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Protein Radicals in the Reaction Between H_2O_2-activated Metmyoglobin and Bovine Serum Albumin

HENRIK ØSTDAL*, STINA G. SØGAARD, EMØKE BENDIXEN and HENRIK J. ANDERSEN

Department of Animal Product Quality, Danish Institute of Agricultural Sciences, Research Center Foulum, P.O. Box 50, Tjele DK-8830, Denmark

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Hydrogen peroxide activation of MMb with and without the presence of BSA gave rise to rapid formation of hyper-valent myoglobin species, myoglobin ferryl radical (·MbFe(IV)=O) and/or ferrylmyoglobin (MbFe(IV)=O). Reduction of MbFe(IV)=O showed first-order kinetics for a 1–2 times stoichiometric excess of H_2O_2 to MMb while a 3–10 times stoichiometric excess of H_2O_2 resulted in a biphasic reaction pattern. Radical species formed in the reaction between MMb, H_2O_2 and BSA were influenced by [H_2O_2] as measured by electron spin resonance (ESR) spectroscopy and resulted in the formation of cross-linking between BSA and myoglobin which was confirmed by SDS-PAGE and subsequent amino acid sequencing. Moreover, dityrosine was formed in the initial phases of the reaction for all concentrations of H_2O_2. However, initially formed dityrosine was subsequently utilized in reactions employing stoichiometric excess of H_2O_2 to MMb. The observed breakdown of dityrosine was ascribed to additional radical species formed from the interaction between H_2O_2 and the hyper-valent iron-center of H_2O_2-activated MMb.

Keywords: Myoglobin; Ferrylmyoglobin; Myoglobin ferryl radical; Protein radicals; Bovine serum albumin; ESR; dityrosine; protein cross-linking

INTRODUCTION

Myoglobin acts as an oxygen storage molecule in muscles, but this protein is also known to perform peroxidase-like activity in the presence of H_2O_2 or other simple peroxides.[1] H_2O_2 is continuously formed *in vivo*, and it has been shown that a wide variety of biomolecules

*Corresponding author. Tel.: +45-89-99-12-48. Fax: +45-89-99-15-64. E-mail: henrik.ostdal@agrsci.dk

including lipids, low-molecular antioxidants and proteins are oxidized by H_2O_2-activated myoglobin.[2–4] Consequently, H_2O_2-activated myoglobin has been proposed to be a potential initiator of oxidative reactions in biological systems.

The reaction between metmyoglobin, MMb and H_2O_2 is believed to proceed through the formation of myoglobin ferryl porphyrin cation radical ($\cdot Por^+$–Fe(IV)=O-globin) which in a subsequent electron transfer process gives rise to myoglobin ferryl radical,[5,6] ·MbFe(IV)=O, a short-lived protein radical (half-life approximately 20 s[7–9]) with iron in the hyper-valent +4 state. Decay of the protein radical by an electron transfer process generates the more long-lived ferrylmyoglobin, MbFe(IV)=O.[5,7,10] The nature of the protein radical in myoglobin ferryl radical is not completely known. However, tryptophan and tyrosine have been suggested to be likely candidates according to electron spin resonance (ESR) spectroscopy studies.[8,11–13]

The reaction between H_2O_2 and metmyoglobin is not an equimolar reaction as 1.4–2-fold excess of H_2O_2 is required for complete conversion of MMb to hyper-valent myoglobin species.[5,14,15] This can be explained by the interaction between myoglobin ferryl porphyrin cation radical and H_2O_2, ($\cdot Por^+$ – Fe(IV) = O–globin + H_2O_2 → MMb + 0_2).[6] In addition, the reformation of MMb through reduction of ferrylmyoglobin makes it possible for MMb to consume H_2O_2 in a cyclic reaction and thus to act as a catalase.[15] However, irreversible modification of MMb will occur after a limited number of cycles, and MMb is often referred to as a pseudo-peroxidase.[1]

Recently, it has been shown that the protein radical in ·MbFe(IV)=O can be transferred onto other proteins inducing the formation of "new" radical species with an increased half-life.[16]

This reaction is believed to proceed through a protein–protein radical transfer with the tyrosine residues on the receiving proteins playing a vital role.[11,17] The role of tyrosine is further stressed by the formation of dityrosine in the reaction between MMb, H_2O_2 and BSA.[16] The objective of the present study was to investigate the reaction between H_2O_2-activated myoglobin and BSA using excess concentrations of H_2O_2. We propose that "new" radical species are formed when the concentration of H_2O_2 is in excess compared to that of MMb due to interaction between H_2O_2 and the hyper-valent iron-center in H_2O_2-activated myoglobin.

MATERIALS AND METHODS

Chemicals

Equine metmyoglobin (MMb), bovine serum albumin (BSA) and bovine catalase (crystallized twice) were obtained from Sigma (St Louis, MO), and H_2O_2 was purchased from Merck (Damstadt, Germany). All other chemicals were analytical grade, and double deionized water (Elgastat Maxima, Elga Ltd, Bucks, UK) was used throughout. All buffers and protein solutions were passed through a chelating resin column (Chelex-100, Sigma) in order to remove any free metal ions.

Reaction Conditions

The reactions between BSA, MMb and a 1–10 times stoichiometric excess of H_2O_2 were performed in 50 mM phosphate buffer (pH 7.4; adjusted to $I = 0.16$ with NaCl) at 25°C. MMb was dissolved in 50 mM phosphate buffer (pH 7.4; adjusted to $I = 0.16$ with NaCl) and purified on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden) equilibrated and eluted with the same buffer. The concentrations of MMb (ε_{525}=7700 l mol^{-1} cm^{-1})[18] and H_2O_2 (ε_{240} = 39.4 l mol^{-1} cm^{-1})[19] were determined spectrophotometrically.

ESR Measurements of the Reaction Between MMb, BSA and H_2O_2

Samples of 500 μl were withdrawn at different time intervals from a typical reaction mixture (0.1 mM MMb, 0.2 mM BSA and 0.1–1.0 mM H_2O_2). The samples were transferred to an ESR flat cell (Wilmad Labglass, Buena, NJ) and mounted in the cavity (ER4119HS) of a Bruker EMX X-band ESR spectrometer (Bruker Analytische Messtechnik, Rheinstetten, Germany). The ESR measurements were conducted at room temperature. The magnetic field was modulated with a frequency of 100 kHz using 15 G field modulation amplitude, 20 mW microwave power, a receiver gain of 2×10^5, a sweep time of 21 s (2.5 ms time constant) and spectra were accumulated through two scans. ESR signal intensity was obtained by double integration of the signal using the routine held within the WINEPR software (Bruker Analytische Messtechnik, Rheinstetten, Germany).

Kinetics of the Reaction Between MMb, BSA and H_2O_2

The reaction between 0.1 mM MMb and 0.1–1.0 mM H_2O_2 with and without the presence of 0.2 mM BSA was followed at 25°C by measuring spectral changes ($450 < \lambda < 700$ nm) using a HP-8453 UV–Vis diode array spectrophotometer equipped with a temperature controlled cuvette compartment (Hewlett Packard Co., Palo Alto, CA). The MMb solution was thermally equilibrated in a quartz cuvette and the reaction was initiated by addition of H_2O_2.

Quantification of Dityrosine in the Reaction Between MMb, BSA and H_2O_2

Samples of 1 ml were withdrawn at different time-intervals from a typical reaction mixture (0.1 mM MMb and 0.1–1.0 mM H_2O_2 with and without the presence of 0.2 mM BSA) and mixed with 1 ml of 12 M HCl. When using ten times excess of H_2O_2 to MMb, withdrawn samples were mixed with 20 μl diluted catalase solution (approximately 7000 units, Sigma, St Louis, MO) and allowed to react for 2 min before stopping the reaction by adding 1 ml of 12 M HCl. The purpose of this was to prevent H_2O_2-induced decreases in the concentrations of dityrosine during the acid hydrolysis which only was observed at this [H_2O_2]. The acidified samples were flushed with N_2, hydrolyzed over night (105°C) and neutralized with 6 M NaOH. Samples were weighed before and after the hydrolysis to compensate for evaporation during this step. The concentration of dityrosine was determined by HPLC (Varian 9012 HPLC pump and a Varian 9100 Auto sampler equipped with a Varian 9075 Fluorescence detector (Ex: 275 nm; Em: 410 nm) (Varian Chromatographic Systems, Walnut Creek, CA, USA)). Twenty microliters of the hydrolyzed sample was loaded onto a C-18 column (Nucleosil 120-5, 250 × 4 mm, Macherey-Nagel Duren, Düren, Germany), using an isocratic flow of 1 ml min^{-1} (4% acetonitrile in aqueous 0.1 M citric acid (pH 2.55)) as described by Daneshvar *et al.*[20] Dityrosine was quantified using a standard curve based on a dityrosine standard prepared according to Nomura *et al.*[21]

SDS-PAGE and Amino Acid Sequencing of Cross-linked Protein in the Reaction Between MMb, BSA and H_2O_2

Samples of 500 μl were withdrawn after 30 min of reaction from a typical reaction mixture (0.1 mM MMb and 0.1–1.0 mM H_2O_2 with and without the presence of 0.2 mM BSA) and mixed with 500 μl SDS sample buffer (10 mM Tris-HCl, 2% SDS, 20% glycerol, pH 6.8). Proteins were analyzed on a 10% SDS-PAGE according to the procedure described by Laemmli.[22] Cross-linking between BSA and myoglobin was observed as a novel protein band in the Coomassie stained gel. Identical, but unstained SDS-PAGE was electroblotted onto a PVDF

(polyvinyldiene difluoride) ProBlott™ membrane (Applied Biosystems, Foster City, CA, USA).[23] The novel protein band was identified by Edman sequencing of the first three amino acids using an Applied Biosystems-477A sequencer (Applied Biosystems, Foster City, CA, USA).

RESULTS

Formation of protein radicals in the reaction between MMb, H_2O_2 and BSA was followed by ESR spectroscopy. Increased excess of H_2O_2 lowered the amount of ESR detectable radicals in the reaction between H_2O_2-activated metmyoglobin and BSA while the length of the period in which radicals could be detected was positively correlated to the concentration of H_2O_2 as illustrated in Fig. 1.

The reduction of ferrylmyoglobin to MMb was followed spectrophotometrically (588 nm) as shown in Fig. 2. The results showed a rapid formation of ferrylmyoglobin, but total conversion of MMb was only observed using stoichiometric excess of H_2O_2 as described previously.[5,15] This conversion was followed by a slow autoreduction of ferrylmyoglobin to metmyoglobin. The autoreduction of ferrylmyoglobin for the experiments using 1 or 2 equiv. of H_2O_2 appeared to follow first-order kinetics, while 3–10 equiv. of H_2O_2 resulted in a biphasic reaction pattern. The initial phase of the biphasic reaction pattern was found to increase with increasing $[H_2O_2]$ and to decrease by the presence of BSA, as illustrated in Table I. In addition, the presence of BSA showed a tendency to increase the rate of ferrylmyoglobin autoreduction slightly (data not shown).

The formation of dityrosine in the reaction between MMb/H_2O_2 or MMb/H_2O_2/BSA is shown in Fig. 3. Rapid formation of dityrosine occurred during the initial phase of a 1:1 stoichiometric reaction between MMb and H_2O_2. Subsequently, a small decrease in the dityrosine concentration was observed until a steady level was reached. Likewise, the use of 2, 5 and 10 times stoichiometric excess of H_2O_2 to MMb resulted in rapid dityrosine formation followed by a more noticeable decrease in the amount of dityrosine with increasing concentrations of H_2O_2. Moreover, a subsequent reformation of dityrosine was observed in experiments using 5 or 10 times stoichiometric excess of H_2O_2 to MMb. Similar patterns were observed when BSA was included in the reaction mixtures, but the maximum dityrosine contents were approximately five times higher. The highest obtained concentration of dityrosine corresponds to a situation were approximately

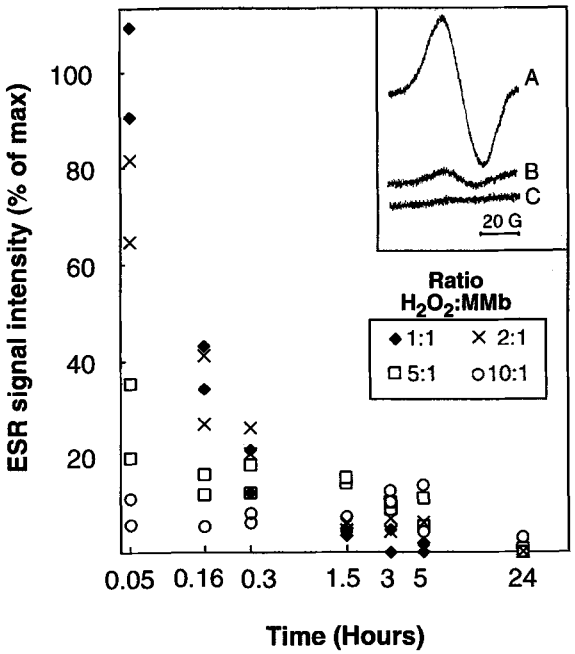


FIGURE 1 Effect of H_2O_2 concentration and reaction time on ESR signal intensity in the reaction between 0.1 mM MMb, 0.1–1.0 mM H_2O_2 and 0.2 mM BSA in 50 mM phosphate buffer (pH 7.4; $I = 0.16$ (NaCl)) at 25°C. ESR signal intensities are corrected for the ESR signal intensity measured for the interaction between MbFe(IV)=O and BSA at specific time points (MbFe(IV)=O was formed in the reaction between 0.1 mM MMb and 0.1 mM H_2O_2 using a 10 min interval before BSA addition). Inset: typical ESR signals obtained 3 min after initiation of the reaction: (A) 0.1 mM MMb, 0.1 mM H_2O_2 and 0.2 mM BSA; (B) 0.1 mM MMb, 0.1 mM H_2O_2 and 0.2 mM BSA (added 10 min after H_2O_2-activation of MMb); (C) 0.1 mM MMb and 0.2 mM BSA. Each data point represents one independent measurement. Spectrometer settings are described in "Materials and methods".

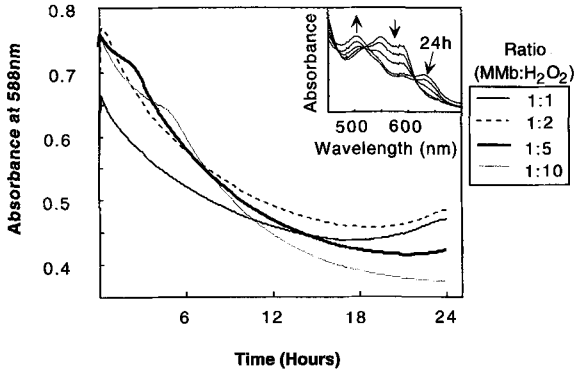


FIGURE 2 Formation and reduction of ferrylmyoglobin followed as absorbance changes at 588 nm during H_2O_2-activation of 0.1 mM MMb using a 1, 2, 5 or 10 times stoichiometric excess of H_2O_2 in the presence of 0.2 mM BSA. Inset: spectra obtained for the reaction using 0.1 mM MMb, 0.2 mM BSA and 0.1 mM H_2O_2 in 50 mM phosphate buffer (pH 7.4; $I = 0.16$ (NaCl)) at 25°C after 10, 30 min, 1.7, 5.8 and 24 h of reaction.

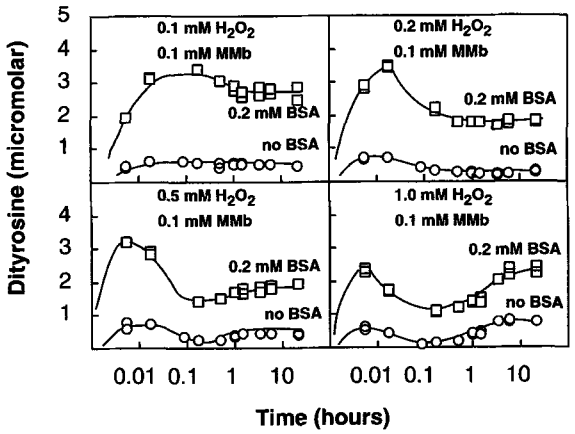
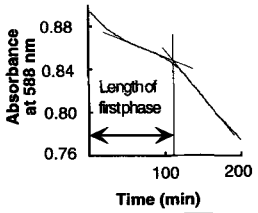


FIGURE 3 Concentration of dityrosine in relation to reaction time for the reaction between 0.1 mM MMb and 0.1, 0.2, 0.5 or 1.0 mM H_2O_2 in the presence (open box) or absence (open circle) of 0.2 mM BSA. Reactions were performed in 50 mM phosphate buffer (pH 7.4; $I = 0.16$ (NaCl)) at 25°C. Samples were withdrawn from the reaction mixture at different time-intervals and mixed with equal amounts of 12 M HCl. The dityrosine content was determined after acid hydrolysis using HPLC-separation and fluorescence detection. Each data point represents one independent measurement.

3–6% (dityrosine can be formed through either the reaction between two tyrosyl radicals or the addition of one tyrosyl radical to a tyrosine residue followed by hydrogen abstraction)[24] of the oxidation equivalents are used in dityrosine formation. Increasing concentrations of BSA increased the concentration of dityrosine formed by a maximum of approximately 25%, which corresponds to 4–8% of the oxidation equivalents in H_2O_2-activated myoglobin are used in dityrosine formation (data not shown). This indicates that other oxidation products are formed, and such oxidation products are currently under investigation in our laboratory. The dityrosine formed originates from the interaction between MMb and H_2O_2 since this protein oxidation product was non-detectable in

TABLE I Length of first phase (min) observed as absorbance changes at 588 nm in the reaction between 0.1 mM MMb and 0.3–1.0 mM H_2O_2 in 50 mM phosphate buffer (pH 7.4; $I = 0.16$ (NaCl)). The length of the first phase was calculated as the intercept between two straight lines fitted to the lowest slope of the first phase and to the highest slope of the second phase, respectively. All values are given as mean ± S.D. of three independent determinations. The presence of BSA was found to significantly decrease the length of first phase using student's *t*-test

	Molecular ratio (H_2O_2:MMb)	Length of first phase (min)	
		With BSA	Without BSA
	3:1	39.2 ± 1.6	41.9 ± 0.3*
	4:1	56.2 ± 4.2	63.5 ± 1.4*
	5:1	75.1 ± 0.4	82.4 ± 5.3
	10:1	142.8 ± 2.7	162.0 ± 0.9†

*$p<0.05$.
†$p<0.01$.

MMb and less than 5 pmol mg^{-1} protein was present in the commercial BSA used (corresponds to less than 0.8% of the dityrosine formed in a typical reaction mixture). Furthermore, the formation of dityrosine was not caused by iron released from myoglobin with subsequent risk of Fenton-induced dityrosine formation as incubation of 0.2 mM BSA, 1.0 mM H_2O_2 and 0.1 mM Fe(II) or Fe(III) produced less than 0.12 μM dityrosine (corresponds to less than 6% of the dityrosine formed in a typical reaction mixture) during a 24 h incubation using the same experimental set-up as illustrated in Fig. 3.

The subsequent decrease in the concentration of dityrosine observed with increasing [H_2O_2] was further investigated by addition of twofold stoichiometric excess of H_2O_2 to a typical reaction mixture (0.1 mM MMb and 0.2 mM BSA). H_2O_2 was added either in one step (0.2 mM) or half the amount twice (2 × 0.1 mM) with a 10 min interval between the additions of H_2O_2. Addition of H_2O_2 in one step represents reaction between excess H_2O_2 and ·MbFe(IV)=O and/or MbFe(IV)=O in the presence of BSA, while the experiment using a 10 min interval between H_2O_2-additions represents reaction between excess H_2O_2 and MbFe(IV)=O in the presence of BSA. The results showed that in the presence of BSA, excess H_2O_2 and either ·MbFe(IV)=O or MbFe(IV)=O will cause a breakdown of initially formed dityrosine (see Fig. 4). This breakdown was not caused by the fact that the dityrosine concentration had reached an upper limit. Triple activation of 0.1 mM MMb with H_2O_2 (3 × 0.1 mM) in the presence of BSA using a 24 h interval between each addition resulted in additional dityrosine formation after each H_2O_2-addition as illustrated in Fig. 5.

SDS-PAGE revealed that an additional band was formed during the reaction of H_2O_2-activated myoglobin and BSA using stoichiometric concentrations of MMb and H_2O_2 (see Fig. 6). The molecular weight of this protein band was higher than BSA and final characterization

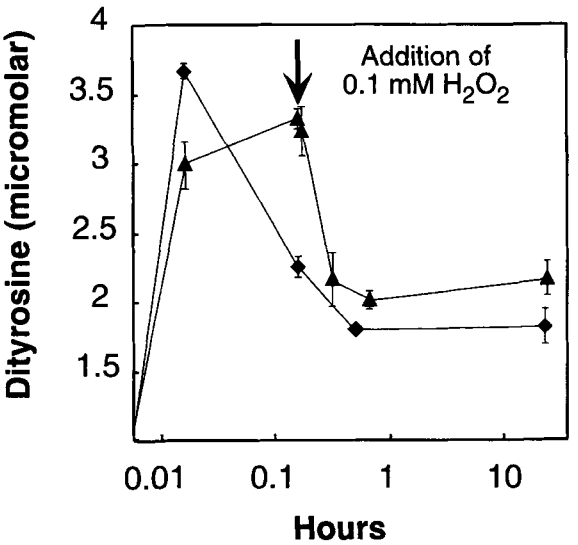


FIGURE 4 Concentration of dityrosine in relation to reaction time for the reaction between 0.1 mM MMb, 0.2 mM BSA and 0.2 mM H_2O_2. H_2O_2 was added either in one step (0.2 mM; filled diamond) or in two steps (2 × 0.1 mM; filled triangle) using a 10 min interval between the additions (second addition indicated by arrow). Reactions were performed in 50 mM phosphate buffer (pH 7.4; I = 0.16 (NaCl)) at 25°C. All data represent the mean±S.D. of three independent determinations.

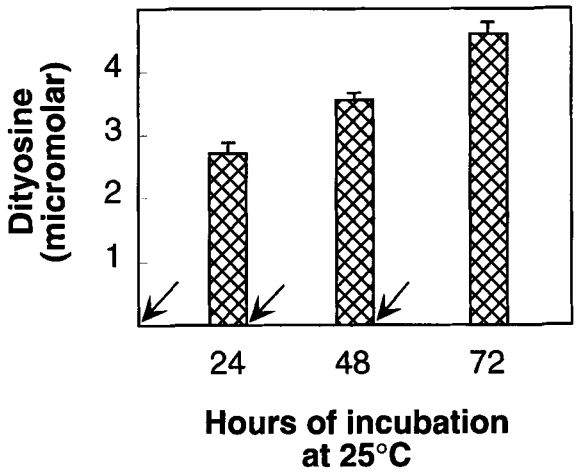


FIGURE 5 Concentration of dityrosine after 24, 48 and 72 h for the reaction between 0.1 mM MMb, 0.2 mM BSA and 0.3 mM H_2O_2. Hydrogen peroxide was added in three steps (3 × 0.1 mM) using a 24 h interval between each addition. Arrows indicate time at which H_2O_2 was added. Reactions were performed in 50 mM phosphate buffer (pH 7.4; I = 0.16 (NaCl)) at 25°C. All data represent the mean±S.D. of three independent determinations.

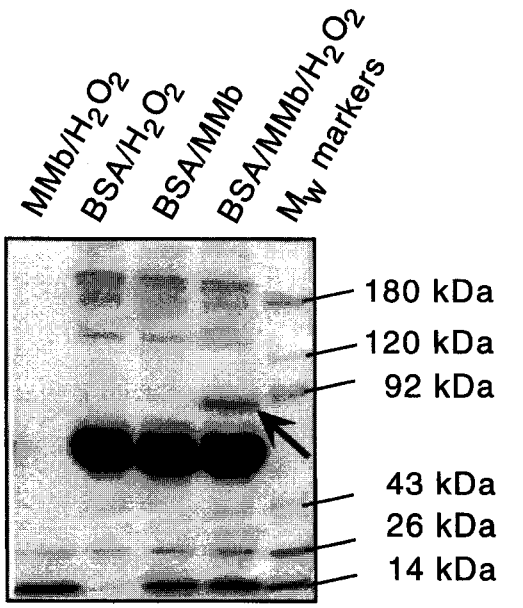


FIGURE 6 Detection of additional protein band by the use of SDS-PAGE. Arrow indicates dimer between BSA and MMb.

was performed by terminal amino acid sequencing showing a 1:1 cross-link between myoglobin and BSA (one to one molar ratio of Gly-Leu-Ser- (myoglobin) and Asp-Thr-His- (BSA)).

DISCUSSION

The results clearly show that the presence of excess H_2O_2 in the present reaction (0.1 mM MMb; 0.2 mM BSA; 0–1.0 mM H_2O_2) affected the subsequent reaction between H_2O_2-activated myoglobin and BSA. The 1:1 stoichiometric reaction between MMb and H_2O_2 in the presence of BSA resulted in radical species with a relatively long half-life and dityrosine formation as previously described.[16] The exact effect of excess H_2O_2 on the present reaction is unknown, but the simplicity of our system facilitates the excess of H_2O_2 to react further with either H_2O_2-activated myoglobin or the radicals formed on BSA.

Interactions between H_2O_2-activated myoglobin and H_2O_2 has been described previously[3,5,8] and corresponds well with the spectrophotometrical data obtained with 3–10 times stoichiometric excess of H_2O_2 to MMb (see Fig. 2 and Table I) showing that the reduction of MbFe(IV)=O to MMb proceeds in two phases. We propose that the first phase continue as long as H_2O_2 is in excess, and the second phase starts when H_2O_2 is depleted, as illustrated in Fig. 2. This second phase represents the reduction of MbFe(IV)=O to MMb. The assumption that [H_2O_2] influence the duration of the first phase was verified as the addition of catalase during the first phase initiated almost instantaneously the second phase (data not shown). Despite excess of H_2O_2 there is still a net formation of MMb during the first phase of the autoreduction of MbFe(IV)=O, as seen by the reduction in absorbance at 588 nm (Fig. 2). This net formation of MMb indicates either the fact that the cyclic reaction between H_2O_2-activated myoglobin and H_2O_2 does not preserve myoglobin in its hypervalent states, or an ongoing protein denaturation at these high H_2O_2/MMb ratios. The final increase in absorbance at low H_2O_2 concentrations can be ascribed to oxidative modifications of the protein structure leading to minor formation of hemichrome.

Further reaction between H_2O_2 and the radical formed on BSA is difficult to predict from the present data, especially, as the formation of protein radicals on BSA and dityrosine production possibly originates from two different reaction pathways. This assumption is based on studies using lactoperoxidase as the heme protein and BSA, β-lactoglobulin or casein as the radical receiving protein.[25] Excess H_2O_2 in the present reaction decreased the amount of ESR detectable radicals, indicating interactions between the BSA radical and H_2O_2. However, the decrease in the concentration of dityrosine using excess H_2O_2 is believed to be related to interactions between the iron-center in H_2O_2-activated MMb and excess H_2O_2 as opposed to the interaction between the BSA radical and H_2O_2 (see below). The interaction between the

BSA radical and H_2O_2 is currently under investigation in our laboratory.

Dityrosine formation during H_2O_2-activation of equine MMb is surprising since it has previously been reported that dityrosine is only formed in sperm whale myoglobin, which contains an additional tyrosine residue (Tyr-151).[26,27] Dityrosine formation proceeds *via* tyrosine radicals[28] and therefore the detection of dityrosine gives unambiguous evidence for the presence of unpaired electrons on the tyrosine residues during H_2O_2-activation of equine MMb. The fact that the observed increase in the dityrosine concentration mainly occurred during the initial phase of a 1:1 stoichiometric reaction between MMb and H_2O_2 in the presence and absence of BSA indicates that the transient protein radical formed on ·MbFe(IV)=O is responsible for the appearance of dityrosine. Participation of the hyper-valent iron-center (MbFe(IV)=O) in the formation of dityrosine was tested in a typical reaction mixture (0.1 mM MMb; 0.1 mM H_2O_2; 0.2 mM BSA) where BSA was added 10 min after H_2O_2-activation of MMb. Based on these results we estimated that 12% of the dityrosine formed in a typical reaction mixture originates from interactions between the hyper-valent iron-center and BSA. The increased concentration of dityrosine formed in the presence of BSA indicates that this protein oxidation product is formed in BSA and/or may be responsible for the observed cross-link between myoglobin and BSA. SDS-PAGE of reaction mixtures employing 5 and 10 times stoichiometric excess of H_2O_2 to MMb in the presence of BSA showed, after 30 min of reaction, a decline in the intensity of the band representing the BSA–myoglobin dimer (data not shown). This indicates that dityrosine is the cross-linking species as the concentration of dityrosine likewise was lowered at this time point as illustrated in Fig. 3.

An increase in the stoichiometric excess of H_2O_2 to MMb showed that dityrosine was not a stable end-product in the present reaction. Subsequent decrease in the dityrosine concentrations observed at molar excess of H_2O_2 to MMb may be explained by further oxidation of dityrosine, similar to what has been described for myeloperoxidase.[29] The higher degree of conjugation in the dityrosine structure compared to that of tyrosine may lead to a very labile structure in relation to the electron transfer processes and thus dityrosine could be more easily oxidized than tyrosine. However, dityrosine oxidation rather than formation of additional dityrosine in the presence of excess H_2O_2 seems surprising as the dityrosine formed accounts for approximately 0.3–0.7% of the number of tyrosine residues present in the reaction mixture.

By comparing the biphasic reaction pattern and the concentrations of dityrosine (5 and 10 times excess, Table I and Fig. 3) it can be seen that the length of the initial phase is comparable to the time span needed before the dityrosine concentration reaches a steady level. This leads to the supposition that the oxidative species reactive towards dityrosine should originate from interactions between the hyper-valent iron-center in H_2O_2-activated myoglobin and H_2O_2. This assumption is based upon the fact that the same degree of dityrosine breakdown was observed regardless whether H_2O_2 could interact with both ·MbFe(IV)=O and MbFe(IV)=O or only with MbFe(IV)=O (Fig. 4) indicating that the protein radical does not contribute significantly to this event. The fact that dityrosine accumulates upon repeating H_2O_2-activation of MMb in a setup that minimizes interaction between H_2O_2 and hyper-valent myoglobin species (24 h interval between stoichiometric H_2O_2-activation of MMb) supports the importance of the hyper-valent iron-center in oxidative breakdown of dityrosine in the presence of excess H_2O_2. These results indicate that in the presence of excess H_2O_2 MMb is not merely cycling between MMb, ·MbFe(IV)=O and MbFe(IV)=O, but novel additional radical species are formed from the interactions between the hyper-valent iron-

center and H_2O_2-radical species with different affinity towards, e.g. dityrosine compared with the radical species formed in the classical peroxidase-cycle of H_2O_2-activated MMb.

The mechanism behind the second increase in the dityrosine concentration using 5 or 10 times stoichiometric excess of H_2O_2 to MMb in the presence or absence of BSA is not known at present. However, this aspect could be explained by modifications of myoglobin during repeating cycles of oxidation and reduction. Modifications which may alter both the surroundings of the iron-center and the tertiary structure of the globin backbone resulting in an altered reaction patterns.

In conclusion, the reaction between MMb and H_2O_2 is influenced by the molar ratios between MMb and H_2O_2. Stoichiometric excess of H_2O_2 will produce novel radical species-an important factor to include when considering the pro-oxidative effects of myoglobin in biological systems.

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