Biochimica et Biophysica Acta, 302 (1973) 475–478

BBA Report

BBA 61272

Generation of superoxide radicals during the autoxidation of mammalian oxyhemoglobin

R. WEVER, B. OUDEGA and B.F. VAN GELDER

Laboratory of Biochemistry, B.C.P. Jansen Institute★*, University of Amsterdam, Amsterdam (The Netherlands)*

(Received February 27th, 1973)

SUMMARY

It is shown that during autoxidation of mammalian oxyhemoglobin to methemoglobin superoxide anions (O_2^-) are generated. We suggest that the physiological role of superoxide dismutase in erythrocytes is to prevent the deleterious action of the superoxide anion.

Red blood cells contain the metalloprotein superoxide dismutase, which has been shown by McCord and Fridovich[1] to catalyze the dismutation of superoxide radicals to hydrogen peroxide and oxygen. The radical is known to be generated during the oxidation of reduced flavins and quinones[2], flavo-proteins[3] and iron-sulphur proteins[4] by oxygen. The presence of the enzyme in red blood cells suggests that superoxide anions may be formed in these cells.

Based mainly on the kinetics of autoxidation of myoglobin and oxyhemoglobin, George and Stratmann[5,6] proposed that superoxide anions are liberated during this process. As pointed out by Weiss[7], the iron atom in oxyhemoglobin can be considered to be trivalent. This has been confirmed by the results obtained from Mössbauer spectroscopy[8], EPR[9] and X-ray fluorescence spectra[10]. A trivalently charged iron atom may suggest that the oxygen bound is in the negatively charged form; that is the superoxide anion radical (O_2^-).

As predicted by Wang *et al.*[11,12], it is possible that in addition to the normal dissociation of oxyhemoglobin into reduced hemoglobin and oxygen, a slow dissociation takes place into methemoglobin and the superoxide anion. The anion can be detected with the aid of

★ Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

cytochrome *c* and superoxide dismutase[1]. It is able to reduce cytochrome *c* by a reaction which is inhibited by superoxide dismutase. In this paper we will present evidence that the superoxide anion is formed during the autoxidation of mammalian hemoglobin.

Hemoglobin was isolated from bovine erythrocytes according to the method of Drabkin[13]. After two crystallizations, the protein was reduced with sodium dithionite. Excess reducing agent was removed by gel filtration on Sephadex G-25. The oxyhemoglobin was prepared from the reduced protein by bubbling oxygen through the solution. CO-hemoglobin was prepared from oxyhemoglobin by bubbling CO (Matheson Gas Products) through the solutions for 2 min. The cuvettes were covered with parafilm. Superoxide dismutase was isolated from bovine blood according to McCord and Fridovich[1]. Horse-heart cytochrome *c* was isolated according to Margoliash and Walasek[14]. Catalase (from beef liver) was obtained from Sigma.

The extinction coefficients used for oxyhemoglobin, cytochrome *c* and superoxide dismutase were 14.6 $mM^{-1} \cdot cm^{-1}$ at 577 nm (ref. 15), 21.1 $mM^{-1} \cdot cm^{-1}$ (reduced *minus* oxidised) at 550 nm (ref. 16) and 0.3 $mM^{-1} \cdot cm^{-1}$ at 680 nm (ref. 1), respectively. Chemicals, analar grade, were obtained from B.D.H. Ltd. Spectrophotometric measurements were performed using a Cary-17 spectrophotometer. The pH of the solution was determined with a Philips 9408 digital pH/mV meter.

On incubation of oxyhemoglobin with cytochrome *c* at neutral pH and low salt concentrations, a very slow reduction of cytochrome *c* occurs, corresponding to the slow autoxidation of oxyhemoglobin. In order to increase this rate, the concentration of the salt (potassium phosphate) was raised to 1 M and the pH of the medium lowered to 6.0. Catalase was present to decompose hydrogen peroxide, which interacts with oxyhemoglobin or cytochrome *c*.

As monitored by the increase in absorbance at 520 nm and 550 nm, cytochrome *c* becomes progressively more reduced with time until a constant reduction level (steady state) is reached (Fig. 1A). This steady state level is caused by the fact that at high salt concentrations, reduced cytochrome *c* is less stable and autoxidizes. The final reduction level of cytochrome *c* in the presence of superoxide dismutase (Fig. 1B) is approximately half that observed in its absence (Fig. 1A).

The inhibition of the reduction of cytochrome *c* in the presence of superoxide dismutase indicates that superoxide anions are formed during the autoxidation of oxyhemoglobin, although an alternative explanation may be that superoxide dismutase accelerates an autoxidation of reduced cytochrome *c*. However, the latter possibility can be ruled out because superoxide dismutase does not stimulate the rate of autoxidation of reduced cytochrome *c* in the absence of hemoglobin.

Under anaerobic conditions a more rapid reduction of cytochrome *c* by deoxygenated hemoglobin is found (not shown). In this case, however, the reduction is insensitive to superoxide dismutase, suggesting a direct reaction between hemoglobin and cytochrome *c* (see also ref. 17). An interaction between hemoglobin and cytochrome *c* probably accounts for the superoxide dismutase-insensitive reduction of cytochrome *c* under the aerobic conditions of Fig. 1. Support for this conclusion comes from the fol-

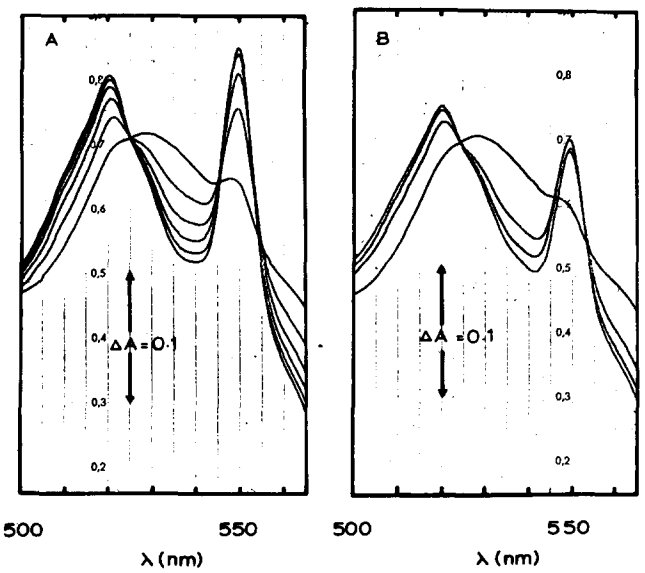

Fig.1. Reduction of cytochrome *c* by oxyhemoglobin in the presence and absence of superoxide dismutase as a function of time. The sample cuvette contained 25 μM ferricytochrome *c*, 26 μM oxyhemoglobin, 100 nM catalase in 1.0 M potassium phosphate buffer (pH 6.0). The reference cuvette contained the same mixture except for cytochrome *c*. Spectra were recorded at 25 °C at regular intervals of 30 min. A, in the absence of superoxide dismutase; B, in the presence of superoxide dismutase (0.8 μM).

lowing experiment. Oxyhemoglobin was converted into its carbon monoxide complex. On incubation of CO-hemoglobin with cytochrome *c* in a medium (1 M potassium phosphate, pH 6.0) saturated with CO, no reduction of cytochrome *c* was observed.

We conclude from our results that *in vitro* a decomposition product of oxyhemoglobin is the superoxide anion. Although the conditions used are non-physiological (1 M potassium phosphate, pH 6.0), it may be possible that *in vivo* during the autoxidation of oxyhemoglobin superoxide anions are formed. Therefore we suggest that the physiological role of superoxide dismutase in erythrocytes is to prevent the deleterious action[18] of the superoxide anions by converting them rapidly into oxygen and hydrogen peroxide. The latter product is then decomposed into water and oxygen by catalase. After completion of these studies on mammalian hemoglobin, Misra and Fridovich[19] reported on the autoxidation of shark hemoglobin. Since hemoglobin of lower animals is particularly prone to autoxidation, they were able to demonstrate the generation of the superoxide anions under physiological conditions.

Thanks are due to Prof. E.C. Slater for his interest; to Prof. P. Borst and Mr A.O. Muijsers for their stimulating discussions and to Dr H.C. Passam for critical reading of the manuscript. This work was supported by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.)

REFERENCES

1 McCord, J.M. and Fridovich, I. (1969) *J. Biol. Chem.* 244, 6049–6055
2 Misra, H.P. and Fridovich, I. (1972) *J. Biol. Chem.* 247, 188–192
3 Massey, V., Palmer, G. and Ballou, D. (1971) in *Flavins and Flavoproteins* (Kamen, H., ed.), pp. 349–361, University Park Press, Baltimore, Butterworth, London
4 Misra, H.P. and Fridovich, I. (1971) *J. Biol. Chem.* 246, 6886–6890
5 George, P. and Stratmann, C.J. (1952) *Biochem. J.* 51, 418–425
6 George, P. and Stratmann, C.J. (1954) *Biochem. J.* 57, 568–573
7 Weiss, J.J. (1964) *Nature* 202, 83–84
8 Maling, J.E. and Weissbluth, M. (1969) in *Solid State Biophysics* (Wyard, S.J., ed.), pp. 327–368, McGraw-Hill, New York
9 Wittenberg, J.B., Wittenberg, B.A., Peisach, J. and Blumberg, W.E. (1970) *Proc. Natl. Acad. Sci. U.S.* 67, 1846–1853
10 Koster, A.S. (1972) *J. Chem. Phys.* 56, 3161–3164
11 Wang, J.H., Nakahara, A. and Fleischer, E.B. (1958) *J. Am. Chem. Soc.* 80, 1109–1113
12 Wang, J.H. (1970) *Acc. Chem. Res.* 3, 90–97
13 Drabkin, D.L. (1946) *J. Biol. Chem.* 164, 703–723
14 Margoliash, E. and Walasek, O.F. (1967) in *Methods in Enzymology* (Estabrook, R.W. and Pullman, M.E., eds), Vol. 10, pp. 339–348, Academic Press, New York
15 Antonini, E. and Brunori, M. (1971) *Hemoglobin and Myoglobin in their Reactions with Ligands*, p. 19, North-Holland Publ. Co., Amsterdam and London
16 Van Gelder, B.F. and Slater, E.C. (1962) *Biochim. Biophys. Acta* 58, 593–595
17 Wu, C.-S. C., Duffy, P. and Brown, W.D. (1972) *J. Biol. Chem.* 247, 1899–1903
18 Fee, J.A. and Teitelbaum, D.H. (1972) *Biochem. Biophys. Res. Commun.* 49, 150–158
19 Misra, H.P. and Fridovich, I. (1972) *J. Biol. Chem.* 247, 6960–6962

The Author and Subject index of this volume can be found on p. 465 and p. 468, respectively.