Biochem. J. (1977) **165**, 141–148
Printed in Great Britain

Oxidation of Human Haemoglobin by Copper

MECHANISM AND SUGGESTED ROLE OF THE THIOL GROUP OF RESIDUE β-93

By CHRISTINE C. WINTERBOURN and ROBIN W. CARRELL*
Department of Clinical Biochemistry, Christchurch Hospital, Christchurch, New Zealand

(*Received* 29 *November* 1976)

Addition of Cu(II) ions to human oxyhaemoglobin caused the rapid oxidation of the haem groups of the β-chain. Oxidation required binding of Cu(II) to sites involving the thiol group of β-93 residues and was prevented when these groups were blocked with iodoacetamide or *N*-ethylmaleimide. Equilibrium-dialysis studies showed three pairs of binding sites, two pairs with high affinity for Cu(II) and one pair with lower affinity. It was the second pair of high-affinity sites that were blocked with iodoacetamide and were involved in haem oxidation. Cu(II) oxidized deoxyhaemoglobin at least ten times as fast as oxyhaemoglobin, and analysis of rates suggested that binding rather than electron transfer was the rate-determining step. No thiol-group oxidation to disulphides occurred during the period of haem oxidation, although it did occur subsequently in the presence of oxygen, or when Cu(II) was added to methaemoglobin. It is proposed that thiol oxidation did not occur because there exists a pathway of electron transfer between the haem group and copper bound to the β-93 thiol groups. The route for this electron transfer is discussed, as well as the implications as to the function of the β-93 cysteine in the haemoglobin molecule.

In the presence of Cu(II) ions, haemoglobin is rapidly oxidized to methaemoglobin. The reaction occurs with both oxy- and deoxy-haemoglobin, and only 50% of the haem groups are oxidized. Cu(II) appears to be the only metal that is effective, and direct binding to the protein is involved (Rifkind, 1974). Cu(II) reacts with cysteine, and the thiol groups of a number of proteins, to catalyse autoxidation to the disulphides (Friedman, 1973). In haemoglobin it appears that the haem groups are oxidized in preference to the exposed thiol groups of the β-93 residues. The present study was undertaken to investigate the reasons why haem oxidation is preferred, and to obtain information about the copper-binding site and the mechanism of electron transfer from the Cu(II) to the haem iron. We have compared rates of haem and thiol oxidation and have examined the effects of thiol-group-blocking agents on Cu(II) binding and oxidation. We have also measured the effects of Cu(II)-ion concentration, oxygenation of the haemoglobin and pH on the reaction rate.

The reaction of Cu(II) with haemoglobin is of considerable significance in normal erythrocyte metabolism. When present at very low concentrations, copper catalyses the autoxidation of oxyhaemoglobin by a mechanism requiring oxygen but otherwise similar to that of direct oxidation (Rifkind, 1974; Winterbourn *et al.*, 1976). One Cu(II) ion per thousand haem groups significantly increases the rate of autoxidation, and the erythrocyte is thought to contain sufficient copper for this to be the main pathway of methaemoglobin formation (Rifkind, 1974). About 2–3% of circulating haemoglobin is oxidized per day, and other products of the reaction are superoxide and H_2O_2 (Winterbourn *et al.*, 1976). As a functional oxygen carrier the erythrocyte must maintain its haemoglobin in a reduced [Fe(II)] form, and also maintain efficient reducing systems for counteracting the oxidant threat of continual production of superoxide and H_2O_2. This requires a significant proportion of the metabolism of the cell, and it is evident that situations such as increased copper concentrations, which increase methaemoglobin and peroxide production, could stress the metabolic capacity of the cell.

* Present address: Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Cambridge CB2 2QR, U.K.

Experimental

Oxyhaemoglobin was purified from normal human erythrocyte haemolysates by column chromatography on DEAE-Sephadex (Huisman & Dozy, 1965). Separations were performed in the cold in the presence of 0.1 mM-EDTA. EDTA was removed just before each set of experiments by passage through a column of Sephadex G-25. Haemoglobin H was prepared from the blood of an α-thalassaemic patient. Free α- and β-chains were isolated by the method of Geraci *et al.* (1969). Reaction of the β-93 cysteine residues of haemoglobin with iodoacetamide was carried out at pH 7.8 for 3 h at 20°C in the dark, with an iodoacetamide/haemoglobin molar ratio of

10. Reaction with *N*-ethylmaleimide was carried out at pH7.0 for 2h at 37°C in the dark with a molar ratio *N*-ethylmaleimide/haemoglobin of 3. Mixed disulphides with glutathione were prepared by treating oxyhaemoglobin with a 30-fold excess of GSSG* at pH7.8 for 20h at 37°C. In each case the modified haemoglobin was separated from excess of reagent on a column of Sephadex G-25, and the absence of free thiol groups was checked by analysis with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959). Iodoacetamide and GSSG were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and *N*-ethylmaleimide was from Pfaltz and Bauer, Flushing, NY, U.S.A.

Rates of haemoglobin oxidation were determined from changes in A_{576} by using a Beckman Acta Spectrophotometer. Circulating cooled water was used to control temperature, which was monitored by a platinum temperature probe in an adjacent cuvette. Small volumes of 1–10mM-$CuSO_4$ were added to cuvettes containing approx. 7μM-haemoglobin in phosphate buffer, pH7.4, stirred magnetically, and A_{576} values recorded from the time of addition. Phosphate buffers were prepared from 0.067M-Na_2HPO_4 and 0.067M-KH_2PO_4. Reactions of deoxyhaemoglobin were carried out in tonometers and A_{560} was monitored. The $CuSO_4$ solution was held in the tonometer lid during evacuation, and the solutions were mixed by inversion. Millimolar extinction coefficients of 66 for oxyhaemoglobin and 18.6 for methaemoglobin at 576nm, and 53.6 for deoxyhaemoglobin and 18.0 for methaemoglobin at 560nm, were used (Benesch *et al.*, 1973). The production of superoxide and H_2O_2 by the reaction was investigated by comprising rates of autoxidation in the presence and absence of catalase (13μg/ml) and superoxide dismutase (2.7μg/ml). The reaction was also carried out in the presence of ferricytochrome *c* (50μM) to detect any superoxide production. The change in A_{560}, relative to a blank containing haemoglobin plus $CuSO_4$, was recorded. The enzymes and cytochrome *c* were obtained from Sigma.

Binding of copper to haemoglobin was examined by equilibrium dialysis. For this 1–12mol of $CuSO_4$ per mol of haemoglobin was added to 5μM solutions of oxyhaemoglobin in 0.02M-sodium/potassium phosphate buffer, pH7.4, prepared by diluting the 0.067M buffer. The solutions were dialysed against a 20-fold excess of the same buffer for 24h at 4°C. Haemoglobin concentrations were measured by the Drabkin method (Dacie & Lewis, 1975) and copper concentrations in the haemoglobin solutions and diffusates determined by atomic absorption. Bound copper was calculated as the difference between the two values.

* Abbreviations: GSSG, oxidized glutathione; Hb, haemoglobin.

Oxidation of the thiol groups of the β-93 residues to disulphides was investigated by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969). Oxidation produces β-chain dimers, which separate from the single chains in this system (Winterbourn & Carrell, 1974). Changes in the thiol groups of the β-93 residues were also monitored (Ellman, 1959).

Results

Stoicheiometry of the reaction of oxyhaemoglobin with Cu(II) and the effect of thiol-blocking agents

As shown in Fig. 1, addition of four Cu(II) ions per haemoglobin tetramer caused the rapid oxidation of nearly 50% of the haem groups. With additional Cu(II), oxidation more closely approached 50%. With less than two Cu(II) ions per tetramer no rapid oxidation was evident. Fig. 1 also shows that blocking the β-93 thiol groups with iodoacetamide completely eliminated the rapid haem oxidation. A similar result was obtained when these groups were blocked by either *N*-ethylmaleimide or by forming a mixed disulphide with glutathione.

Cu(II) caused the rapid oxidation of purified β-chain haemoglobin (haemoglobin H) but not α-chain haemoglobin. There was no significant oxidation with four Cu(II) ions per β-chain tetramer, but, with further addition of Cu(II), more than 75% of the haem groups were oxidized. Methaemoglobin concentrations above 75% could not be measured because of haemichrome formation and precipitation of the highly unstable oxidized chains.

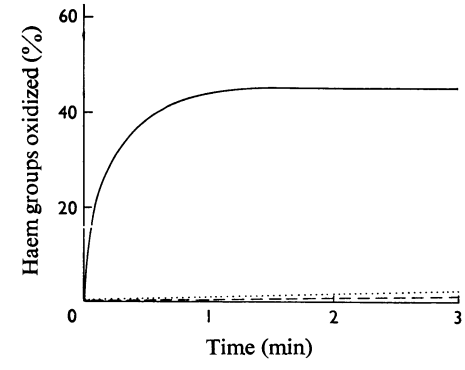

Fig. 1. *Oxidation by Cu(II) ions of human oxyhaemoglobin with and without β-93 thiol groups blocked with iodoacetamide*

——, 7μM-Oxyhaemoglobin + four Cu(II) ions/tetramer; ····, oxyhaemoglobin+two Cu(II) ions; – – – –, blocked oxyhaemoglobin+four or more Cu(II) ions. The percentage of oxyhaemoglobin converted into methaemoglobin was calculated from the change in A_{576}.

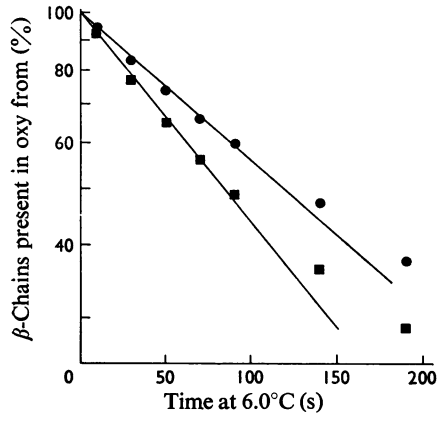

Fig. 2. *Semi-log plot showing the rate of oxidation of oxyhaemoglobin by Cu(II) ions*
●, 6.7 μM-HbO_2+33 μM-$CuSO_4$; ■, 6.7 μM-HbO_2+67 μM-$CuSO_4$. Details are given in the Experimental section. The amount of oxidation was calculated from the change in A_{576}. No β-chains present in the oxy-form corresponds to the maximum oxidation attainable, i.e. 50% of the total haemoglobin oxidized.

Table 1. *Effect of Cu(II)-ion concentration on rate of oxidation of oxyhaemoglobin*
Oxyhaemoglobin solutions (6.7 μM) were incubated at pH7.4 at 6.0°C as described in the Experimental section.

Concn. of $CuSO_4$ (μM)	Hb/Cu	$t_{\frac{1}{2}}$ (s)
33	1:5	115
67	1:10	80
134	1:20	65
268	1:40	55

Superoxide dismutase and catalase inhibit the rate of autoxidation of haemoglobin catalysed by Cu(II), indicating that the reaction produces superoxide and H_2O_2 (Winterbourn *et al.*, 1976). However, direct oxidation of haemoglobin by Cu(II) was found to produce no superoxide or H_2O_2. The rate of reaction was unaffected by superoxide dismutase or catalase, and the reaction did not cause any reduction of cytochrome *c*. If superoxide were produced, it would be expected to reduce cytochrome *c* (McCord & Fridovich, 1969).

Rates of reaction of Cu(II) with haemoglobin

Fig. 2 shows semi-log plots of the percentage of unoxidized β-chains remaining versus time for two different Cu(II) concentrations. The plots were linear for at least 50% of the reaction, representing a pseudo-first-order reaction. The curvature towards the end was partly due to further reaction of the oxidized chains to haemichromes. Half-lives ($t_{\frac{1}{2}}$) were calculated for each set of conditions and used for comparing rates.

Table 2. *Effect of pH on the rate of oxidation of oxyhaemoglobin by Cu(II) ions*
Oxyhaemoglobin solutions (7 μM) were incubated at 6.0°C with 28 μM-$CuSO_4$ in 0.067M-phosphate buffers. The reaction was carried out in duplicate at each pH, and agreement between duplicates was within ±2s.

pH	$t_{\frac{1}{2}}$ (s)
6.0	115
7.0	100
7.4	105
8.0	115

Table 1 shows that the reaction rate increased with increasing Cu(II) concentration. The reaction rate was fairly insensitive to pH change over the range 6–8 (Table 2). It was a maximum at pH7 and declined very slightly on either side.

Comparison of oxy- and deoxy-haemoglobin

Oxidation by Cu(II) was much faster for deoxy- than for oxy-haemoglobin. With 6.7 μM-haemoglobin and five Cu(II) ions per tetramer at 6°C, three experiments with deoxyhaemoglobin gave $t_{\frac{1}{2}}$ values of 12–17s, compared with 102s for oxyhaemoglobin. H9wever, with deoxyhaemoglobin the reaction was so fast that the time for mixing is significant, and the measured $t_{\frac{1}{2}}$ values are less accurate and probably higher than the true values. Cu(II) can therefore oxidize deoxyhaemoglobin about 10 times faster than oxyhaemoglobin. Deoxyhaemoglobin with β-93 thiol groups blocked with iodoacetamide reacted 100–500 times more slowly than did normal deoxyhaemoglobin.

Effects of Cu(II) ions on the β-93 thiol groups

Table 3 summarizes the results of experiments investigating changes to the β-93 thiol groups of haemoglobin on reaction with Cu(II). With four Cu(II) ions per haemoglobin tetramer, there was a rapid decrease in the number of reactive thiol groups, that were not regenerated by extended dialysis against 0.2mM-EDTA, or in the presence of sodium dodecyl sulphate. This loss of thiol groups did not occur with two Cu(II) ions, but occurred more rapidly with six. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed on the samples to detect β-chain dimers that would be produced if the β-93 thiol groups were oxidized to disulphides. With complete oxidation the

Table 3. *Effect of Cu(II) ions on the thiol groups of haemoglobin*

Oxyhaemoglobin or methaemoglobin (6μM) was incubated at 20°C with various amounts of $CuSO_4$ for the times indicated. Maximum oxidation corresponds to 50% of total oxyhaemoglobin. Thiol-group loss was determined by analysis with 5,5'-dithiobis-(2-nitrobenzoic acid). β-Dimers were determined by scanning polyacrylamide gels. 50% dimer bond corresponds to complete oxidation to disulphides. Abbreviation: n.d., none detectable.

	Cu/Hb	Reaction time (min)	Hb oxidation (% of maximum)	Thiol groups lost (%)	β-Dimer band (%)
OxyHb	2	5	n.d.	n.d.	2
		10	n.d.	n.d.	2
	4	1	79	40	2
		10	84	50	4.5
		20		50	7
		30	92	70	15
	6	20	100	80	14
MetHb	4	1		20	6
		10		53	18
		30		87	22

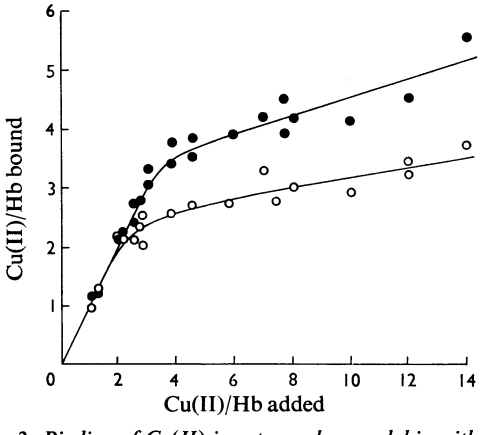

Fig. 3. *Binding of Cu(II) ions to oxyhaemoglobin with and without β-93 thiol groups blocked with iodoacetamide*

Bound Cu(II) ions were measured after 24h dialysis as described in the Experimental section. ●, Oxyhaemoglobin; ○, blocked oxyhaemoglobin.

sample should contain 50% dimer. The amount of dimer increased during the reaction, but there was insufficient formed to account for the loss of measurable thiol groups and production was only detectable after the first minute of reaction (Table 3). By this time haem oxidation was almost complete. The haem groups were therefore oxidized in preference to the thiol groups, and the absence of free thiol groups was probably a consequence of copper binding.

With methaemoglobin and four Cu(II) ions per tetramer, however, measurable thiol groups decreased to near-zero over a period of 30min, and there was a parallel rise in the amount of dimer band on electrophoresis (Table 3). The amount of dimer was almost enough to account for all of the thiol groups lost.

Oxygen appeared to be necessary for thiol-group oxidation in oxyhaemoglobin. Deoxyhaemoglobin or oxyhaemoglobin that was deoxygenated 1 min after addition of Cu(II) produced no dimers during 30min reaction with four Cu(II) ions per tetramer. That O_2 rather than the oxy or *R* configuration of haemoglobin was required for disulphide formation was indicated from the behaviour of a solution containing haemoglobin A and about 30% haemoglobin Heathrow, a high-oxygen-affinity variant that is 'frozen' in the *R* configuration even when deoxygenated. Deoxyhaemoglobin prepared from this mixture and treated as described above also produced no dimers. With methaemoglobin, disulphides were produced in air and under vacuum, although the reaction appeared to be faster when O_2 was present.

Copper–haemoglobin binding studies

Dialysis experiments showed that human oxyhaemoglobin has four sites that bind Cu(II) firmly (Fig. 3). There was very little dissociation from these sites during dialysis against 20vol. of buffer. Weaker binding of additional Cu(II) ions was also detected. There are probably two weaker binding sites, but it was not possible to determine this number accurately because increasing amounts of Cu(II) promoted precipitation of the haemoglobin. Fig. 3 also shows that blocking the β-93 thiol groups with iodoacetamide decreased the amount of Cu(II) binding to haemoglobin. No difference was detected until after the first pair of Cu(II) ions were bound, and the total number bound approached four rather than six. This suggests that the binding sites for the second pair are on the β-chains and require free thiol groups at position 93. Cu(II) binding to methaemoglobin was very similar to binding to oxyhaemoglobin.

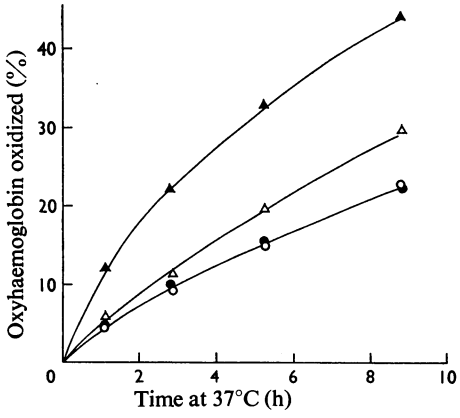

Fig. 4. *Effect of Cu(II) ions on the rate of autoxidation of oxyhaemoglobin with and without β-93 thiol groups blocked with iodoacetamide*

Solutions were incubated at 37°C in 0.067M-phosphate buffer, pH7.4, containing 0.1M-NaCl. Concentrations of oxyhaemoglobin, methaemoglobin and haemichrome were determined by special analysis at three wavelengths (Winterbourn *et al.*, 1976). ●, 6.7μM-oxyhaemoglobin+0.2mM-EDTA; ○, 6.7μM-blocked oxyhaemoglobin+0.2mM-EDTA; ▲, 6.7μM-oxyhaemoglobin+1μM-$CuSO_4$; △, 6.7μM-blocked oxyhaemoglobin+1μM-$CuSO_4$.

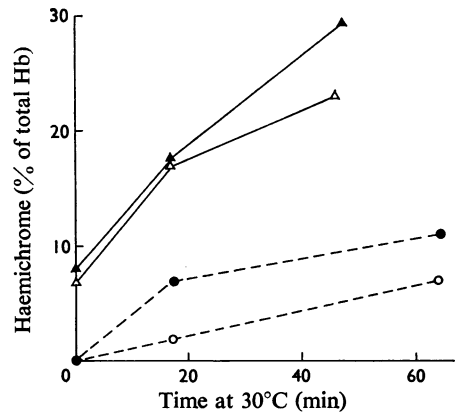

Fig. 5. *Rate of haemichrome formation from methaemoglobin with and without β-93 thiol groups blocked with iodoacetamide*

Solutions were incubated at 37°C in 0.067M-phosphate buffer, pH7.4. △, 10μM-Methaemoglobin+four Cu(II) ions/tetramer; ▲, 10μM-methaemoglobin+six Cu(II) ions/tetramer; ○, blocked methaemoglobin + four Cu(II) ions/tetramer; ●, blocked methaemoglobin + six Cu(II) ions/tetramer. Total haemoglobin concentrations and percentages of haemichrome were calculated from absorbances at 560 and 576nm. Millimolar extinction coefficients of 18.0 and 36.5 at 560nm, and 18.6 and 28.6 at 576nm, for methaemoglobin and haemichrome respectively were used (Winterbourn *et al.*, 1976). Almost no precipitation occurred during the course of the reaction. The haemichrome present at zero time in the unblocked methaemoglobin solutions was formed between the time of adding the $CuSO_4$ and taking the first measurements.

Comparison of copper- and zinc-binding sites

The human haemoglobin molecule contains two Zn(II)-binding sites (Gilman *et al.*, 1975). To see if these correspond to any of the Cu(II)-binding sites, the effects of two and four Zn(II) ions per tetramer on haem oxidation by two and four Cu(II) ions per tetramer were examined. Addition of Zn(II) did not result in any oxidation by two Cu(II) ions, and did not prevent or decrease the amount of oxidation by four Cu(II) ions.

Copper-catalysed autoxidation of haemoglobin

The rate of autoxidation of oxyhaemoglobin is considerably increased in the presence of Cu(II). This is shown in Fig. 4, in which the effects of Cu(II) on the rates of autoxidation of haemoglobin with and without β-93 thiol groups blocked with iodoacetamide are compared. In the presence of EDTA, modified and unmodified haemoglobin autoxidized at the same rate, but with one Cu(II) ion per 28 haem groups the rate was increased 3.5 times with unmodified haemoglobin, but only by 10% of this when the thiol groups were blocked.

Effect of Cu(II) on haemichrome formation and haemoglobin precipitation

The rate of conversion of methaemoglobin into haemichrome, which is normally extremely slow, is accelerated by the addition of Cu(II) (Winterbourn *et al.*, 1976). The effects of various amounts of Cu(II) and of blocking the β-93 thiol groups on the rates of haemichrome formation and precipitation of methaemoglobin are shown in Figs. 5 and 6. No significant haemichrome formation occurred in the presence of two Cu(II) ions per haemoglobin tetramer, but addition of a further two Cu(II) ions resulted in rapid conversion into haemichrome (Fig. 5). Additional Cu(II) ions caused only a slight increase in rate. Haemichrome formation was very much less with methaemoglobin blocked with iodoacetamide. Precipitation occurred much more rapidly in the presence of six rather than four Cu(II) ions per methaemoglobin tetramer (Fig. 6). Addition of two Cu(II) ions caused no precipitation during the time of the experiment. Blocking the β-93 thiol groups shortened the incubation time before precipitation, and the blocked methaemoglobin started to be precipitated at approximately the same time as unblocked methaemoglobin in the presence of two additional

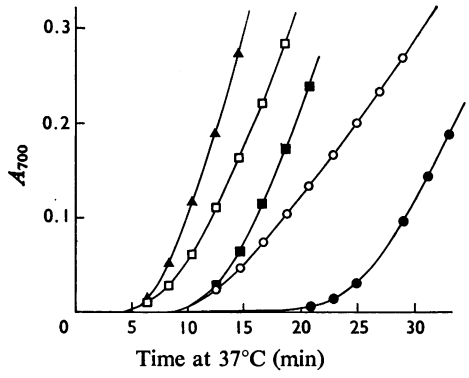

Fig. 6. *Rate of precipitation of methaemoglobin with and without β-93 thiol groups blocked with iodoacetamide*
Methaemoglobin solutions (6μM) were incubated at 37°C in 0.067M-phosphate buffer, pH7.4. The increase in A_{700} was monitored as a measure of turbidity. ●, MetHb + four Cu(II) ions/tetramer; ■, metHb + six Cu(II) ions/tetramer; ▲, metHb + eight Cu(II) ions/tetramer; ○, blocked metHb + four Cu(II); □, blocked metHb + six Cu(II).

Cu(II) ions per tetramer. Once precipitation had started, it occurred more rapidly with unblocked than blocked haemoglobin.

Discussion

Nature of the Cu(II)-binding sites

The results of our equilibrium-dialysis studies, and the effects of thiol-blocking agents on haemoglobin oxidation, suggest that the human haemoglobin tetramer binds six Cu(II) ions, four strongly and two more weakly. The second pair bind to the β-chains and require free thiol groups at position 93. Binding of this pair of Cu(II) ions causes rapid oxidation of 50% of the haem groups in oxy- or deoxy-haemoglobin, presumably those of the β-chains to which the Cu(II) is bound. This conclusion is supported by experiments with blocked haemoglobin and β_4 tetramers. The requirement for more than four Cu(II) ions per β_4 tetramer for oxidation suggests that the first binding sites are also on the β-chains.

The kinetic oxidation data suggest that the deoxy conformation is more favourable than the oxy conformation for the binding of the second pair of Cu(II) ions. A possible reason for this is that in deoxyhaemoglobin the thiol group at position 93 and the negatively charged terminal carboxyl group of the β-chain are aligned so that they could both be liganded to the copper.

It would be expected that Cu(II) would preferentially bind to an ionized thiol group, and therefore that binding would occur more readily at higher pH. The relative insensitivity of the rate of haemoglobin oxidation to pH, and apparent maximum rate around pH 7.4, implies that another factor that influences binding may have the opposite pH-dependence.

Rifkind (1974) has also observed 50% oxidation of the haem groups of horse haemoglobin by Cu(II) ions. However, he observed oxidation with only two Cu(II) ions per tetramer, which suggests that the first pair of binding sites in human haemoglobin may not exist in horse haemoglobin. Binding sites for Cu(II) have also been demonstrated in myoglobin (Breslow, 1973). It has been shown crystallographically that the first binding site in sperm-whale myoglobin involves histidine residue 12 and asparagine residue 122 (Banaszak *et al.*, 1965). Since human haemoglobin has alanine and glutamic acid in the homologous positions in both α- and β-chains, it is unlikely to contain this binding site. Cu(II) and Zn(II) binding is competitive in myoglobin. With human haemoglobin Cu(II) binding to the first and second pair of sites was not affected by adding comparable concentrations of Zn(II). The binding sites for the two ions are therefore either quite distinct, or they exhibit a much higher affinity for Cu(II).

Autoxidation

The stimulation of autoxidation of oxyhaemoglobin by catalytic amounts of Cu(II) also involves binding to the β-93 thiol groups, as low concentrations of Cu(II) have very little effect on the rate of autoxidation of iodoacetamide-blocked haemoglobin. The mechanisms of direct oxidation and copper-catalysed autoxidation appear to be similar. Superoxide is produced during autoxidation, evidently as a result of the reoxidation of Cu(I) to Cu(II) by molecular oxygen, with the copper remaining bound to the protein (Rifkind, 1974; Winterbourn *et al.*, 1976).

$$Cu(I)\cdots Hb^{III}O_2 \rightarrow Cu(I)\cdots Hb^{III} + O_2 \quad (1)$$

$$Hb^{III}\cdots Cu(I) + O_2 \rightarrow Hb^{III}\cdots Cu(II) + O_2^{-\bullet} \quad (2)$$

where Hb is haemoglobin.

One consequence of the existence of a binding site on human haemoglobin with a higher affinity for Cu(II) than the oxidation site is that this molecule should be less sensitive to copper-catalysed autoxidation than haemoglobins such as that of horse, which appear to lack this site. Comparison of our results on human haemoglobin with those of Rifkind (1974) on horse haemoglobin suggest that this is likely to be the case. Cu(II) would be expected to have some catalytic effect, however, because the affinity of the second site appears to be almost as high as the first, and some occupancy of the second sites would be expected.

Denaturation and precipitation

As well as causing its oxidation to methaemoglobin, addition of Cu(II) results in denaturation and precipitation of haemoglobin (Winterbourn *et al.*, 1976). This process appears to involve a combination of two structural changes associated with binding to two different sites. One structural change gives rise to haemichrome and requires free β-93 thiol groups at the second or 'oxidation' binding site. This site lies close to the haem group, and presumably Cu(II) binding alters the haem environment and favours haemichrome formation. The other structural change appears to be caused by Cu(II) binding at the third (weaker) binding site. It occurs more readily in haemoglobin with β-93 thiol groups blocked, when this site is occupied at lower Cu(II) concentrations. It does not result in haemichrome formation, and is probably remote from the haem group, but it appears to be the main initiator of precipitation. Haemichrome formation appears to play only a secondary role in the precipitation process, contributing to the rate and not to the time interval before it starts.

Oxidation and electron transfer

One of the most interesting features of the binding of Cu(II) to haemoglobin is the rapid electron transfer from the haem group to Cu(II) occupying the second binding sites. Some information about this process can be obtained from kinetic data on the oxidation reaction. We have established that one of the Cu(II) ligands is the thiol group of the β-93 cysteine residue which is adjacent to the haem-linked proximal histidine residue. Oxidation can be considered to take place in two steps: (1) binding of the Cu(II) to the haemoglobin and (2) electron transfer from the haem group to the Cu(II). The increase in reaction rate with Cu(II) concentration above that required to saturate all these sites indicates that binding of Cu(II) is the slow step, and that, once bound, electron transfer occurs rapidly.

Although Cu(II) binds to the β-93 thiol groups of oxyhaemoglobin, it does not directly oxidize these groups to disulphides, or catalyse their oxidation by molecular oxygen. This contrasts with the behaviour of small thiols, such as cysteine, and other proteins, such as taka-amylase, all of which form disulphides (Cavallini *et al.*, 1969; Takagi & Isemura, 1964). It is possible to form disulphides from oxyhaemoglobin, but this only occurs subsequently to haem oxidation and only in the presence of O_2, presumably after Cu(II) has been regenerated from O_2 and Cu(I). However, Cu(II) oxidizes the thiol groups of methaemoglobin to disulphides. An explanation must lie in the mobility of the electrons in haemoglobin. With most thiols, reactions (3)–(5) occur (Friedman, 1973).

$$Cu(II)+RS^- \rightarrow Cu(I)+RS^\cdot \quad (3)$$

$$2RS^\cdot \rightarrow RSSR \quad (4)$$

$$Cu(I)+O_2 \rightarrow Cu(II)+O_2^{-\cdot} \quad (5)$$

With oxy- or deoxy-haemoglobin, however, it appears that donation of an electron from the haem group neutralizes the free radical $RS^\cdot$ and regenerates RS^-, and reaction (4) does not occur. With methaemoglobin there would be no electron available for transfer from the haem group and disulphides should be formed.

The demonstration of electron transfer from the β-chain haem group to the cysteine residue at position 93 raises two questions. The first is the route taken by the electron. Steric considerations make transfer by direct contact very unlikely and the most probable route is that of a hydrophobic channel as proposed in general terms by Moore & Williams (1976). A channel of this type, between the haem group and the β-93 cysteine, is demonstrable in the molecular model of haemoglobin (M. F. Perutz, personal communication).

The second question is the physiological role of this electron-transfer mechanism. A hint as to its importance is the fact that all known β-chain globins have a cysteine residue in position 93. This is externally situated and must be of some disadvantage to the molecule owing to its liability to oxidize with consequent risk of polymerization and denaturation. Yet there is no known balancing favourable role of this residue in the processes of oxygenation, deoxygenation or the other co-operative effects of haemoglobin. The most attractive proposition is that it could provide a route whereby free radicals released in the haem pocket, and potentially reactive with other sites in the protein, could be neutralized by electrons donated from the β-93 cysteine residue. Production of the superoxide free radical in the haem pocket is now well documented (Misra & Fridovich, 1972; Wever *et al.*, 1973), and haemoglobin is a well-known catalyst in other free-radical-producing reactions. The free radical $RS^\cdot$, formed as a result, could be reduced again by glutathione (GSH). Protection of the protein against denaturation would then be a consequence of the presence of this cysteine.

This investigation was supported by a grant from the Medical Research Council of New Zealand. We are grateful to Mrs. D. Hinton for carrying out the atomic absorption analyses.

References

Banaszak, L., Watson, H. C. & Kendrew, J. C. (1965) *J. Mol. Biol.* **12**, 130–137

Benesch, R. E., Benesch, R. & Yung, S. (1973) *Anal. Biochem.* **55**, 245–248

Breslow, E. (1973) in *Inorganic Biochemistry* (Eichhorn, G. I., ed.), vol. 1, p. 238, Elsevier, Amsterdam

Cavallini, D., de Marco, C., Duprè, S. & Rotilio, G. (1969) *Arch. Biochem. Biophys.* **130**, 354–361

Dacie, J. V. & Lewis, S. M. (1975) *Practical Haematology*, 5th edn., pp. 32–34, Churchill–Livingstone, Edinburgh

Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77

Friedman, M. (1973) *The Chemistry and Biochemistry of the Sulfhydryl Group in Amino Acids, Peptides and Proteins*, p. 36, Pergamon, Oxford

Geraci, G., Parkhurst, L. J. & Gibson, Q. H. (1969) *J. Biol. Chem.* **244**, 4664–4667

Gilman, J. G., Oelshlegel, F. J. & Brewer, G. J. (1975) *Prog. Clin. Biol. Res.* **1**, 85–106

Huisman, T. H. J. & Dozy, A. M. (1965) *J. Chromatogr.* **19**, 160–169

McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049–6055

Misra, H. P. & Fridovich, I. (1972) *J. Biol. Chem.* **247**, 6960–6962

Moore, G. E. & Williams, R. J. P. (1976) *Abstr. Int. Congr. Biochem. 10th* abstr. 04-4-L012

Pryor, W. A. (1970) *Sci. Am.* **223**, 70–83

Rifkind, J. M. (1974) *Biochemistry* **13**, 2475–2481

Takagi, T. & Isemura, T. (1964) *J. Biochem. (Tokyo)* **56**, 344–350

Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412

Wever, R., Oudega, B. & Van Gelder, B. F. (1973) *Biochim. Biophys. Acta* **302**, 475–478

Winterbourn, C. C. & Carrell, R. W. (1974) *J. Clin. Invest.* **54**, 678–689

Winterbourn, C. C., McGrath, B. M. & Carrell, R. W. (1976) *Biochem. J.* **155**, 493–502