A Stopped-Flow Study of the Cupric Ion Oxidation of Adult-Human Haemoglobin

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The addition of excess Cu^{2+} to adult human haemoglobin leads to the production of $\alpha_2^{2+}\beta_2^{3+}$, in both the oxy and deoxy forms of the protein. Stopped-flow studies of the oxidation process yields apparent second-order rate constants of $196\text{ M}^{-1}\cdot\text{s}^{-1}$ and $41\text{ M}^{-1}\cdot\text{s}^{-1}$ for the deoxy and oxy forms respectively. The rate of the deoxy-form oxidation is linearly dependent on $[\text{Cu}^{2+}]$, whereas that of the oxy form is rate-limited above 2 mM to 0.11 s^{-1} . Arrhenius activation energies of the two processes are almost identical at $91\text{ kJ}\cdot\text{mol}^{-1}$, as are the activation enthalpies of $89\text{ kJ}\cdot\text{mol}^{-1}$. The activation entropies show small differences, being 31 entropy units and 48 entropy units for the oxy and deoxy forms respectively. ATP and glycerate 2,3-bisphosphate at saturating concentrations do not affect the rate of oxidation of the oxy form, but halve the rate found for the deoxy form. These data are discussed in terms of the previously proposed mechanism of oxidation in which slow Cu^{2+} binding is followed by rapid electron transfer.

During the 120-day circulatory lifetime of erythrocytes, approx. 2–3% of the haemoglobin is oxidized per day. Thus to maintain the haemoglobin in the oxygen-carrying ferrous form, much of the cells metabolic energy is directed towards the process of re-reduction. It has been shown that cupric (Cu^{2+}) ions have a significant role to play in the oxidation process (Rifkind, 1974; Winterbourn *et al.*, 1976). The reaction of Cu^{2+} with haemoglobin leads only to the oxidation of the β -chains of the protein tetramer via an interaction with the β -93 cysteine residues (Winterbourn & Carrell, 1977).

Winterbourn & Carrell (1977) previously showed, in somewhat qualitative terms, that Cu^{2+} oxidation of the oxy form of haemoglobin is slower than that of the deoxy form. However, owing to technical difficulties, no quantitative determination could be made of the rate of oxidation for the deoxy form, and the concentration range of Cu^{2+} accessible to these investigators was severely limited.

A number of papers have appeared concerned with the reverse situation in which fully oxidized methaemoglobin is reduced to an intermediate 'valence hybrid' analogous to that found in the Cu^{2+} oxidation of ferrous haemoglobin. It has been found that reaction of methaemoglobin with ascorbate leads solely to the reduction of the β -chains (Tomoda *et al.*, 1978*a,b*), whereas reaction with the NADPH-flavin reductase system yields both $\alpha_2^{3+}\beta_2^{2+}$ and $\alpha_2^{2+}\beta_2^{3+}$.

The present paper reports a more detailed study carried out to characterize more fully the reaction that occurs during the oxidation of adult human haemoglobin by Cu^{2+} , over a wider range of conditions than previously reported, and to investigate further the mechanism whereby oxidation takes place.

Experimental

Haemoglobin was purified from normal adult erythrocyte haemolysates essentially as described by Drabkin (1946). The final haemoglobin solution was dialysed for 2 days at 4°C against four changes of 0.1 M-sodium phosphate, pH 7.2. All haemoglobin solutions were reduced with an excess of sodium dithionite before use to remove any methaemoglobin present, and then run through a column (25 cm $\times$ 2 cm) of Sephadex G-25 to remove reaction products. The concentration of the stock solution was determined spectrophotometrically by using a millimolar absorption coefficient of 66 cm^{-1} for the oxy form at 576 nm (Benesch *et al.*, 1973). Deoxygenation was achieved by evacuation followed by re-equilibration of solutions with O_2 -free N_2 (New Zealand Industrial Gases, Auckland, New Zealand).

All solutions were made up in doubly deionized water, which had a resistivity of $>180\text{ M}\Omega\cdot\text{cm}^{-1}$ (Millipore, Bedford, MA, U.S.A.). Kinetic experiments were performed in unbuffered solution, as it

was observed that, in phosphate buffers, mixing with Cu^{2+} leads to precipitation, and most other buffering systems lead to Cu^{2+} complex-formation, which might affect the mechanism of oxidation. In these cases the pH of the protein solution was measured before and after mixing with CuSO_4 ; in no case did the pH alter by more than 0.3 pH unit. Thus in no case did the starting pH of 6.9 move out of the region for which it has been previously shown the reaction is pH-insensitive (Winterbourn & Carrell, 1977).

Static spectra were recorded by using an Aminco DW2a spectrophotometer equipped with a magnetic-stirrer accessory (American Instrument Co., Silver Spring, MD, U.S.A.). Rapid kinetic experiments were performed by using an Aminco-Morrow stopped-flow apparatus with a dead time of 3 ms for the 1-cm cell, in combination with the DW2a spectrophotometer. Wavelength pairs used in kinetic dual-wavelength experiments were determined from static difference spectra. Temperature control for both static and kinetic studies was provided by a circulating water bath (Grant Instruments, Cambridge, U.K.).

For the analysis of kinetic traces the output from the DW2a spectrophotometer was put into a Data Lab 901 transient recorder (Data Laboratories, Mitcham, Surrey, U.K.). Each reaction trace was digitized into 1000 data points and viewed on a Telequipment S61 oscilloscope (Tektronix, Beaverton, OR, U.S.A.) and permanent records obtained by output to a Varian Techtron chart recorder (Varian Ltd., Los Angeles, CA, U.S.A.). Digital output from the transient recorder was interfaced to a Compucorp 610 mini-computer (Compucorp, Los Angeles, CA, U.S.A.) and the reaction traces were analysed by using in-house exponential 'peel' computer programs. All analyses presented are for data for which the calculated correlation coefficients were better than 0.98.

Glycerate 2,3-bisphosphate and ATP were obtained from May and Baker (Dagenham, Essex, U.K.).

Results

Static studies

When excess Cu^{2+} is added either aerobically to oxyhaemoglobin or anaerobically to deoxyhaemoglobin then a product is formed that has the spectral characteristics easily identifiable as arising from the oxidation of half of the haem centres present (Figs. 1a and 2a). As previously reported (Winterbourn & Carrell, 1977), prolonged exposure of haemoglobin solutions to excess Cu^{2+} in the presence of phosphate leads to precipitation. However, if unbuffered solutions are used, then no precipitation occurs over the same time range (~30 min) and solutions

Fig. 1. Static and kinetic difference spectra of the Cu^{2+} oxidation of oxyhaemoglobin

(a) the static spectra of oxy-(---), met-(—) and valence-hybrid-(···)haemoglobin. Protein was $16\mu\text{M}$ in 0.1 M-sodium phosphate, pH 7.2, in a 1-cm cell. A 100-fold molar excess of Cu^{2+} was added to oxyhaemoglobin to give the valence hybrid. (b) Comparison of static (—) and kinetic (O) difference spectra for the reaction of excess Cu(II) with $12.5\mu\text{M}$ -oxyhaemoglobin in a 1-cm cell.

appeared stable for up to 6 h. This observed precipitation is at least in part due to the formation of insoluble copper phosphate, which seems to have been overlooked in previous studies.

Kinetic studies

A comparison of the kinetic and static difference spectra (Figs. 1b and 2b) shows that no process is being overlooked in the kinetic experiments.

When mixed with excess Cu^{2+} , oxyhaemoglobin showed a simple reaction trace consisting of a single exponential decay (Fig. 3).

Deoxyhaemoglobin when made to react with excess Cu^{2+} anaerobically shows a simple reaction trace consisting of a single exponential decay, as in the case of the oxy form (Fig. 3). The oxy form shows a linear dependence on $[\text{Cu}^{2+}]$ up to 2.5 mM, where it rate limits at 0.11s^{-1} , with an apparent

Fig. 2. Static and kinetic difference spectra of Cu(II) oxidation of deoxyhaemoglobin

(a) The static spectra of deoxy-(---), met-(—) and valence-hybrid(-.-.)haemoglobin. (b) Comparison of static (—) and kinetic (O) difference spectra of deoxyhaemoglobin. In both (a) and (b) conditions were as in Figs. 1(a) and 1(b).

Fig. 3. Progress curves of the Cu^{2+} oxidation of oxy- and deoxy-haemoglobin

(a) A typical reaction trace obtained for the oxidation of 5.1 μ M-oxyhaemoglobin by 4 mM- Cu^{2+} in a 1-cm stopped-flow cell at 15°C. (b) A typical reaction trace obtained for the oxidation of 6 μ M-deoxyhaemoglobin by 2.5 mM- Cu^{2+} in a 1-cm cell of the stopped-flow apparatus at 15°C.

second-order rate constant, derived from the linear slope of $41 \text{ M}^{-1} \cdot \text{s}^{-1}$. The deoxy form, however, shows a linear dependence on $[\text{Cu}^{2+}]$ over the entire range investigated, up to 5 mM. (Figs. 4 and 5) with an associated apparent second-order rate constant of $196 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Effect of temperature

To characterize further the two reactions, the temperature-dependence of the rates of oxidation was studied. Figs. 6(a) and 6(b) show the effect of temperature on the rate of oxidation of oxy and deoxy forms respectively over the range 5–35°C, cast in the form of Arrhenius plots. The activation energies obtained from these plots are $91.6 \text{ kJ} \cdot \text{mol}^{-1}$ for the oxy form and $91.8 \text{ kJ} \cdot \text{mol}^{-1}$ for the deoxy form. The difference in the rates of oxidation of the two forms together with the virtual identity of the Arrhenius activation energies suggested a closer investigation of the effect of temperature on the reaction rates was warranted. The data was recast in

Fig. 4. $[\text{Cu}^{2+}]$ -dependence of the oxidation of oxyhaemoglobin

Oxyhaemoglobin (6.2 μ M) was made to react with Cu^{2+} at 17°C in a 1-cm cell. The reaction was monitored by measuring the change in absorbance difference between 576 and 525 nm.

the form of Eyring plots (Atkins, 1978) of $\ln(k/T)$ versus $1/T$. This reploting yields activation enthalpies of $89.1 \text{ kJ} \cdot \text{mol}^{-1}$ and $89.5 \text{ kJ} \cdot \text{mol}^{-1}$ for the oxy and deoxy forms respectively. Substitution of these data into eqn. (1)

$$\ln k = \ln(kT/h) + \Delta S^\ddagger/T^\ddagger - \Delta H^\ddagger/RT \quad (1)$$

(k is the observed rate constant, k is Boltzmann's constant, h is Planck's constant and $\Delta S^\ddagger$ and $\Delta H^\ddagger$ the activation entropy and enthalpy of the reaction respectively) yields values of the activation entropy for the oxidations of 31.2 and 48.1 entropy units for the oxy and deoxy forms respectively.

Effects of allosteric effectors

Table 1 summarizes the effects of the allosteric effectors glycerate 2,3-bisphosphate and ATP on the rate of Cu^{2+} oxidation of both oxy- and deoxy-haemoglobin. The addition of excess glycerate 2,3-bisphosphate and ATP clearly does not affect the rate of oxidation of the oxy form, but does lead

Fig. 5. $[\text{Cu}^{2+}]$ -dependence of the oxidation of deoxy-haemoglobin

Deoxyhaemoglobin ($6 \mu\text{M}$) was made to react anaerobically with Cu^{2+} at 17°C in a 1-cm cell. The reaction was monitored by measuring the change in absorbance difference between 556 and 527 nm.

to a decrease in the rate of oxidation of the deoxy form by a factor of approx. 2.

Discussion

By comparison with published spectra (Tomoda *et al.*, 1978a; Banerjee & Cassoly, 1969) of valence-

Fig. 6. Effect of temperature on the rate of Cu^{2+} oxidation of oxy- and deoxy-haemoglobin

(a) $5.5 \mu\text{M}$ -Oxyhaemoglobin was reduced with 2 mM - Cu^{2+} in the 1-cm cell of the stopped-flow apparatus. The reaction was monitored by measuring the difference in absorbance between 576 and 588 nm. (b) The anaerobic oxidation of deoxyhaemoglobin. Conditions were as in (a), except that the reaction was monitored at 556 and 527 nm.

Table 1. Comparison of the effect of ATP and glycerate 2,3-bisphosphate (DPG) on the rate of Cu(II) oxidation of oxy- and deoxy-haemoglobin

In all cases $6.8 \mu\text{M}$ -haemoglobin was made to react with 2 mM - Cu^{2+} at 17°C in the 1-cm cell of the stopped-flow apparatus.

Effector	Concn. (mg/ml)	Rate (s^{-1})	
		Oxyhaemoglobin	Deoxyhaemoglobin
None	—	0.02	0.29
DPG	0.5	0.017	0.145
ATP	0.05	0.0192	0.139
DPG	0.1	0.0185	0.14

hybrid haemoglobins, prepared by combination of isolated chains, it is clear that the treatment of human adult haemoglobin with excess Cu^{2+} leads to the production of the valence hybrid $\alpha_2^{2+}\beta_2^{3+}$. The production of this species has been shown to require four Cu^{2+} ions per haemoglobin tetramer for the human protein, two of which bind initially to the cysteine residues at position β -93. It has been proposed that this process is followed by electron transfer through a hydrophobic channel, from the haem group to the copper atom, by a mechanism analogous to that proposed by Moore & Williams (1976).

The rates of formation of the valence hybrid found in the present study ($196 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $41 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the deoxy and oxy forms respectively) are very low by comparison with other previously reported electron-transfer rates between metal ions and haem proteins (Brittain & Greenwood, 1975; Brittain *et al.*, 1974).

The following speculative scheme explains the kinetic findings:

In terms of the above scheme the rapid binding of copper to the haemoglobin molecule is followed by internal electron transfer. Assuming the first equilibrium is rapid, a rate limit would be observed at high $[\text{Cu}^{2+}]$, equivalent to $k_{+2} + k_{-2}$, which approximates to k_{+2} when k_{-2} (intercept of Fig. 4) is small.

In the case of the oxy form the rate limit is observed at 0.12 s^{-1} . It should be noted, however, that this rate limit cannot be due to the necessary reaction of Cu^{2+} with the unliganded form, as the O_2 'off-rate' has been measured as 10 s^{-1} at pH 9.0 and 30 s^{-1} at pH 5.5 (Antonini & Brunori, 1971). Previously evidence has been found for the oxidation of liganded haemoglobin by ferricyanide at a rate higher than the ligand-dissociation rate (Wittenberg *et al.*, 1967).

The absence of a rate limit in the case of the deoxy form could then be a consequence of the altered structure in the deoxy form (Perutz, 1970), leading to a more rapid internal electron-transfer rate that is not rate-limiting over the Cu^{2+} concentration range explored.

The study of the effect of temperature on the rate of oxidation shows that the only significant difference in activation is between the activation entropies. The higher activation entropy ($\Delta S^\ddagger$) found for the deoxy form shows that the process proceeds through a more ordered transition state. What molecular configuration actually represents the transition state, in either form of the haemoglobin molecule, remains open to speculation for such a complex reaction.

The possibility that, after the initial binding of Cu^{2+} , electrons are transferred through a hydrophobic channel, is difficult to verify experimentally if the rate of the process is limited by the much slower binding process.

It has been suggested that the physiological role of the electron-transfer system operates in a protective manner, removing the potentially destructive free radicals, such as superoxide, formed in the haem pocket on catalytic oxygen autooxidation (Winterbourn *et al.*, 1976; Wever *et al.*, 1973). To test this hypothesis, experiments are required to investigate any changes in electron transfer to such acceptors as glutathione from normal and cysteine-blocked haemoglobins under both normal conditions and those which are known to enhance free-radical production (Misra & Fridovitch, 1972).

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