EVIDENCE FOR THE GENERATION OF EXCITED METHYLGLYOXAL IN THE MYOGLOBIN CATALYZED OXIDATION OF ACETOACETATE

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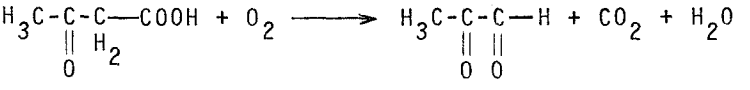
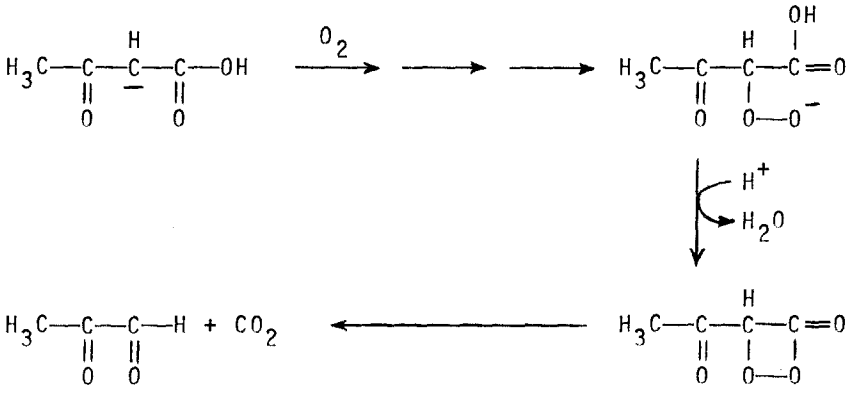
SUMMARY: At acidic pHs the myoglobin catalyzed oxidation of acetoacetate results in the concomitant destruction of the hemeprotein. The spectral changes are similar to those observed on irradiating myoglobin alone at 270 nm. The intermediacy of the species $^1O_2(^1\Delta g)$, $O_2^-/HO_2^\cdot$ and $OH^\cdot$ was excluded. Methylglyoxal sensitizes the photodestruction of myoglobin. It appears that methylglyoxal may be generated in an electronically excited state.

Work from this laboratory (1-3) indicated that there are dark biochemical systems which may be mechanistically similar to bioluminescent processes. In such dark systems, various paths other than radiative emission would be open to the electronic energy. One such system is the myoglobin catalyzed conversion of acetoacetate or derivatives into methylglyoxal (4):



Thus, the substrate has the potentiality of a luciferin, inasmuch as it can easily lose a methylene proton and has a good leaving group in a properly located position (5):



Orbital symmetry considerations (5) suggest that methylglyoxal may be formed in an electronically excited state as a result of the dioxetane ring cleavage.

We report here that at acidic pHs, as a result of the reaction, myoglobin is altered, the spectral changes being similar to those observed by irradiating myoglobin with 270 nm light, either alone or in presence of methylglyoxal.

Materials and Methods

Whale myoglobin and methylglyoxal were obtained from Sigma Chemical Company. Deuterium oxide (99.7%) was from BDH Chemical Ltd.

Sodium acetoacetate was prepared by the method of Krebs and Eggleston (6) as described by Milligan and Baldwin (4). Superoxide dismutase was obtained as reported by McCord and Fridovich (7).

The reactions were run in a Warburg apparatus at 25°C, the buffer being sodium acetate, 0.14M. The concentrations were: myoglobin, 6.0×10^{-5}M; sodium acetoacetate, 1.5×10^{-3}M and $MnCl_2$, 8.5×10^{-3}M. Potassium hydroxide was present in the central well.

Spectra were taken on a Zeiss DMR-21 Recording Spectrophotometer using 1 cm path cells.

Myoglobin was irradiated with 270 nm light with a 150 watt xenon lamp at 90 cm distance.

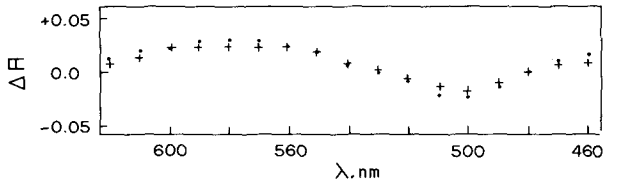


Figure 1. Differential spectra of myoglobin at pH 5.3 relatively to a freshly prepared solution: (+++++) after 4.25 hours irradiation with 270 nm light; (•••••) after catalyzing the conversion of acetoacetate into methylglyoxal.

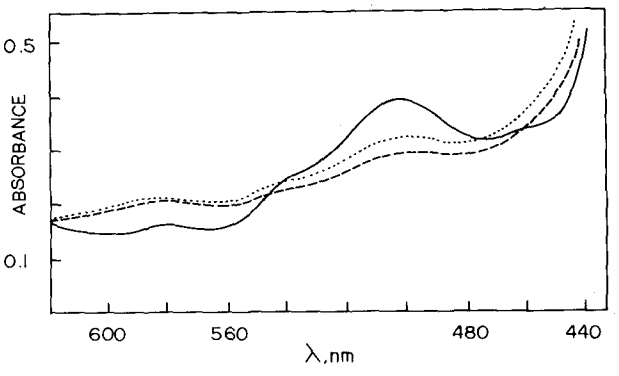


Figure 2. The spectrum of myoglobin before (———) and after (– – – – –) 4.25 hours irradiation with 270 nm light at pH 4.6. The curve (·····) represents the spectrum of the final reaction mixture, that is, after the myoglobin catalyzed conversion of acetoacetate into methylglyoxal. For comparative purposes the spectrum of the irradiated solution is lowered by 0.030 in the absorbance scale.

Results and Discussion

As a consequence of the reaction which catalyzes the conversion of acetoacetate into methylglyoxal myoglobin becomes altered spectrally. The alteration increases markedly on running the reaction at lower pHs, that is, going from pH 5.2 to pH 4.6 and down to pH

4.2. This spectral effect almost matches that obtained by irradiating myoglobin alone with ultraviolet light (Figs. 1 and 2).

To ascertain if the destruction of myoglobin is due to the hydroxyl radical, $OH^{\cdot}$, the conversion of acetoacetate was also studied in the presence of 5×10^{-3}M benzoate (8) as well as in the presence of 0.01M formate (9); however no protection whatsoever was observed. Nor was the myoglobin destruction due to the superoxide ion or perhydroxyl radical, because 3.3×10^{-7}M superoxide dismutase (7) did not protect the hemeprotein. Actually the participation of the $O_2^-/HO_2^{\cdot}$ species is also excluded in the destruction of myoglobin brought about by 270 nm light. Thus the spectral alterations, at least during the first two hours, were the same in the presence or absence of oxygen; moreover the alterations were essentially similar at pH 5.2 and 4.2, that is at both sides of $pK_{HO_2^{\cdot}}$ which is 4.8 (9).

From these facts the possibility arises that methylglyoxal is generated in an electronically excited state and then transfers its energy to or reacts with myoglobin. Consistent with this view, when the reaction was run at pH 5.2 and the final reaction mixture brought and kept at 4.6, the spectral changes were still those observed at pH 5.2. This clearly indicates that the destruction of myoglobin is concomitant with the reaction which is taking place. One possibility is the formation of an excited product, another -less likely in the present case- is the involvement of radical intermediates.

One variation would be the transfer of electronic energy to oxygen with formation of $^1\Delta g$ singlet oxygen which would then attack myoglobin. This can be ruled out however because the same spectral differences were observed in H_2O and D_2O buffer, despite the fact that the lifetime of $^1O_2$ is extended by one order of magnitude in deuterium oxide (10). Not even in the alteration of myoglobin produced by radiation should $^1O_2$ be involved. Thus, the

alteration is the same in the presence or absence of O_2, whether in H_2O or D_2O; moreover visible light was practically ineffective in altering myoglobin.

In accord with the hypothesis that methylglyoxal is formed in an excited state and then transfers its energy to myoglobin, we found that methylglyoxal sensitizes the photochemical destruction of myoglobin.

As a result of its destruction, myoglobin loses its catalytic activity, whereby the reactions at lower pHs are not quantitative. From the fact that at pH 4.2 the reaction is only half complete; from the concentrations of myoglobin and acetoacetate employed; and assuming that the destruction of myoglobin is due to excited methylglyoxal formation, one can calculate an approximate yield of 0.08 for excited state generation. This is a minimum value inasmuch as it is assumed that (a) the efficiency of transfer of electronic energy from methylglyoxal to the heme is unity (b) every molecule of excited myoglobin undergoes decomposition (c) the resulting molecule is catalytically inactive.

The fact that the yield is increased on lowering the pH could be due either to an increased photochemical sensitivity of myoglobin or to an increased yield of excited product.

Preliminary experiments suggest that the first possibility is rather unlikely. The second possibility seems more feasible and might tentatively be connected with protonation of acetoacetic acid required for the removal of water in the dioxetane ring formation step.

The direct and relatively great efficiency of excited methylglyoxal in altering myoglobin would be due to two favourable factors. First, that methylglyoxal sensitizes the photochemical destruction of myoglobin. Second, that methylglyoxal should be generated very near the heme crevice. Thus, the system would be prone to a "photochemistry

without light" (11) process.

The acetoacetate myoglobin system, as stated by Milligan and Baldwin (4) shows similarities with the α-peroxidase systems of higher plants in which a C_n long chain fatty acid is converted into the C_{n-1} aldehyde with CO_2 release (12). In an earlier paper from this laboratory (1), it was proposed that in the α-peroxidase system also a dioxetane might be an intermediate. Two indications in favour of this suggestion are now available: the very likely intermediacy of a α-hydroperoxy acid (13) and a very weak chemiluminescence emission.

We conclude that the large amount of energy which must be released in the acetoacetate-myoglobin-O_2 system and in the α-peroxidase system probably appears as electronic energy, presumably by way of formation and cleavage of a dioxetane intermediate.

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