BIOCHEMICAL JOURNAL LETTERS

A controversy on the mechanism of autoxidation of oxymyoglobin and oxyhaemoglobin: oxidation, dissociation, or displacement?

The stability properties of the iron(II)–dioxygen bond in Mb and Hb are of particular importance, since the oxygenated form is known to be oxidized easily to the ferric met-form, which cannot be oxygenated and is therefore physiologically inactive. Since the early work of Brooks (1931, 1935) on HbO_2 and that of George & Stratmann (1952, 1954) on MbO_2, it has been observed that the autoxidation rate increases with decreasing partial pressure of O_2 and increases with increasing H^+ concentration. Several proposals have therefore been made concerning the mechanism of this autoxidation reaction. These can be classified into the following three types that provide an interesting basis for future study.

(*a*) *Oxidation.* Along with the early work, Brown & Mebine (1969) and Wallace *et al.* (1982), among others, also agree that the first step in autoxidation of MbO_2 and HbO_2 is the dissociation of the oxygen ligand followed by the oxidation of the deoxy species by free O_2 to produce metMb or metHb and the superoxide anion. In the case of myoglobin, therefore, one may write the autoxidation reaction as

$$\mathrm{MbFe(II)(O_2) \rightleftharpoons MbFe(II) + O_2} \qquad (1)$$

$$\mathrm{MbFe(II) + O_2 \xrightarrow{H^+} metMbFe(III) + O_2^{-\cdot}} \qquad (2)$$

In this scheme, however, the differences in the deoxy species which allow them to react with oxygen in one instance to become oxygenated (eqn. 1) and in another instance to become oxidized (eqn. 2) are completely unknown (Snyder, 1963). Wallace *et al.* (1974, 1982) presented the idea that anion binding to the deoxy species mediates one-electron transfer from iron(II) to free O_2 through the porphyrin or aromatic amino acid residues of the protein, and that under physiological conditions one of the most potential anions is Cl^-.

At this point it should be noted that free dioxygen is a poor electron acceptor with a lower redox potential, $E'_0(O_2/O_2^{-\cdot}) = -0.27$ to -0.33 V (Sawada *et al.*, 1975), than that, $E'_0 = +0.046$ V, for the MbFe(III)/MbFe(II) system (Taylor & Morgan, 1942). Furthermore, evidence for free dioxygen as the real and the sole oxidant for Mb and Hb at low O_2 levels still remains indirect.

(*b*) *Dissociation.* Weiss (1964) proposed that oxyHb could be described as a superoxo-ferrihaem complex formed by electron transfer from iron to bound dioxygen. Therefore, it has been suggested that a superoxide anion may be split off directly from the iron during autoxidation of MbO_2 or HbO_2 to its met-form, i.e. that autoxidation is essentially the dissociation of $O_2^{-\cdot}$:

$$\mathrm{MbFe^{3+}O_2^{-\cdot} \longrightarrow metMbFe(III) + O_2^{-\cdot}} \qquad (3)$$

In fact, generation of $O_2^{-\cdot}$ has been demonstrated during autoxidation of shark HbO_2 (Misra & Fridovich, 1972), bovine HbO_2 (Wever *et al.*, 1973), isolated α- and β-chains of human HbO_2 (Brunori *et al.*, 1975), and bovine MbO_2 (Gotoh & Shikama, 1976). As a direct electron-acceptor from iron(II), the co-ordinated O_2 would be much more acceptable than free O_2. Nevertheless, this scheme seems to be too simple to provide any basis for interpretation of the pH- or dioxygen-dependence of the autoxidation rate. Since the spontaneous dissociation of $O_2^{-\cdot}$ from the FeO_2 centre in hydrophobic haem environments is an energetically unfavourable process (George, 1961), there must be involved some specific mechanism that makes it possible to produce $O_2^{-\cdot}$ from the FeO_2.

(*c*) *Displacement.* Under air-saturated conditions, the rate of autoxidation of MbO_2 to metMb increases rapidly with increasing H^+ concentration, a rate minimum appears at about pH9 and a small increase occurs at higher pH values (Yamazaki *et al.*, 1964; Gotoh & Shikama, 1974; Shikama & Sugawara, 1978). Kinetic and thermodynamic analyses of this pH-dependence have recently revealed that the following three types of displacement processes are involved in the autoxidation reaction:

$$\mathrm{MbFe(II)(O_2) + H_2O + H^+ \xrightarrow{k^H_{H_2O}} MbFe(III)(OH_2) + HO_2} \qquad (4)$$

$$\mathrm{MbFe(II)(O_2) + H_2O \xrightarrow{k_{H_2O}} MbFe(III)(OH_2) + O_2^{-\cdot}} \qquad (5)$$

$$\mathrm{MbFe(II)(O_2) + OH^- \xrightarrow{k_{OH}} MbFe(III)(OH^-) + O_2^{-\cdot}} \qquad (6)$$

Abbreviations used: Hb, haemoglobin; Mb, myoglobin.

where for bovine MbO_2 the values of $k^{H}_{H_2O} = 0.25 \times 10^4 M^{-2} \cdot h^{-1}$, $k_{H_2O} = 0.47 \times 10^{-4} M^{-1} \cdot h^{-1}$, and $k_{OH} = 0.18 \times 10^2 M^{-1} \cdot h^{-1}$ were obtained in the neutral pH range at 25°C (Sugawara & Shikama, 1980). The extent of contribution of these elementary processes can vary with changes in concentrations of H^+ and OH^- ions, and can give rise primarily to a strong pH-dependence of the overall autoxidation rate.

It becomes thus quite evident that the proton-catalysed process with the rate constant $k^{H}_{H_2O}$ promotes mainly the autoxidation reaction of MbO_2 above the spontaneous process in water with the rate constant k_{H_2O}. In fact, the catalytic proton enhances the rate by a factor of 5×10^7/mol. In this proton catalysis (eqn. 4), the distal histidine, which forms a hydrogen bond to the bound dioxygen (Phillips & Schoenborn, 1981), appears to participate in facilitating the effective movement of a catalytic proton from the solvent to the bound dioxygen via its imidazole ring by a proton-relay mechanism (Sugawara & Shikama, 1980; Suzuki & Shikama, 1983). This proton transfer can lead to a favorable displacement of $O_2^{-\cdot}$ as the hydroperoxyl radical HO_2, which departs and, since its pK_a is 4.8 (Fridovich, 1975), then dissociates into the superoxide anion and a catalytic proton again.

To elucidate further the molecular mechanism of these substitution reactions (eqns. 4–6) leading to metMb formation *in vivo*, Satoh & Shikama (1981) have studied the oxidation of MbO_2 induced by excess anion. The anions examined were SCN^-, F^-, OCN^-, N_3^- and CN^-, whose nucleophilicity differs from that of H_2O and OH^-. In each case, the observed oxidation rate was linearly dependent upon the concentration of an added anion. A Brønsted plot for the series showed that the rates correlated with the pK_a of the conjugate acid, a measure of the nucleophilicity of the anion. These results clearly indicate that the mechanism of autoxidation is not a simple, dissociative loss of $O_2^{-\cdot}$ from MbO_2. Rather, the oxidation of MbO_2 proceeds by way of a nucleophilic attack of anions at the iron centre; only in the presence of attacking nucleophiles is a full charge transfer from Fe(II) to O_2 produced. They also concluded that, as the most common nucleophiles *in vivo*, both H_2O and OH^- can react with MbO_2, and thereby displace the bound dioxygen in the form of $O_2^{-\cdot}$ so that the iron is converted to the ferric form.

Unfortunately it seems that there is no provision in this scheme for the inverse dependence of the autoxidation rate upon oxygen pressure. In this respect, however, it is very interesting to note that H_2O_2 can oxidize deoxyMb more than 100 times more easily than can oxyMb (Yusa, 1984). Since H_2O_2 may be produced by dismutation of the superoxide anion generated from autoxidation of the oxy-form, it must act as at least one of the potent oxidants of the deoxy-form that increases with decreasing O_2 pressures. This idea also seems to be attractive for a possible interpretation of the oxygen dependence, and remains open to future study.

Keiji SHIKAMA
Biological Institute, Tohoku University, Sendai 980, Japan

(*Received 17 May 1984*)

Brooks, J. (1931) *Proc. R. Soc. London Ser. B* **109**, 35–50
Brooks, J. (1935) *Proc. R. Soc. London Ser. B* **118**, 560–577
Brown, W. D. & Mebine, L. B. (1969) *J. Biol. Chem.* **244**, 6696–6701
Brunori, M., Falcioni, G., Fioretti, E., Giardina, B. & Rotilio, G. (1975) *Eur. J. Biochem.* **53**, 99–104
Fridovich, I. (1975) *Annu. Rev. Biochem.* **44**, 147–159
George, P. (1961) in *Haematin Enzymes* (Falk, J. E., Lemberg, G. & Morton, R. K., eds.), p. 103, Pergamon, Oxford
George, P. & Stratmann, C. J. (1952) *Biochem. J.* **51**, 418–425
George, P. & Stratmann, C. J. (1954) *Biochem. J.* **57**, 568–573
Gotoh, T. & Shikama, K. (1974) *Arch. Biochem. Biophys.* **163**, 476–481
Gotoh, T. & Shikama, K. (1976) *J. Biochem. (Tokyo)* **80**, 397–399
Misra, H. P. & Fridovich, I. (1972) *J. Biol. Chem.* **247**, 6960–6962
Phillips, S. E. V. & Schoenborn, B. P. (1981) *Nature (London)* **292**, 81–82
Satoh, Y. & Shikama, K. (1981) *J. Biol. Chem.* **256**, 10272–10275
Sawada, Y., Iyanagi, T. & Yamazaki, I. (1975) *Biochemistry* **14**, 3761–3764
Shikama, K. & Sugawara, Y. (1978) *Eur. J. Biochem.* **91**, 407–413
Snyder, H. E. (1963) *Biochim. Biophys. Acta* **69**, 200–202
Sugawara, Y. & Shikama, K. (1980) *Eur. J. Biochem.* **110**, 241–246
Suzuki, T. & Shikama, K. (1983) *Arch. Biochem. Biophys.* **224**, 695–699
Taylor, J. F. & Morgan, V. E. (1942) *J. Biol. Chem.* **144**, 15–20
Wallace, W. J., Maxwell, J. C. & Caughey, W. S. (1974) *FEBS Lett.* **43**, 33–36
Wallace, W. J., Houtchens, R. A., Maxwell, J. C. & Caughey, W. S. (1982) *J. Biol. Chem.* **257**, 4966–4977
Weiss, J. J. (1964) *Nature (London)* **202**, 83–84
Wever, R., Oudega, B. & Van Gelder, B. F. (1973) *Biochim. Biophys. Acta* **302**, 475–478
Yamazaki, I., Yokota, K. & Shikama, K. (1964) *J. Biol. Chem.* **239**, 4151–4153
Yusa, K. (1984) M.Sc. Thesis, Tohoku University