Effect of Peroxodisulphate Ions on Functional Behaviour of Hemoglobin

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Abstract. The influence of ammonium peroxodisulphate on the functional state of human hemoglobin was studied. The investigation techniques included: laser photolysis, spectrophotometry and pH measurements. Two different actions of the peroxodisulphate ions upon hemoglobin seem to occur; a) a proton consuming process and an increase of the 4th CO-binding rate constant, l'_4, but not yet oxidation of the heme iron (when $S_2O_8^{2-}$ and heme concentrations were of the same order), and b) a marked oxidation effect (when $S_2O_8^{2-}$ was added in excess).

Key words: Hb ligand kinetics — Laser photolysis — Hb oxidation — Peroxodisulphate action

Introduction

Reversible oxygenation of human hemoglobin (Hb) occurs only for the reduced state of the heme iron (see Antonini and Brunori 1971; Imai 1982). In the erythrocyte, a low level of the ferric state is maintained by enzymatic reduction (Bishop 1964). Hb purification generally results in an enhanced autooxidation rate. It was established that Hb autooxidation involves the participation of superoxide radical anion and H_2O_2 as intermediates (Winterbourn et al. 1976). These species were also found to appear during the Hb oxidation in the presence of chemical agents, most of them being cations (Rifkind 1974; Winterbourn and Carrell 1977; Brittain 1980) and only a few anions (Tomoda et al. 1981a; Tarburton and Metcalf 1986). In some cases, the oxidizing reactants act directly on protein sites; in others they influence the heme iron oxidation process by changing Hb conformation (Tomoda and Yoneyama 1979).

In view of these findings, peroxodisulphate anions ($S_2O_8^{2-}$) seem to be interesting reactants for Hb oxidation studies, due to their known oxidative properties, to their relationship with H_2O_2 and to their capability to dissociate into radicals

(e.g. Gupta 1989), as well as to their use in certain industries: production of sugars, oils for soaps etc. (see Pascal 1960). Some metabolic effects of these salts were also found (e.g. Mellor 1956).

This paper presents our preliminary results, obtained by means of laser photolysis in the microsecond time range, concerning the influence of ammonium peroxodisulphate upon functional state of Hb. This influence was also studied using static optical spectroscopy and pH measurements. Our data indicate modifications to occur in kinetic parameters of ligand binding, as well as structural changes of Hb. This *in vitro* behaviour of peroxodisulphate anions against Hb is intended to be subsequently used to experimentally support a model of the electron transfer pathway through which the heme iron is oxidized.

Materials and Methods

Oxyhemoglobin (HbO_2) was obtained from normal human erythrocyte hemolysates and purified by a method described previously (Serbănescu and Turcu 1987). HbO_2 solution was deoxygenated under vacuum and then converted into carbonmonoxyhemoglobin (HbCO) by CO bubbling up to saturation ([CO]=1 mmol/l). Unbuffered solutions were generally used. In a few experiments, dilutions were made with phosphate buffer (pH 7). Final concentrations (50–60 μmol/l in heme) were spectrophotometrically checked using the extinction coefficients given by van Kampen and Zijlstra (1983). Aminoacids and all other chemicals were of p.a. grade.

Ligand recombination kinetics of HbCO was studied after CO photodissociation by a nitrogen laser (337.1 nm) delivering pulses of 0.5 mJ and 10 ns, under low photolysis level (5–6%) conditions. Observations of these kinetics were performed at 438 nm, using a quartz cuvette with 2 mm optical path length. Data were collected on a Tektronix oscilloscope (Serbănescu et al. 1989), each trace used for analysis consisting of 30 superimposed curves obtained by laser shot repetitions. These traces were graphically smoothed. Total absorbance change values $\Delta A^{total}(t)$ were calculated at different moments t after CO photolysis as logarithm of the ratio between the photovoltage measured before excitation and its transient value at time t. Since the observed recombination process was biphasic, $\Delta A^{total}(t)$ represented the sum of the two stages. Further data analysis resulted in two rate constants l'_{fast} and l'_{slow} for the two stages of the recombination kinetics. These rate constants were assigned to l'_4 and l'_3 respectively (Sharma 1983; Serbănescu et al. 1989). Photolysis experiments were carried out up to 40 minutes during HbCO reaction with $S_2O_8^{2-}$, and each time the kinetic parameters were calculated.

Optical absorption changes accompanying HbO_2 and HbCO reaction with the peroxodisulphate ions were registered with a Specord UV-VIS (Jena) spectrophotometer in order to obtain some reaction parameters and to identify the end products. The spectra were recorded at a fixed repetition rate in the range 450–700 nm or as a function of time at a fixed wavelength.

pH of HbO_2 and HbCO samples in the presence of peroxodisulphate was measured with a Corning EEL 10 pH-meter equipped with a glass electrode. pH values were not corrected for the small influence of protein concentration on the response of the pH-meter.

Details on the reactant concentrations are mentioned in the legends to the respective Figures.

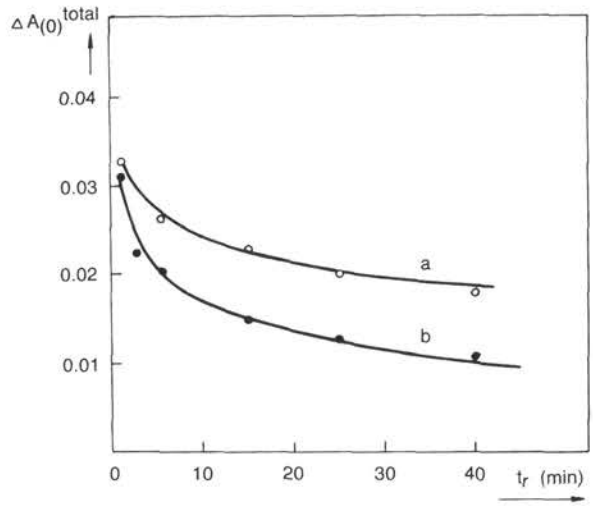

Figure 1. Dependence of total absorbance change $\Delta A^{total}(0)$ (at zero photolysis time) on the reaction time, t_r, after the mixing of HbCO with $(NH_4)_2S_2O_8$. $[HbCO]_{t_r=0}$ = 52 μmol/l. $[HbCO]/[S_2O_8^{2-}]$: *a*) 1/5; *b*) 1/20.

Results

Laser photolysis

The total absorbance change following HbCO photolysis, calculated at different moments during HbCO interaction with $S_2O_8^{2-}$ is presented in Fig. 1. A decrease of ΔA^{total} down to 1/3 from the value at zero reaction time can be observed after 40 minutes, indicating a diminution of the number of HbCO molecules participating in the ligand rebinding process, as an effect of the peroxodisulphate ions action.

The fourth CO molecule rebinding rate constant l'_4, obtained during HbCO - peroxodisulphate reaction is plotted in Fig. 2 at two molar ratios $[HbCO]/[S_2O_8^{2-}]$. l'_4 was found to markedly increase by 43% when $[HbCO]/[S_2O_8^{2-}]=1/5$, and by 58% when the ratio was 1/20, as compared to l'_4 value in the absence of $S_2O_8^{2-}$ ions.

Since catalase is known to break down H_2O_2, its influence upon CO recombination rate constant l'_4 was also checked. As can be seen from Fig. 2, in the presence of this enzyme, the l'_4 increase during $HbCO+S_2O_8^{2-}$ reaction was less important (42%). Buffering of the medium was also found to diminish l'_4 variation.

It should be mentioned that l'_3 (the 3rd CO rebinding rate constant) remains nearly constant at the same reaction time, having a R - like value of $(1.64\pm0.08) \cdot 10^6 mol^{-1} l\, s^{-1}$.

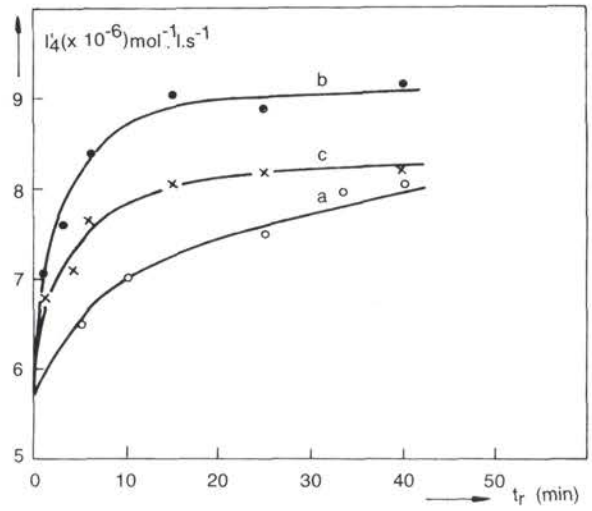

Figure 2. Variation of l'_4 rate constant with the reaction time, t_r, during HbCO interaction with $(NH_4)_2S_2O_8$.$[HbCO]_{t_r=0}$ = 52 μmol/l.$[HbCO]/[S_2O_8^{2-}]$: *a*) 1/5; *b*) 1/20.; *c*) 1/20, but in the presence of catalase (1.3 μg/ml).

Optical spectroscopy

The effects of peroxodisulphate upon HbO_2 and HbCO were studied by recording the absorption spectra in the 450–700 nm range. Spectral modifications were observed only under conditions of excess $S_2O_8^{2-}$ ions ($[Hb]/[S_2O_8^{2-}]$ = 1/40). Figs. 3*a* and 3*b* present these spectral changes recorded at fixed repetition rates.

The peaks which appeared at 500 and 630 nm during $S_2O_8^{2-}+HbO_2$ reaction (Fig. 3*a*) indicate the presence among the reaction products of high spin methemoglobin (metHb) (Uchida et al. 1970), while the spectral evolution in the case of HbCO (Fig. 3*b*) seems to rather reveal the presence of valence hybrids after 12 minutes (Tomoda and Yoneyama 1979; Brittain 1980; Tomoda et al. 1981b). No destruction of the heme group sets in, as revealed by the remaining of the isosbestic points at 525 and 590 nm (Fig. 3*a*) or at 519 and 585 nm (Fig. 3*b*) (Ribarov et al. 1985). The reaction advances more rapidly for HbO_2 than for HbCO under the same concentration conditions.

An analysis of the kinetic curves yielded the percent composition of reaction products 10 min after the mixing of peroxodisulphate with HbO_2 or HbCO. In the first case, it showed 80% metHb, and in the second one, only 45%. The composition found in the last case might indicate the formation of valency hybrids, as expected.

It must be noted that for the same molar ratio ($[Hb]/[S_2O_8^{2-}]$=1/5) as that used in the photolysis studies, no spectral changes were observed after 4 hours.

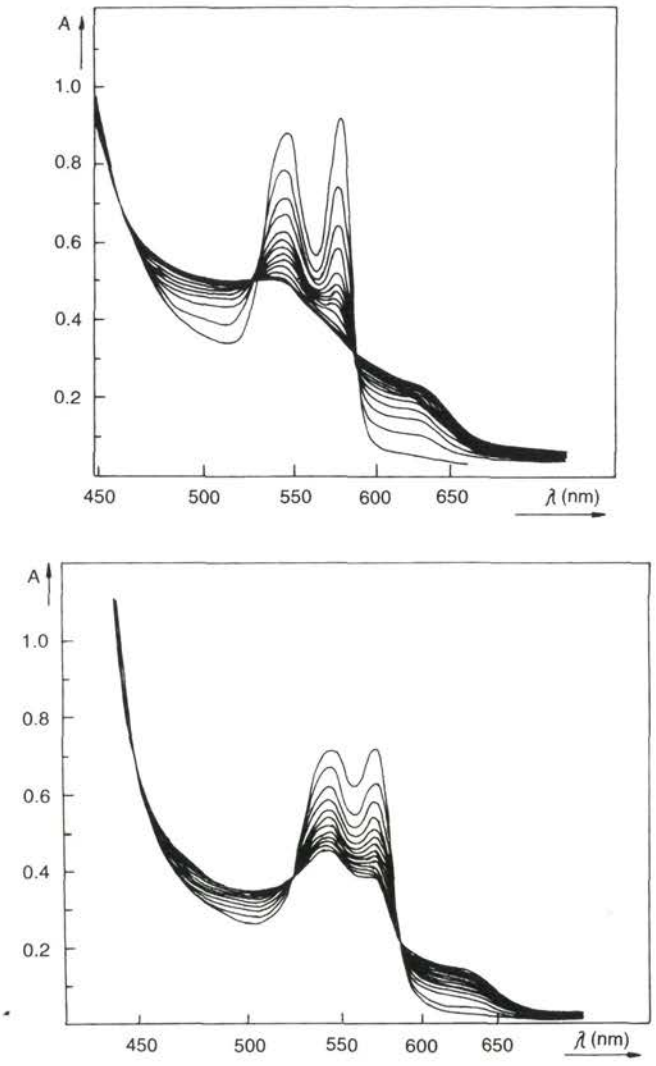

Figure 3. Changes in the absorption spectrum of: *a*) HbO_2; *b*) HbCO during the interaction with $S_2O_8^{2-}$ ions in water. $[HbO_2]_{t_r=0} = 63$ μmol/l; $[HbCO]_{t_r=0} = 53$ μmol/l; $[Hb]/[S_2O_8^{2-}] = 1/50$. Time interval between two successive recordings: *a*) 67 s; *b*) 57 s.

The semilogarithmic plots from Fig. 4 refer to the variation of Hb concentration as a function of reaction time of HbO_2 and HbCO oxidation by $S_2O_8^{2-}$ ions. The plot is linear for HbCO, indicating pseudo-first order reaction, while for $HbCO_2$, the plot is more complex. Consequently, the calculation of partial reaction order with respect to HbO_2 by vant' Hoff differential method gave a value of 2.1 ± 0.1.

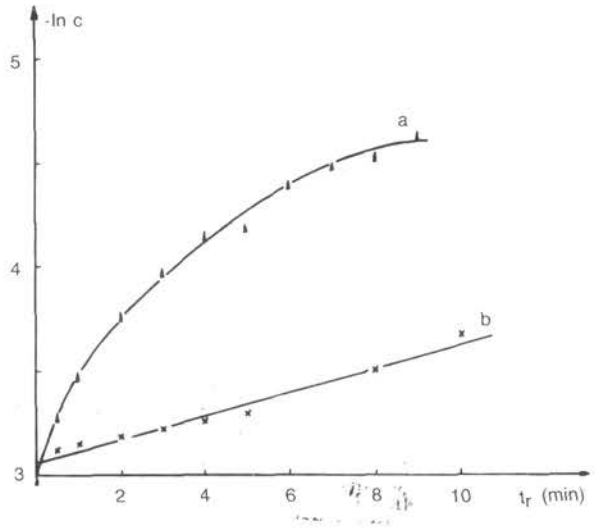

Figure 4. Decrease of hemoglobin concentration c during the interaction of HbCO or HbO_2 with $S_2O_8^{2-}$ ions. *a*) $[HbO_2]_{t_r=0} = 63$ μmol/l; $[HbO_2]/[S_2O_8^{2-}] = 1/50$ *b*) $[HbCO]_{t_r=0} = 53$ μmol/l; $[HbCO]/[S_2O_8^{2-}] = 1/50$.

Table 1. Half-lives of HbO_2 and HbCO concentrations following the interaction with different oxidizing agents

	Oxidant			
Hb form	$NaNO_2$ (Tomoda et.al 1981*a*)	Cu^{2+} (Winterbourn and Carrell 1977)	$S_2O_8^{2-}$ (this paper)	$S_2O_8^{2-}$ (catalase) (this paper)
HbO_2	240 s	115 s	120 s	150 s
HbCO	–	–	480 s	–

Since the establishment of the rate law and of the stoichiometry for both oxidation reactions need further experiments, we estimated their half life time, $t_{1/2}$, and compared its values with those obtained for other oxidation processes. The results are listed in Table 1.

By means of optical absorption spectroscopy, we also examined the $S_2O_8^{2-}$ induced oxidation reaction for some Fe^{2+} complex ions, used as models for protein embedded hemes: the aqueous complex, the tris-phenanthroline complex, and the hexacyanoferrate ion. These complexes have respectively the following values for the Fe^{2+}/Fe^{3+} redox potential: 0.771 V, 1.141 V and 0.350 V (George et al. 1966),

whereas the value for HbO_2 is 0.168 V (Santucci et al. 1984).

The spectra in the visible range showed that only the aqueous complex has undergone an oxidation process within few minutes after reagent mixing. We concluded that HbO_2 and HbCO oxidation by $S_2O_8^{2-}$ ions cannot be accounted for only on the basis of redox potential values but probably by considering the electric double layer. Protein configuration must play a significant role in the reaction mechanism.

In order to find other possible Hb sites playing the role of targets for $S_2O_8^{2-}$ ions, we studied the reactions of these ions with some aminoacids: tryptophane, tyrosine, lysine and cysteine. Only cysteine in alkaline medium showed spectral changes which were related to SH group modifications, though not up to its total oxidation (e.g. Lehninger 1975).

pH measurements

pH values of HbO_2 and HbCO aqueous solutions were shown to decrease under the influence of $S_2O_8^{2-}$ anions. pH variation curves vs. reaction time are presented in Fig. 5.

We observed that pH lowering is directly related to the concentration of $S_2O_8^{2-}$ ions, as it would be expected if the ammonium ion hydrolysis was the proton source. However, the pH decrease is much smaller than that assumed at the hydrolysis expense. Consequently, we assumed that a proton consumption process took place.

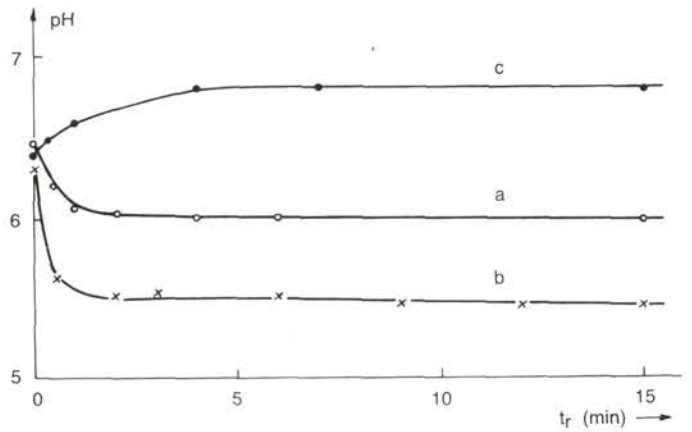

Figure 5. pH variation during HbCO and HbO_2 interaction with the oxidizing reagents *a*) $[HbCO]_{t_r=0} = 58$ μmol/l; $[HbCO]/[S_2O_8^{2-}] = 1/3.3$; *b*) $[HbCO]_{t_r=0} = 50$ μmol/l; $[HbO_2]/[S_2O_8^{2-}] = 1/3.6$; *c*) $[HbO_2]_{t_r=0} = 50$ μmol/l; $[HbO_2]/[NO_2^-] = 1/1000$.

Our calculations indicated that up to 3.7 H^+/HbO_2 chain and 4.5 H^+/HbCO chain disappeared.

These results were indirectly checked by comparing them with those obtained for $HbO_2+NO_2^-$ system, as already mentioned. pH was found to increase during this reaction (Fig. 5, curve c). This behaviour can be well explained by the established mechanism, which, according to the overall equation, needs protons (Tomoda et al. 1981 a):

$$Hb(O_2)_4 + 4\,NO_2^- + 4\,H^+ \rightarrow \text{met Hb} + 4\,NO_3^- + 2\,H_2O + O_2 \qquad (1)$$

Discussion

The results presented suggest the complexity of the actions of peroxodisulphate ions upon human HbO_2 and HbCO. Complex actions have also been described for other reagents such as Cu^{2+} ions, which enhanced the rate of Hb autooxidation process when added in small amounts (Rifkind 1974), but lead to oxidized Hb forms at equimolar or greater concentrations (Winterbourn and Carrell 1977).

The peroxodisulphate ions may act as oxidizing reagents on Hb, following the oversimplified equations:

$$2\,Fe^{2+} + S_2O_8^{2-} \rightarrow 2\,Fe^{3+} + 2\,SO_4^{2-} \qquad (2)$$

$$2\,R-S^- + S_2O_8^{2-} \rightarrow R-S-S-R + 2\,SO_4^{2-} \qquad (3)$$

where Fe^{2+} could be the heme iron and RS^-, the exposed thiol group in sulphide form.

In order to assure the stoichiometric conditions so that at least these two reactions take place, it is sufficient to keep the molar ratio of Hb (as heme) to the oxidant smaller than 1/1. Considering the broad range of $S_2O_8^{2-}$ concentrations used in our experiments, the anion effects on the ligand affinity and on tetramer dissociation into dimers could be rejected in the cases studied by us, due to the small ionic strength introduced by the used concentrations of ammonium peroxodisulphate. For the same reason, we supposed that the acid-base properties of hemoglobin were not perceptibly modified (Antonini and Brunori 1971).

It was pointed out that fast oxidation process of Hb occurs only when $S_2O_8^{2-}$ is added in excess. Under these conditions, the initial oxidation rate follows the order HbO_2 > HbCO also observed for other oxidative actions (Rifkind 1974), and it accounted for the same order of the 4th ligand dissociation rate constants for HbO_2 and HbCO. Moreover, the products of the oxidation process depend on the nature of the heme ligand.

When the molar reactant ratio is near 1/5 the oxidation process is very slow. Instead, a proton consuming process proceeds. It is very well known that protons

are taken up upon oxygenation, below pH = 6.3 (Imai 1982). Any oxidation process needs ligand releasing as a preliminary step, so that proton consumption observed by us cannot be explained on the basis of the acid Bohr effect.

On the other hand, the kinetics of ligand binding are modified, and a part of HbCO molecules are inactivated for laser photolysis.

The rise of l'_4 by approx. 40% in the presence of $S_2O_8^{2-}$ ions cannot merely be explained as being due to the pH decrease, as previous experiments (Serbănescu et al. 1989) indicated a l'_4 increase by only 3% upon dropping pH from 6.5 to 5.5. The observed increase of l'_4 is influenced by catalase and by the solutions being buffered, somehow relating the participation of H_2O_2 and protons to the peroxodisulphate action upon Hb-CO ligand kinetics. In the trame of the two states allosteric model (Shulman et al. 1975), the 4th CO binding rate constant l'_4 describes the kinetic propperties of the Hb R state. The raised l'_4 values could indicate conformational modifications of this quaternary state. On the other side, l'_4 increase could reflect a modification of the positive charge density in the vicinity of Fe^{2+} as suggested by extensive discussions (Paul et al. 1985) regarding the axial Fe-ligand line as an important pathway for protein-heme interactions. A relationship was also established between the protonation state of the iron coordinated proximal imidazol and CO-binding rate constant to porphyrin systems (Valentine et al. 1979; Stanford et al. 1980). In this sense, a hydrophobic electron transfer channel from Fe^{2+} through proximal imidazole to the exposed cys(β93) was proposed in general terms by Moore and Wiliams (see Winterbourn and Carrel 1977).

Free cysteine spectral modifications observed by us in the presence of $S_2O_8^{2-}$ ions seem to be an indication of the action of these ions upon the exposed β93 SH groups. The pathway through which this perturbation could modify heme iron reactivity towards the gaseous ligand remains to be clarified.

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Final version accepted July 29, 1991