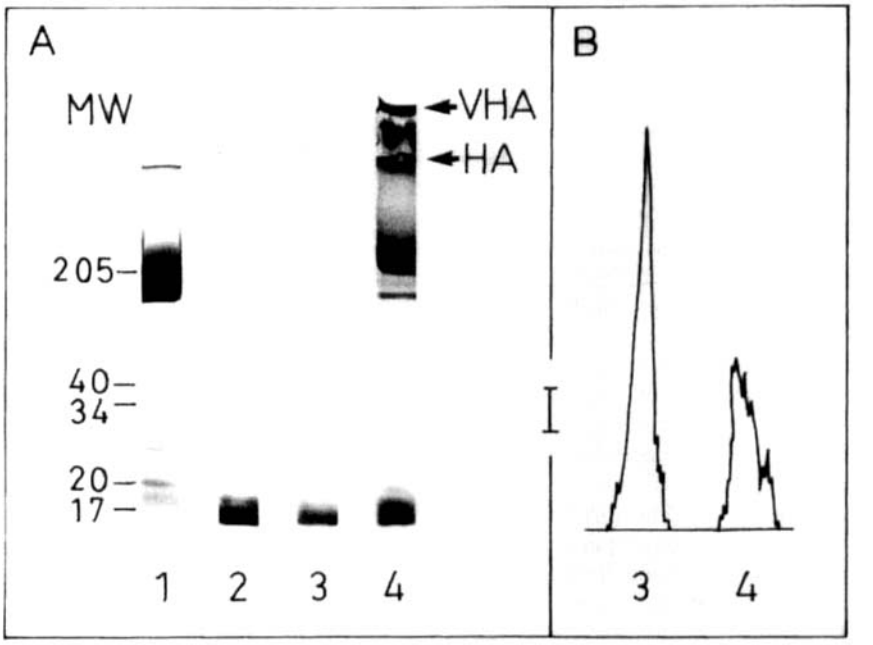

FIGURE 6 Myoglobin crosslinking by H_2O_2 in the presence and absence of myosin. A-Crosslinking reaction on double layer gel of 6% & 12% acrylamide. Concentrations: myoglobins - 30 μM; myosin-5 μM; H_2O_2-200 μM; incubation time - 30 min. 0.1 M pyrophosphate buffer, pH-9.5; Temp-37°C - 1. My alone. 2. Mb alone. 3. Mb with H_2O_2. 4. My Mb and H_2O_2. B-Light absorption scan of the marked, **I**, area in lanes 3 & 4. 3. The area of the Mb dimers in the reaction of Mb with H_2O_2. 4. The area of the Mb dimers in the reaction of My Mb and H_2O_2. The calculated ratio of the size of the absorption bands scanned: lane 3/lane 4 = 2.2.

Figure 6 for experimental details). In the presence of H_2O_2 alone (compare lanes 2 & 3 in Figure 6A), a new 34K band representing the 17 K globin dimer was formed. Since myoglobin dimerization is poor, reactions were performed at pH 9.5 to yield dimerization under our experimental conditions of limited H_2O_2 (note that at neutral pH of Figure 1, unlike results in reference 22, myoglobin completely failed to dimerize). In the presence of myosin, crosslinking products of the myosin heavy chains were formed (compare lanes 1 & 4 in Figure 6A), but the amount of WMb dimers was reduced (compare lanes 3 & 4 in Figure 6A). To quantitate the differences in the levels of Mb dimers in the presence and absence of myosin, the gel sections in lanes 3 & 4 were scanned in the 34 K bands (Figure 6B). The quantity of WMb dimers formed in the presence of myosin was reduced to about half the level formed without myosin. Thus, despite the lack of any effect by myosin on WMb ferryl state (Figure 5), the presence of myosin in the H_2O_2/Mb mixture had an impact on globin dimerization.

DISCUSSION

Our data clearly indicate that the action of myoglobin as a peroxidase differs from that of a typical enzyme like HRP. A hemoprotein concentration in the range expected for a specific enzymatic activity was actually employed only in peroxidation of dianisidine catalyzed by HRP (Table 1). The low reactivity of HRP for oxidation of N-acetyl tyrosine to bityrosines expressed by the higher HRP concentration required to yield the standard rate correlates with the documented low activity of HRP as a catalyzer of tyrosines peroxidation, especially L emanations used in the current study.[27,28] The lower activity of Mb compared to HRP as a mediator of dianisidine and tyrosine oxidation (Table 1) is in agreement with the well documented poor peroxidase activity of this hemoprotein.[5,6] That the gap in the concentrations of the two hemoproteins required to reach the same rate was narrowed from five to two orders of magnitude by replacing of dianisidine with N-acetyl tyrosine, indicates that tyrosines are relatively preferred substrates for Mb. Moreover, the lower activity of HRP on myosin tyrosines compared to the isolated amino acid is obvious in view of obstructed access of a large size substrate to the enzyme active site. The surprise was that Mb was a more efficient mediator of both myosin crosslinking and peroxidation (X10) than HRP (Figures 1 & 2). The correlation between bityrosine formation and crosslinking is attributed to the formation of inter-molecular bityrosines in the process of crosslinking.

Why is the relatively low Mb activity on free tyrosines compared to HRP reversed once these amino acids are anchored in a protein? It is clear by now that when peroxidases are activated by H_2O_2, two reducing equivalents are usually removed. In a classical heme peroxidase like HRP, an oxidized compound I complex is formed in which one of the two extra oxidizing equivalents is confined to the the ferryl iron center ($Fe^{+4}=O$ cation), and the second resides in the porphyrin macrocycle as a Π cation radical.[29] Although myoglobin/H_2O_2 interaction served as a model for the active center of heme peroxidases in earlier studies, it is clear by now that the reaction of myoglobin with H_2O_2 differs from that of HRP. In some peroxidases the charge equivalents are not confined to the prosthetic group, but exist as a protein centered stable radical. Pyruvate formate-lyase and ribonucleotide reductase, which contain iron as a part of their reaction mechanism, are examples of such enzymes.[30,31] Transfer of unpaired electrons has long been known in basic processes involving hemoproteins, such as mitochondrial or chloroplasts functions. Cytochrome c oxidase is a well characterized heme peroxidase shown to have a stable transient protein radical as part of its peroxidative mechanism.[32] Because a protein radical is formed in the Mb/H_2O_2 reaction, myoglobin might be related to the latter group of peroxidases.[33] Unlike the above-mentioned peroxidases, peroxidised myoglobin has in addition to an unstable heme, a fast dissipating globin radical.[4] Mb tyrosine radicals were shown to dissipate in several ways: via heme-protein crosslinking, reaction with molecular oxygen, and formation of bityrosines.[9,22,34] Although not all the amino acids involved in the globin radical are defined, it is clear from available studies that the globin radical is mainly tyrosine centered.[35] The nature of the tyrosine radicals is controversial, with some groups suggesting peroxyl radicals and other carbon centered radicals.[36,33] The latter view is supported by the appearance of bityrosines, which can be formed only by transient carbon centered tyrosine radicals.[19] Tyr-103, which is close to the active heme center and present in all myoglobins, has been identified as the primary

locus of the globin free radical.[22] However, in WMb which has an additional tyrosine in position 151, the globin radical resides in tyr-151 as well, due to intra-molecular electron transfer between the two tyrosines.[22]

As stated, the low reactivity of HRP as a peroxidizer and crosslinker of myosin is clear in view of the steric hindrance that renders the electron transport from myosin to the heme active center inefficient. Being a much weaker peroxidase, Mb heme active center should be completely incapable of accepting electrons from the myosin substrate. This expectation is fulfilled, as reflected by the inability of myosin to quench Mb ferryl (Figure 5). Thus, crosslinking of myosin under these conditions (insert to Figure 5) must be affected by an alternative route, which can only be the transfer of electrons from myosin surface tyrosines to globin tyrosines. The efficiency of the globin radical as an oxidizer of myosin is even more pronounced in light of the fast distraction of its' source – the Mb heme moiety.[20] The conclusion of an efficient electron transport from myosin to globin is also supported by the ability of myosin to interfere with the self-coupling of globin radicals to dimers (Figure 6).

That HMb triggers myosin peroxidative crosslinking (Figure 4), albeit less efficiently than WMb, points to the ability of electrons to transfer from myosin to both tyr-103 and tyr-151 in myoglobin. Thus, unlike Mb where only intra-molecular electron transport takes place,[22] our results indicate that myosin surface tyrosines are more efficient as electron donors to the short-lived myoglobin radical. This conclusion gains support from our findings that Mb dimers completely failed to form at neutral pH under conditions of massive myosin crosslinking (Figure 1), and elevating the pH resulted in only minor dimerization of myoglobin.

The cascade of reactions that lead to Mb induced myosin crosslinking may be summarized as follows:

1) $Mb + H_2O_2 \rightarrow Mb(Ferryl\cdot) + Mb(tyr\cdot)^*$

2) $Mb(tyr\cdot) + My(tyr) \rightarrow My(tyr\cdot) + Mb$

3) $nMy(tyr\cdot) \rightarrow My(tyr)n$

$\cdot$ stands for any oxidative equivalent.

*Mb(tyr·) relates to both 151 and 103 since, as stated, myosin transfers electrons to both radicals.

Finally, this study demonstrates that a globin-assisted peroxidase activity is the key to the efficiency of myoglobin as a mediator of protein peroxidation and crosslinking. Since myoglobin and myosin share residence in muscle cells, under peroxidative conditions which follow ischemia,[37] myoglobin may turn from a physiological oxygen transporter to a trigger of pathological events.

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