J. Biosci., Vol. 16, Numbers 1 & 2, June 1991, pp 43–53. © Printed in India.

Haemoglobin: A scavenger of superoxide radical

ASOKE MAL, ANURADHA NANDI and I B CHATTERJEE*
Department of Biochemistry, University College of Science, 35, Ballygunge Circular Road, Calcutta 700 019, India

MS received 31 July 1990; revised 22 April 1991

Abstract. Superoxide is continuously generated in the erythrocytes, and oxyhaemoglobin from different animals including fish, amphibians, reptiles, birds, flying mammals, mammals and human beings acts as a scavenger of superoxide. The approximate rate constants of the reaction between superoxide and oxyhaemoglobin of different animals are $0{\cdot}32–1{\cdot}6 \times 10^7 M^{-1}\ s^{-1}$. Results obtained with anion ligands like CN^- and N_3^- indicate that superoxide preferentially reacts with anion ligand-bound deoxyhaemoglobin. Carbonmonoxyhaemoglobin and methaemoglobin are ineffective. Work with photochemically generated oxyradical indicate that oxyhaemoglobin may also act as a scavenger of singlet oxygen. The rate constant of the reaction between superoxide and human oxyhaemoglobin is $K_{app} = 6{\cdot}5\times10^6\ M^{-1}\ s^{-1}$, which is about three orders less than K_{SOD} ($2\times 10^9\ M^{-1}\ s^{-1}$). Thus, in the erythrocytes, oxyhaemoglobin would appear to act as a second line of defence. Oxyhaemoglobin appears to be as effective as superoxide dismutase for scavenging superoxide in the erythrocytes.

Keywords. Oxyhaemoglobin; deoxyhaemoglobin; methaemoglobin; ligand; superoxide; superoxide dismutase; erythrocyte.

1. Introduction

In our study on the assay of superoxide dismutase (SOD) activity in the Tsuchihasi extract of human erythrocyte haemolysate by the pyrogallol autoxidation method (Nandi and Chatterjee 1988), we observed that the SOD activity was about 1000–1100 units per g haemoglobin (Hb). On the other hand, when the untreated whole erythrocyte haemolysate was used, the apparent SOD activity was about 6000-8000 units per g Hb. Later, we observed that this high superoxide ($O_2^{\cdot-}$) scavenging activity of the whole haemolysate was present in the oxyhaemoglobin (HbO_2) isolated from the haemolysate by ion-exchange chromatography over phosphocellulose (Mal and Chatterjee 1991). The $O_2^{\cdot-}$ scavenging activity was also observed in HbO_2 isolated from erythrocytes of other animals including fish, amphibians, reptiles, birds and mammals. Scarpa *et al* (1984) have shown that $O_2^{\cdot-}$ is continuously generated in human erythrocytes. Demma and Salhany (1977) demonstrated that $O_2^{\cdot-}$ produced by photolytic dissociation of HbO_2 reattacks HbO_2 to produce methaemoglobin (MetHb) and H_2O_2. That $O_2^{\cdot-}$ reacts with HbO_2 has also been shown by Sutton *et al* (1976), Lynch *et al* (1977) and Watkins *et al* (1985). We have also demonstrated that $O_2^{\cdot-}$ produced during autoxidation of HbO_2, apparently by dissociation of Hb^{3+} $O_2^{\cdot-}$, reattacks HbO_2 to produce MetHb (Mal and Chatterjee 1991). In this communication, we present evidence indicating that HbO_2 is a

*Corresponding author.

potential scavenger of $O_2^{\cdot-}$. We also present further evidence to show that the ligand-bound deoxyhaemoglobin is the preferential form which reacts with $O_2^{\cdot-}$.

2. Materials and methods

2.1 *Chemicals*

Cellulose phosphate P-ll was purchased from Whatman, England. Xanthine, xanthine oxidase, bovine erythrocyte superoxide dismutase and riboflavin were obtained from Sigma Chemical Company, St. Louis, Mo, USA. Catalase (free of SOD) was purchased from the CSIR Centre for Biochemicals, New Delhi. All other chemicals and reagents used were of analytical grade. All solutions were made with double distilled water.

2.2 *Isolation and estimation of HbO_2*

The procedure for the isolation and estimation of haemoglobin has been described earlier (Mal and Chatterjee 1991).

2.3 *Quantitative determination of HbO_2 and MetHb*

These were done according to the method described by Mal and Chatterjee 1991.

2.4 *Assay of erythrocyte SOD*

SOD activity of erythrocyte haemolysate was measured in the Tsuchihasi extract (Crapo *et al* 1978) by the pyrogallol autoxidation method (Nandi and Chatterjee 1988) as described below.

2.5 *Assay of $O_2^{\cdot-}$ scavenging activity of HbO_2*

This was done by the pyrogallol autoxidation method (Nandi and Chatterjee 1988). The assay system contained 1 mM DTPA, 40 μg catalase, 50 mM air-equilibrated Tris-cacodylate buffer, pH 8·5, and HbO_2 solution as needed, in a final volume of 2 ml. The reaction was initiated by the addition of 100 μl of freshly prepared 2·6 mM pyrogallol solution in 10 mM HCl to attain a final concentration of pyrogallol of 0·13 mM in the assay mixture. One unit of $O_2^{\cdot-}$ scavenging activity of HbO_2 represents the amount of HbO_2 required to produce 50% inhibition in 3 ml assay mixture.

Results

Oxyhaemoglobin isolated from erythrocytes of different animals including fish, amphibians, reptiles, birds, flying mammals, mammals and human beings acts as a scavenger of $O_2^{\cdot-}$ (table 1). The $O_2^{\cdot-}$ scavenging activity has been assayed by the pyrogallol autoxidation method (Nandi and Chatterjee 1988). At the pH of the

Table 1. Superoxide scavenging activity of Tsuchihasi extract of erythrocyte haemolysate and HbO_2 of different animals.

Animal	Activity in unit/g Hb		
	Tsuchihasi extract (SOD)	HbO_2	$K_{app} \times 10^7\ M^{-1}\ s^{-1}$
Fish			
Labeo rohita (6)	1,072[a]	6,525 ± 900[b]	0·34
Amphibians			
Toad[c] (*Bufo melanostictus*) (18)	1,144	13,452 ± 2000	1·60
Frog[c] (*Rana tigrina*) (18)	1,100	10,600 ± 2000	0·90
Reptiles			
Blood sucker[c] (*Caloter versicolor*) (18)	1,067	7,214 ± 950	0·56
Anjani (*Mabuya carinata*) (8)	1,050	10,884 ± 1000	1·28
Turtle (*Lissemys punctata*) (4)	1,014	4,471 ± 500	0·36
Common Indian monitor (*Varanus monitor*) (4)	1,050	8,071 ± 1000	0·68
Birds			
Pigeon (4)	1,007	6,731 ± 600	0·45
Chicken (4)	1,060	10,506 ± 1100	0·95
Flying mammals			
Indian fruit bat (4)	3,360	5,346 ± 500	0·32
Mammals			
Rabbit (4)	1,012	5,178 ± 450	0·47
Rat (12)	1,020	5,750 ± 550	0·48
Guinea pig (6)	1,550	5,102 ± 500	0·44
Goat (4)	1,029	5,146 ± 550	0·50
Cattle (4)	1,026	5,036 ± 400	0·39
Human (4)	1,074	7,692 ± 800	0·65

One unit represents the amount of HbO_2 required to produce 50% inhibition of the autoxidation of pyrogallol.

$O_2^{\cdot-}$ scavenging activity was assayed by the pyrogallol autoxidation method (Nandi and Chatterjee 1988). The assay system contained 1 mM DTPA, 40 μg catalase, 50 mM air-equilibrated Tris-cacodylate buffer, pH 8·5, and Hb solution and Tsuchihasi extract as needed, in a final volume of 2 ml. The reaction was initiated by the addition of 100 μl of freshly prepared 2·6 mM pyrogallol solution in 10 mM HCl to attain a final concentration of pyrogallol of 0·13 mM in the assay mixture. The details of the procedure are described elsewhere (Nandi and Chatterjee 1988). The final concentration of Hb in the assay mixture varied from 0·2 to 1 μM depending on the $O_2^{\cdot-}$ scavenging activity.

Numbers in parentheses indicate the number of animals used.

[a]Average values.

[b]Mean±SD

[c]Each blood sample was pooled from 3 animals.

assay system used (pH 8·5), the autoxidation of pyrogallol is essentially fully inhibited by SOD and hence this method can be used as an effective assay for the determination of $O_2^{\cdot-}$ scavenging activity (Nandi and Chatterjee 1988; Mårklund and Marklund 1974; McCord *et al* 1977). The $O_2^{\cdot-}$ scavenging activity cannot be assayed by the xanthine-xanthine oxidase ferricytochrome c (cyt c^{3+}) method (McCord and Fridovich 1969) because as indicated in the previous paper (Mal and

Chatterjee 1991), HbO_2 directly reduces cyt c^{3+} to cyt c^{2+} at a very fast rate and about 79% of this reduction is insensitive to SOD. The direct reaction between HbO_2 and cyt c^{3+} has also been shown by Tomoda *et al* (1980). Also, the $O_2^{\cdot-}$ scavenging activity of HbO_2 cannot be assayed by xanthine-xanthine oxidase-nitroblue tetrazolium (NBT) method (Beauchamp and Fridovich 1971) because we have observed that NBT directly oxidizes HbO_2 to MetHb. In the 2 ml assay system containing 2 μM HbO_2, 0·1 mM EDTA in 50 mM potassium phosphate buffer, pH 6·8, 0·25 μM NBT produced 15% MetHb in 10 min at 37°C.

In table 1 the $O_2^{\cdot-}$ scavenging activities of HbO_2 isolated from different animals have been expressed in unit per g Hb. Since one unit of SOD also represents the amount of SOD needed to produce 50% inhibition of the autoxidation of pyrogallol, one unit of $O_2^{\cdot-}$ scavenging activity of HbO_2 is apparently equivalent to one unit of SOD. Since the amount of SOD needed to produce 50% inhibition in the pyrogallol autoxidation system is about half of that needed to produce 50% inhibition in the xanthine-xanthine oxidase—ferricytochrome c system (Nandi and Chatterjee 1988), one unit of SOD in the pyrogallol system may be taken to be 165 ng of SOD. This is approximately equivalent to $2{\cdot}58\times10^{-9}$ M. Taking K_{SOD} to be equal to 2×10^{9} M^{-1} s^{-1} (McCord *et al* 1977), the approximate rate constants of the reaction between HbO_2 and $O_2^{\cdot-}$ in the pyrogallol system have been calculated for HbO_2 of different animals to be $0{\cdot}32–1{\cdot}6\times10^{7}$ M^{-1} s^{-1} (table 1).

Figure 1 shows the effect of variation of concentration of the human HbO_2 on the $O_2^{\cdot-}$ scavenging activity. The graph is linear for about 24 to 75% inhibition. The $O_2^{\cdot-}$ scavenging activities of HbO_2 and SOD are additive. When 0·5 unit equivalent of HbO_2 is added to the pyrogallol autoxidation system containing 0·5 unit of SOD, 50% inhibition of the autoxidation is obtained. When one unit equivalent of HbO_2 is added to the pyrogallol system containing one unit of SOD, the

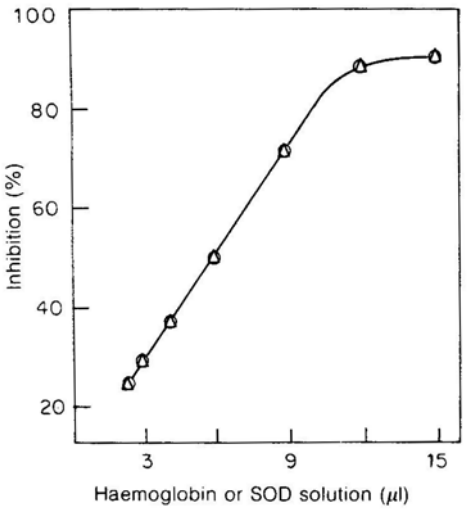

Figure 1. Per cent inhibition as a function of concentration in human HbO_2 solution/ pure SOD solution.

$O_2^{\cdot-}$ scavenging activity was assayed by the pyrogallol autoxidation method (Nandi and Chatterjee 1988). The detailed procedure is described under 'materials and methods'. (Δ) and (O) represent HbO_2 solution and SOD solution respectively.

autoxidation is inhibited about 85%. This 85% inhibition is also observed when either 2 units equivalent of HbO_2 or 2 units of SOD are added to the pyrogallol system. In contrast to the erythrocyte SOD, the $O_2^{\cdot-}$ scavenging property of HbO_2 is completely lost on treatment with heat (5 min at 65°), SDS (2%), H_2O_2 (50 μM) and chloroform-methanol mixture (Tsuchihasi procedure).

Figure 2 shows that in the pyrogallol autoxidation system, human HbO_2 undergoes oxidation to MetHb. An adequate amount of catalase is present in the system to prevent any oxidation of HbO_2 by H_2O_2. The formation of MetHb is completely inhibited in the presence of excess SOD (figure 2) indicating that in the pyrogallol autoxidation system the oxidation of HbO_2 is mediated by $O_2^{\cdot-}$. Pyrogallol or preoxidized pyrogallol does not oxidize $HbO_2^{\cdot-}$ to MetHb. At pH 6 autoxidation of pyrogallol is very slow and there is practically neither production of $O_2^{\cdot-}$ nor oxidation of HbO_2.

Figure 3 shows that photochemically-generated reactive oxygen species oxidizes human HbO_2 to MetHb very fast as evidenced by the increase of A_{630} and decrease of A_{575}. However, this production of MetHb is not inhibited by SOD (15 μg). This would indicate that the active species is probably singlet oxygen rather than $O_2^{\cdot-}$ because irradiation of riboflavin is known to produce singlet oxygen (Hodgson and Fridovich 1976; Michelson 1977; Foote 1982). It was also observed that 2 mM NaN_3, a scavenger of singlet oxygen (Klebanoff and Rosen 1979), completely inhibited the oxidation of HbO_2 in the photochemical system.

Figure 4 shows that when HbO_2 is allowed to react with $O_2^{\cdot-}$ generated in the xanthine-xanthine oxidase system, no MetHb is formed. However, MetHb is produced when HbO_2 is added to the xanthine-xanthine oxidase system containing 2 mM KCN (figure 4). A large excess of catalase was present to scavenge the H_2O_2 produced, which would otherwise oxidize HbO_2 to MetHb. The amount of catalase

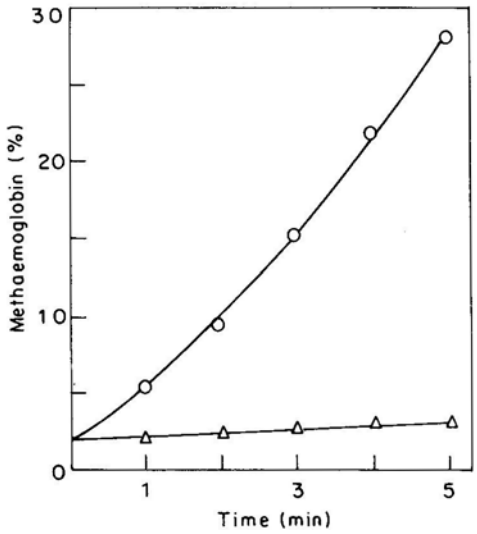

Figure 2. Oxidation of human HbO_2 to MetHb in the pyrogallol autoxidation method. Reaction mixture was the same as indicated in figure 1 except that the concentration of HbO_2 was 2 μM. (O) represents the oxidation of HbO_2 in the absence of SOD and (Δ) represents the oxidation in the presence of SOD (10 μg).

Figure 3. Spectral changes obtained during the oxidation of human HbO_2 to MetHb in the photochemical system.

Two ml reaction mixture contained 2 μM Hb O_2, 50 mM potassium phosphate buffer, pH 6·8, 0·1 mM EDTA, catalase (80 μg), 1 μM riboflavin. The reaction mixture was illuminated 6 cm away by a 40 W fluorescence lamp. Spectra were obtained at 0, 5 and 10 min elapsed time.

used was over and above that which could be inhibited by CN^-. This would indicate that $O_2^{\cdot-}$ reacts with $HbCNT^-$. In other words, $O_2^{\cdot-}$ reacts with anion ligand-bound deoxyhaemoglobin rather than HbO_2. Figure 5 shows the spectral changes obtained during the oxidation of $HbCN^-$ in the pyrogallol autoxidation system. That $O_2^{\cdot-}$ reacts with anion ligand-bound deoxyhaemoglobin is also confirmed by the observation that in the presence of a large excess of catalase, HbN_3^- reacts with $O_2^{\cdot-}$ to produce MetHb (figure 6). Figure 7 shows that oxidation of HbN_3^- is a function of $O_2^{\cdot-}$ concentration. The rate of oxidation increases with the increased concentration of $O_2^{\cdot-}$.

While $HbCN^-$ and HbN_3^- react readily with $O_2^{\cdot-}$, HbCO is completely inactive both in the pyrogallol autoxidation system and the xanthine-xanthine oxidase system.

The product of the reaction of $O_2^{\cdot-}$ with HbO_2 is MetHb. However, MetHb is ineffective as a scavenger of $O_2^{\cdot-}$ either in the pyrogallol system or in the xanthine-xanthine oxidase system. We have observed that when MetHb (2–5 μM) from different sources (human bovine, toad) is allowed to react with $O_2^{\cdot-}$ (4nmol/min,

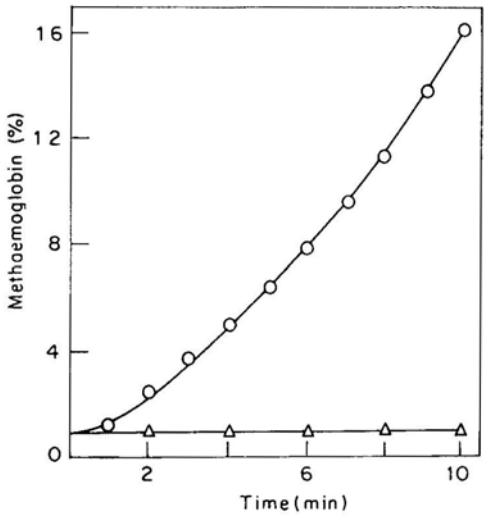

Figure 4. Oxidation of human HbO_2 by $O_2^{\cdot-}$

Two ml reaction Mixture contained 2 μM HbO_2 preincubated with 2 mM KCN when needed, 50 mM potassium phosphate buffer, pH 7·4, 0·1 mM EDTA, catalase (100 μg), 0·4 mM xanthine and 4·5mU (*5 μ*) xanthine oxidase. The amount of catalase was more than sufficient to scavenge the H_2O_2 produced in the presence of 2 mM KCN. The amount of $O_2^{\cdot-}$ produced in this mixture was 2 nmol/min as determined by the reduction of ferricytochrome c (McCord and Fridovich 1969). (Δ) represents oxidation of HbO_2 in the absence of KCN and (O) represents oxidation in the presence of 2mM KCN.

generated by the xanthine-xanthine oxidase system) there is no decrease of A_{630} or increase of $A_{575,}$ indicating that MetHb does not react with $O_2^{\cdot-}$ However, the $O_2^{\cdot-}$ scavenging property is regained when MetHb is reduced to $HbO_2^{\cdot-}$ with sodium dithionite (100 μg/ml for 2 μM MetHb). Figure 8 shows that MetHb is also reduced by the physiological concentration of ascorbic acid. The rate of reduction of MetHb by ascorbic acid is $K_{app} = 2{\cdot}64\ min^{-1}$.

4. Discussion

Respiration, while providing numerous biochemical advantages, imposes the need for extra protection against free radical toxicity. This is needed at the very first moment when Hb binds O_2 in the erythrocytes, because part of the HbO_2 dissociates into MetHb and $O_2^{\cdot-}$ (Misra and Fridovich 1972; Mal and Chatterjee 1991) resulting in a continuous generation of $O_2^{\cdot-}$ in the erythrocytes (Scarpa *et al* 1984). The rate of formation of $O_2^{\cdot-}$ in the erythrocytes has been calculated to be $3{\cdot}25\times10^{-8}$ M s^{-1} (Mal and Chatterjee 1991). This $O_2^{\cdot-}$, unless scavenged properly, would lead to irreversible oxidative damage of the red cells (Fridovich 1972, 1979; Kellog and Fridovich 1977). There are present in the erythrocytes, scavengers of $O_2^{\cdot-}$ namely, SOD (about $1{\cdot}63\times10^{-6}$ M; K_{SOD} = $2\times109\ M^{-1}\ s^{-1}$) and ascorbic acid (about 4×10^{-5} M; $K_{AH_2} = 8{\cdot}2\times10^{7}\ M^{-1}\ s^{-1}$) (Nandi and Chatterjee 1987) as well as scavengers of H_2O_2, namely, catalase and glutathione peroxidase. However, the presence of SOD and ascorbic acid need not necessarily mean that these

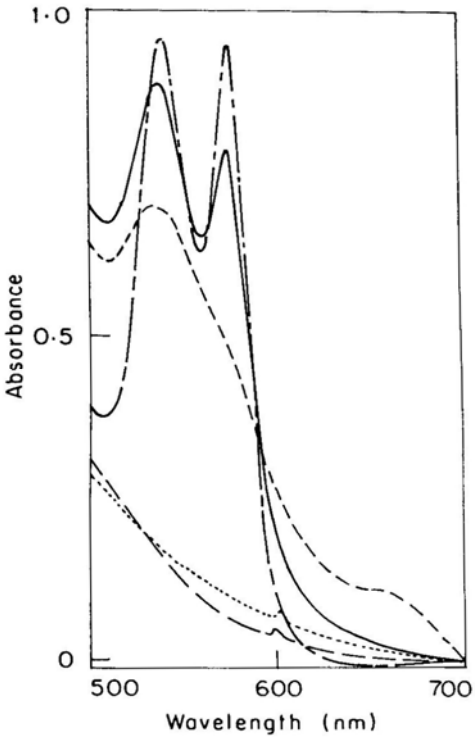

Figure 5. Spectral changes obtained during the oxidation of human Hbo_2 in the pyrogallol autoxidation system in the presence of KCN.

Reaction mixture was the same as in figure 1 except that the concentration of Hbo_2 was 2 μM ,KCN 2 mMand catalase 100 μg. Spectra were obtained at 0, 5 and 10 min elapsed time. Bottom lines represent the spectraL changes of only pyrogallol in the absence of Hbo_2 at 0 min ad 10 min.

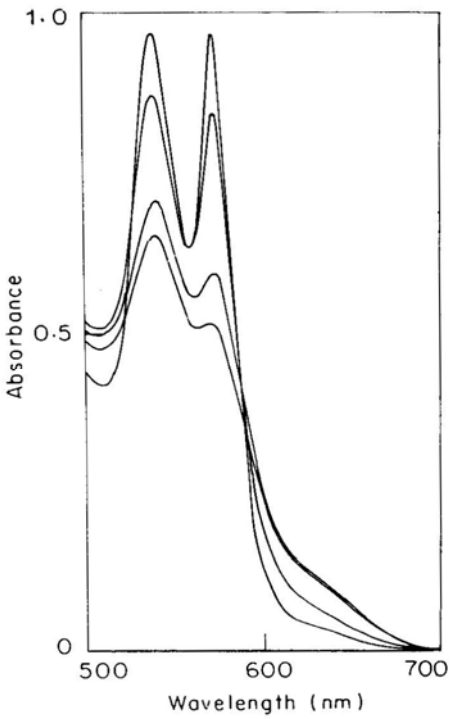

Figure 6. Spectral changes obtained during the oxidation of human Hbo_2 by $O_2^{\cdot-}$ generated in the xanthine-xanthine oxidase system in the presence of NaN_3.

Reaction mixture was the same as in figure 4 except that Hbo_2 was preincubated with 2 mM NaN_3 when needed instead of 2 mM KCN.The amount of catalase was more than sufficient to scavenge the H_2O_2 produced in the presence of 2 mM NaN_3. Spectra were obtained at 0, 5, 15 and 30 min elapsed time

scavengers succeed totally. A fraction of the $O_2^{\cdot-}$ generated in the erythrocytes may always escape and, if they do, they will cause oxidative damage to the red cells. Evidences presented in this communication indicate that in addition to SOD and ascorbic acid, HbO_2 is also a potential scavenger of $O_2^{\cdot-}$ The rate constant of the reaction between human HbO_2 and $O_2^{\cdot-}$ in the pyrogallol system is approximately $6{\cdot}5 \times 10^6$ M^{-1} s^{-1}. Results obtained with $HbCN^-$ and HbN_3^- indicate that $O_2^{\cdot-}$ preferentially reacts with anion ligand-bound deoxyhaemoglobin (HbL^-) rather than HbO_2. In the pyrogallol system also, pyrogallol presumably acts as an anion ligand to Hb. It has been shown that the hydrophobic cluster on the side of E_7 (distal histidine), which accommodates the heme-linked O_2, contains enough room beneath His E_7 for the entry of a heme ligand as large as n-butylisocyanide (Perutz 1979). However, under physiological conditions, only Cl^-, H_2O and OH^- appear to serve as anion ligands (Watkins *et al* 1985).

The reaction of HbL^- with $O_2^{\cdot-}$ produces MetHb. However, MetHb is ineffective

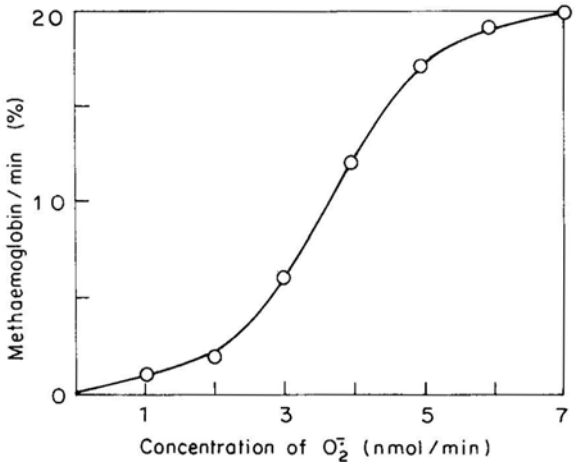

Figure 7. Oxidation of human HbO_2 as a function of O_2^- concentration in the presence of NaN_3.

Reaction mixture was the same as in figure 6 except that the increased concentration of O_2^- was obtained by using an increased amount of xanthine oxidase (5-15 μl).

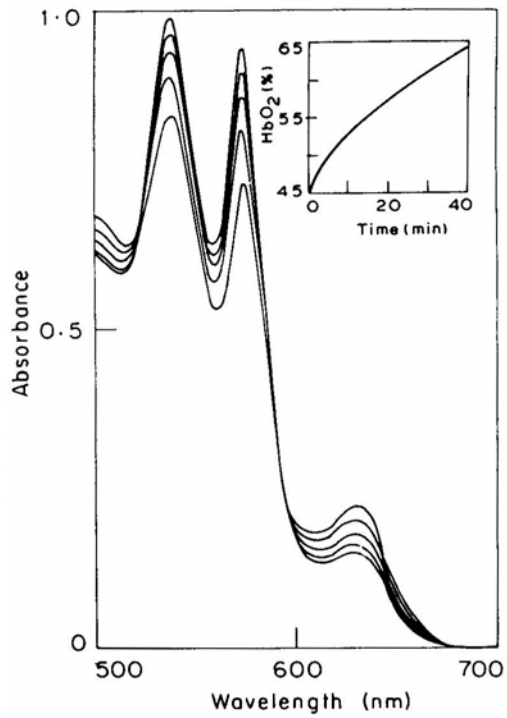

Figure 8. Spectral changes obtained during the reduction of human MetHb in the presence of ascorbic acid

Two ml reaction mixture contained 1·5 μM human MetHb, 50 mM potassium phosphate buffer, pH 6·8, 0·1 mM ascorbic acid and catalase (80 μg), The reaction was carried out at 37° using a temperature controlled cell holder. Spectra were obtained at 0, 10, 20, 30 and 40 min elapsed time. Inset of the figure represents the rate of reduction of MetHb.

as a scavenger of $O_2^{\cdot-}$. The activity is, of course, regained by reducing MetHb to HbO_2. *In vivo,* this reduction of MetHb is accomplished by erythrocyte NADH MetHb reductase as well as ascorbic acid (see figure 8) to achieve a steady state HbO_2 level, which is 0·5 to 1% in normal blood (Rodkey and O'Neal, 1974).

It has already been shown that the rate constant of the reaction between $O_2^{\cdot-}$ and human HbO_2 is K_{app} = 6·5× 10^6 M^{-1} s^{-1}, which is about three orders smaller than that of K_{S0D} (2×10^9 M^{-1} s^{-1}). Thus, in the erythrocyte, HbO_2 would appear to act as a second line of defence. However, the concentration of HbO_2 in the erythrocyte is approximately $4{\cdot}65\times10^{-3}$ M (Mal and Chatterjee 1991) which is about three orders greater than that of SOD ($1{\cdot}6\times10^{-6}$ M). Thus, considering both the rate constants and the concentrations, HbO_2 appears to be as effective as SOD for scavenging $O_2^{\cdot-}$ in the erythrocytes.

Acknowledgements

This work was supported by the Department of Science and Technology, New Delhi (grant No. 22(9P–47)/84–STPII). The assistance of Dr A C Banerjee for technical help is gratefully acknowledged.

References

Beauchamp C and Fridovich I 1971 Superoxide dismutase: Improved assay and an assay applicable to acrylamide gel; *Anal. Biochem.* **44** 276-287

Crapo J D, McCord J M and Fridovich I 1978 Preparation and assay of superoxide dismutases; *Methods Enzymol.* **53** 382-393

Demma L S and Salhany J M 1977 Direct generation of superoxide anions by flash photolysis of human oxyhaemoglobin; *J. Biol. Chem.* **252** 1226-1231

Foote C S 1982 Light, oxygen and toxicity; in *Pathology of oxygen* (e .) A P Autor (New York: Academic Press) pp 21-42

Fridovich I 1972 Superoxide radical and superoxide dismutase; *Acc. Chem. Res.* **5** 321-326

Fridovich I 1979 *Oxygen free radicals and tissue damage*, Ciba Foundation Symposium 65 (New Series) (Amsterdam: Excerpta Medica) pp 77-93

Hodgson E K and Fridovich I 1976 The mechanism of the activity dependent luminescence of xanthine oxidase; *Arch. Biochem. Biophys.* **172** 202-205

Kellog E W III and Fridovich I 1977 Liposome oxidation and erythrocyte lysis by enzymically generated superoxide and hydrogen peroxide; *J. Biol. Chem.* **252** 6721-6728

Klebanoff S J and Rosen H 1979 *Oxygen free radicals and tissue damage,* Ciba Foundation Symposium 65 (New Series) (Amsterdam: Excerpta Medica) pp 263-272

Lynch R E, Thomas J E and Lee G R 1977 Inhibition of methemoglobin formation from purified oxyhemoglobin by superoxide dismutase; *Biochemistry* **16** 4563-4567

Mal A and Chatterjee I B 1991 Mechanism of autoxidation of oxyhemoglobin; *J. Biosci,* **16** 55-70

Marklund S and Marklund G 1974 Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase; *Eur. J. Biochem.* **47** 469-474

McCord J M, Crapo J D and Fridovich I 1977 Superoxide dismutase assays: A review of methodology; in *Superoxide and superoxide dismutase* (eds) A M Michelson, J M McCord and I Fridovich (New York: Academic Press) pp 11-17

McCord J M and Fridovich I 1969 Superoxide dismutase: An enzymic function of erythrocuprein (hemocuprein); *J. Biol. Chem.* **244** 6049-6055

Michelson AM 1977 Toxicity of superoxide radical anions; in *Superoxide and superoxide dismutase* (eds) A M Michelson, J M McCord and I Fridovich (New York: Academic Press) pp 245-255

Misra H P and Fridovich I 1972 The generation of superoxide radical during the autoxidation of hemoglobin; *J. Biol. Chem.* **247** 6960-6962

Nandi A and Chatterjee I B 1987 Scavenging of superoxide radical by ascorbic acid; *J. Biosci.* **11** 435-441

Nandi A and Chatterjee I B 1988 Assay of superoxide dismutase activity in animal tissues; *J. Biosci.* **13** 305-315

Perutz M F 1979 Regulation of oxygen affinity of hemoglobin: Influence of structure of the globin on the heme iron; *Annu. Rev. Biochem.* **48** 327-386

Rodkey F L and O'Neal J D 1974 Effects of carboxyhemoglobin on the determination of methemoglobin in blood; *Biochem. Med.* **9** 261

Scarpa M, Viglino P, Contri D and Rigo A 1984 Generation of superoxide ion in the human red blood cell lysates; *J. Biol. Chem.* **259** 10657-10659

Sutton H C, Robert P B and Winterbourn C C 1976 The rate of reaction of superoxide radical ion with oxyhaemoglobin and methaemoglobin; *Biochem. J.* **155** 503-510

Tomoda A, Tsuji A and Yoneyama Y 1980 Mechanism of hemoglobin oxidation by ferricytochrome c under aerobic and anaerobic conditions; *J. Biol. Chem.* **255** 7978-7983

Watkins J A, Kawanishi S and Caughey W S 1985 Autoxidation reactions of hemoglobin A free from other red cell components: A minimal mechanism; *Biochem. Biophys. Res. Commun.* **132** 742-748.