Further Studies on the Thiol Groups of Human Haemoglobin

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When the SH groups of various derivatives of human haemoglobin are converted into their mercuric mercaptides small changes are observed in the optical rotatory dispersion in the range 385–294 m μ . These changes are proportionately greater for the reactive than for the unreactive SH groups. These observations have been interpreted in terms of the conformational changes that are likely to occur in the haemoglobin molecule on modification of the SH groups.

The haemoglobins contain two types of SH groups that are normally termed 'reactive' and 'unreactive'. The reactive SH groups show a chemical reactivity comparable with that of simple thiols; the unreactive SH groups show a very low reactivity but become indistinguishable from the reactive groups when the protein is denatured. Human haemoglobin has two reactive and four unreactive SH groups/molecule.

The reactive SH groups react rapidly with heavy-metal reagents, such as mercuric salts and organic mercury compounds of the type RHgX, to give the corresponding mercaptides. Riggs (1953) has shown that this reaction produces changes both in the affinity of haemoglobin for oxygen and in the degree of haem-haem interaction. These SH groups do not appear to be involved in the Bohr effect (Snow, 1962).

The unreactive SH groups react slowly with heavy-metal reagents and Cecil & Snow (1962*a,b*) concluded that they contribute to the structural stability of the protein although the type of bond involved was then uncertain. Cecil & Thomas (1965) studied the kinetics of the reaction of the unreactive SH groups in human HbCO $\dagger$ with mercuric chloride in the presence of various concentrations of simple monohydric alcohols. They concluded that these groups were involved in hydrophobic interactions with other non-polar groups in the protein and that this was the reason for their low reactivity. They confirmed the observation by Cecil & Snow (1962*b*) that conversion of all six SH groups in HbCO into their mercaptides produced no change in the

absorption spectrum but they found that there was a small difference in the optical rotatory dispersion.

We have continued the study of the optical rotatory dispersion, using different derivatives of haemoglobin, as this appears to be the most sensitive method at present available for determining small changes in conformation. Modification of the reactive SH groups alone was found to produce similar changes and so the effect of various ethanol concentrations on the reactivity of these groups was also studied.

EXPERIMENTAL

Preparation of haemoglobin and its derivatives

Preparation. Blood was obtained by venepuncture and HbO $_2$ prepared by a procedure devised by G. T. Stevenson & M. A. W. Thomas (unpublished work). HbCO was obtained by bubbling CO through a solution of HbO $_2$. Hb was obtained by bubbling N $_2$ through HbO $_2$ for 10–15 min.; it was then transferred anaerobically to stoppered cells for measurement of absorption spectra or optical rotatory dispersion. Solutions were estimated as HbCO by using 10 $^{-3}$ ϵ 59.6 at 540 m μ for four Fe atoms/molecule. MetHb was prepared and estimated as described by Cecil & Snow (1962*b*).

Substitution of the reactive SH groups. The mercuric mercaptides of HbCO and HbO $_2$ were prepared by adding 2 equiv. of HgCl $_2$. The Hb derivative was prepared by deoxygenation of the HbO $_2$ derivative. The reactive SH groups of HbCO were converted into their *S*-carbamoyl-methyl derivatives by reaction with 2 equiv. of iodoacetamide for 2 hr. at pH 9.6 and 37°.

Substitution of the unreactive SH groups. HbCO and HbO $_2$ were allowed to react with 10 equiv. of HgCl $_2$ at pH 9.2 for 15 hr. at 37°. The excess of HgCl $_2$ was removed by dialysis against 0.05 M-Na $_2$ S $_2$ O $_3$ –0.05 M-Na $_2$ B $_4$ O $_7$, pH 9.2 (Cecil & Snow, 1962*a*). The Hb derivative was prepared by deoxygenation of the HbO $_2$ derivative.

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$\dagger$ Abbreviations: HbCO, carboxyhaemoglobin; HbO $_2$, oxyhaemoglobin; Hb, reduced haemoglobin; MetHb, methaemoglobin.

METHODS

Optical rotatory dispersion. Dispersion curves were measured at 25° in the range 227–588 m μ in a Bellingham-Stanley Polarmatic 62 recording spectropolarimeter fitted with automatic correction for the Verdet constant. The cell had a path length of 1 cm. and the entrance slit was set at 0.5 mm., giving a band width of 2 m μ at 350 m μ . Automatic scanning was used except in the region of the 420 m μ absorption peak of haemoglobin when readings had to be taken manually.

The buffer used was either 0.05 M-Na₂HPO₄-0.05 M-NaH₂PO₄-0.2 M-KCl, pH 6.8, or 0.1 M-Na₂B₄O₇-0.2 M-KCl, pH 9.2. Solutions were centrifuged before use to remove all traces of particulate matter. Haemoglobin concentrations were about 10 μ M, except for studies near 420 m μ and below 270 m μ when they were 2 μ M. All dispersion curves were run at least twice and rotations were reproducible $\pm 0.0002^\circ$.

Kinetics of the reaction with iodoacetamide. The reaction of the reactive SH groups of Hb and HbCO with iodoacetamide was followed polarographically in a Radiometer type PO4 recording polarograph as described by Cecil & Snow (1962a). The polarographic reduction of iodoacetic acid has been described by Elving, Rosenthal & Kramer (1951) and Gergely & Iredale (1951), and iodoacetamide was found to behave similarly. The buffers used in these experiments

consisted of 0.1 M-tris-0.1 M-KCl, the pH being adjusted as required with HCl.

Absorption spectra. Spectra were recorded on an Optika model CF4 recording spectrophotometer. A Hilger Uvispek instrument was also used for extinction measurements.

RESULTS

Cecil & Thomas (1965) reported that a change occurs in the optical rotatory dispersion of HbCO between 385 and 294 m μ when all six SH groups/molecule are converted into the mercaptide. These observations have now been extended to HbO₂ and Hb with both the reactive and unreactive SH groups substituted. It has been found that, in each case, changes occur in the same wavelength range. No differences were observed in the dispersion curves below 260 m μ or above 450 m μ and so these are not shown.

Fig. 1 shows the dispersion curves for HbCO, HbO₂, Hb and MetHb at pH 6.7 in the region 385–294 m μ expressed as the reduced specific molar rotation, $[M']_{\text{are}}$. Fig. 2 shows the same curve for HbCO together with that obtained after conversion

Fig. 1. Optical rotatory dispersion of various derivatives of human haemoglobin at pH 6.7 and 25°. ●, HbCO; ○, HbO₂; ■, Hb; ▲, MetHb. The curves for HbCO and MetHb are in good agreement with Beychok's (1964) results with the corresponding horse proteins.

Fig. 2. Optical rotatory dispersion of human HbCO at pH 6.7 and 25° before and after reaction with HgCl₂ (see the text for the conditions of reaction). Curve I, HbCO; curve II, HbCO with all six SH groups/molecule converted into the mercury mercaptide.

Table 1. *Changes in the optical rotatory dispersion of haemoglobin*

The changes that occur in HbCO, HbO₂, Hb and MetHb are given under two headings: (a) on conversion of the two reactive SH groups into the mercuric mercaptide and (b) on conversion of all six SH groups into mercaptide. In each case the mean value of $\Delta[M']_{495}$ between 294 and 385 m μ is given.

	$10^{-4} \times \Delta[M']_{495}$			
	+2Hg	+6Hg	Change/ reactive SH group	Change/ unreactive SH group
HbCO	-1.9	-4.0	-1.0	-0.5
HbO ₂	-2.8	-3.9	-1.4	-0.3
Hb	-1.8	-3.0	-0.9	-0.3
MetHb	-1.6	—	-0.8	—

of all six SH groups/molecule into their mercuric mercaptides. Comparable results were obtained with the same derivatives of HbO₂ and Hb. Only the dimercuric derivative of MetHb was used, as further reaction with mercuric chloride caused precipitation. As the displacement of the dispersion curve is approximately constant in the wavelength range used the average change in $[M']$ can conveniently be used as a measure of the effect. The values are given in Table 1.

To see whether the effect was dependent on the reagent used the reactive SH groups of HbCO were allowed to react with iodoacetamide (see the Experimental section). The change in $[M']$ was the same as when mercuric chloride was used.

HbCO is known to dissociate into half-molecules ($\alpha\beta$ -sub-units) at high ionic strength (Rossi-Fanelli, Antonini & Caputo, 1961; Kirshner & Tanford, 1964). The dispersion curve was therefore measured in the presence of M-magnesium chloride but no change was observed.

Absorption spectra. Measurements of the absorption spectra of HbCO, HbO₂ and Hb between 240 and 600 m μ showed that no change was produced by reaction of the reactive SH groups with mercuric chloride or iodoacetamide or by reaction of all six SH groups with mercuric chloride.

Reaction with iodoacetamide. Since the effect of substituting the reactive SH groups was proportionately greater than that of substituting the unreactive groups, it was decided to see whether the former, like the latter, were involved in any form of non-covalent bonding. If a group, such as SH, is involved in hydrogen or hydrophobic bonding its tendency to ionize will be decreased (Laskowski & Scheraga, 1954; Scheraga, 1961), and so any deviation of its acid dissociation constant from the normal range gives a measure of its involvement in non-covalent bonding.

Fig. 3. Current-voltage curve of 0.25 mM-iodoacetamide at 25° in 0.2 M-tris-0.2 M-KCl, pH 8.65. Curve I, buffer only; curve II, buffer plus iodoacetamide. The potentials refer to the saturated calomel electrode.

Lindley (1960) used the reaction with a compound with an active halogen atom, chloroacetamide, to study the ionization of the SH group of cysteine. The reaction is second-order and takes place with the ionized form, RS⁻, of the thiol. At any given pH the true rate constant k and the apparent rate constant k_{app} are related by the equation:

$$k_{app} = \frac{K'_{SH}k}{[H^+] + K'_{SH}}$$

Iodoacetamide was chosen for these studies because it reacts at a convenient rate with haemoglobin. It gives a well-defined polarographic reduction curve (see Fig. 3) and this provides a convenient method of measuring its concentration. The reactions were carried out in the polarographic cell at 25° and the current at -1.0 V (versus the saturated calomel electrode) was measured against time. The reaction times were varied from 15 to 45 min. and the pH between 7.24 and 9.58 (in the course of an experiment the change in pH did not exceed 0.02 unit). Under these conditions the amount of hydrolysis of iodoacetamide was negligible. Cysteine and GSH were used as model compounds to check the method. Reactions were then carried out with HbCO and Hb. The polarographic method cannot be used with HbO₂ as the reduction curve of oxygen interferes with that of iodoacetamide.

There was the possibility that some of the iodoacetamide was reacting with groups other than SH, e.g. NH₂, particularly with haemoglobin. Accordingly the amount of SH remaining at various

Table 2. *Reaction of iodoacetamide with HbCO at 25°*

The concentrations of the reactants were in the range 0.12–0.50 mM (for HbCO this refers to the reactive SH groups). The buffer consisted of 0.1 M-tris–0.1 M-KCl adjusted to the required pH with HCl; the pH was varied between 7.24 and 9.58. k_{app} refers to the apparent second-order rate constant at any given pH. k refers to the true second-order rate constant with RS^- .

pH	$10^{10} \times [H^+]$	k_{app} (l. mole ⁻¹ sec. ⁻¹)	k (l. mole ⁻¹ sec. ⁻¹)	$10^{10} \times K'_{SH}$
7.24	576	0.115		
7.68	209	0.198		
8.20	63.1	0.47	4.8	7.3
8.63	23.4	0.88		
9.13	7.42	2.95		
9.58	2.63	5.03		

Table 3. *Reaction of iodoacetamide with cysteine GSH, HbCO and Hb*

The reactions were carried out at 25° and the concentrations of the reactants were 0.25 mM (this refers to the concentration of SH). The buffer consisted of 0.1 M-tris–0.1 M-KCl. adjusted to the required pH with HCl; the pH was varied between 7.24 and 9.58.

Substance	k (l. mole ⁻¹ sec. ⁻¹)	pK'_{SH}
Cysteine	7.9	8.2 ₈
Cysteine*	—	8.2 ₇
GSH	13.8	8.6 ₉
HbCO	4.8	9.1 ₄
Hb	0.70	8.7 ₄

* Lindley (1960).

Table 2 gives the results obtained with HbCO. The rate constant, k , and the acid dissociation constant of the reactive SH groups, K'_{SH} , were obtained by plotting $1/k_{app}$ against $[H^+]$. Table 3 summarizes the rate constants and pK'_{SH} values obtained with all the compounds. Lindley's (1960) value for cysteine is given for comparison.

In view of the finding by Cecil & Thomas (1965) that the rate of reaction of the unreactive SH groups with mercuric chloride was increased in the presence of alcohols, the experiments with iodoacetamide were repeated in the presence of ethanol. The results given in Table 4 show that, although the rate constants are lower, the pK'_{SH} values are unchanged.

DISCUSSION

This work was undertaken in an attempt to interpret the changes in dispersion that were observed by Cecil & Thomas (1965) when the SH groups of HbCO were converted into their mercuric mercaptides. The evidence obtained may be summarized as follows:

(i) The changes in dispersion are confined to a limited wavelength range, namely 385–294 mμ.

(ii) There is no corresponding change in the absorption spectrum.

(iii) Changes in dispersion of approximately the same magnitude were observed when HbCO, HbO₂, Hb and MetHb were treated with mercuric chloride.

(iv) The changes are proportionately twice as great for the reactive as for the unreactive SH groups.

(v) The acid dissociation constants of the reactive SH groups of HbCO and Hb are in the same range as those of simple thiols.

It is convenient to discuss it in this order.

The fact that there is no change in dispersion below 250 mμ after modification of the SH groups suggests that this does not induce a change in the helical content of the molecule. The absence of any change in the region of the 420 mμ and 540 mμ

Table 4. *Reaction of iodoacetamide with cysteine, GSH and HbCO in the presence of ethanol*

The reactions were carried out at 25° and the concentrations of the reactants were 0.25 mM (this refers to the concentration of SH). The buffer consisted of 0.1 M-tris–0.1 M-KCl adjusted to the required pH with HCl; the pH was varied between 8.20 and 9.58.

Substance	Concn. of ethanol (%, w/w)	k (l. mole ⁻¹ sec. ⁻¹)	pK'_{SH}
Cysteine	0	7.9	8.2 ₈
Cysteine	13	7.2	8.2 ₄
GSH	0	13.8	8.6 ₉
GSH	13	12.7	8.6 ₈
HbCO	0	4.3	9.1 ₄
HbCO	6.5	4.3	9.1 ₈
HbCO	13	3.0	9.1 ₃

stages of the reaction was estimated by amperometric titration with chloromercuribenzenes-*p*-sulphonate (this reagent can be used in the presence of iodide) and was found to agree, within 3%, with the amount of iodoacetamide remaining.

Fig. 4. Curves showing the change in absorption spectrum and in the optical rotatory dispersion that occurs when HbO_2 is converted into Hb. Curve I, difference in absorption spectrum; curve II, difference in optical rotatory dispersion.

absorption bands is harder to explain. These bands are present in the porphyrin and haemin spectra but the $345\text{ m}\mu$ band only appears when the haem group is combined with a protein. It is strong in HbCO and HbO_2 but weak in Hb and MetHb. Unfortunately, the direction of polarization of this band in relation to the plane of the haem group and to the position of the SH group is not known and so no detailed interpretation can be attempted.

The type of change in dispersion that is induced by a change in the absorption spectrum of the haem group is illustrated by the conversion of HbO_2 into Hb. Fig. 4 shows the difference curves of both the absorption spectra and the dispersion of the two proteins. A change in dispersion appears in the region of each absorption band.

Table 1 shows the change in dispersion that occurs per reactive and unreactive SH group in HbCO_2 , HbO_2 , Hb and MetHb. The fact that the magnitude of these changes is the same in the different derivatives of haemoglobin suggests that the changes in conformation may be limited to the immediate vicinity of the cysteine side chain concerned; it could be confined to the rotation of the side chain about the α -carbon atom.

This view is consistent with the evidence obtained by Cecil & Thomas (1965) from the slow reaction of the unreactive SH groups of HbCO with the complex, $\text{Hg}(\text{SO}_3)_2^{2-}$. They found that the reaction was first-order with respect to the concentration of SH and zero-order with respect to $\text{Hg}(\text{SO}_3)_2^{2-}$ and so the limiting process can be represented by the equation:

The increase in the rate of the reaction in the

presence of alcohols suggested that, in $[\text{RSH-bound}]$ the SH groups were involved in hydrophobic interactions whereas in $[\text{RSH-free}]$ they were unbound and able to react rapidly to form mercaptides. Since the mercaptide group is polar the cysteine side chain would be unable to return to its original position in a non-polar environment.

Perutz (1965) has shown that, of the four unreactive SH groups in human haemoglobin, two (at 104α) lie in the interior cavity of the α -chains between helices and two (at 112β) lie in the area of contact between the α - and β -chains. The first pair must therefore contribute to the stability of the α -chains and the second pair to the linkage between the α - and β -chains. This is consistent with the observation that dissociation of HbCO into $\alpha\beta$ -subunits at high ionic strength produces no change in dispersion or in the behaviour of the SH groups.

The finding that the dispersion change associated with modification of the reactive SH groups was greater than with the unreactive groups is surprising in that there is no reason to suppose that the reactive groups are involved in hydrophobic bonding. As an additional check the acid dissociation constants were measured, since any involvement in non-covalent bonding will result in an abnormally high $\text{p}K'_{\text{SH}}$. However, the constants obtained with HbCO and Hb (Table 3) were comparable with those of cysteine and glutathione. This agrees with Perutz's (1965) finding that these SH groups (at 93β), in contrast with the unreactive groups, lie on the surface of the molecule (close to their respective haem groups) and therefore in a polar environment.

It is still possible that substitution of these SH groups, either with mercuric chloride or iodoacetamide, could induce a change in position of the cysteine side chain for steric reasons. Since they are closer to the haem group than the unreactive SH groups this could produce a proportionately larger change in dispersion. This proximity to the haem group is sufficient to account for the previous findings by Riggs (1953), Riggs & Wolbach (1956) and Gibson & Roughton (1955) that modifications of the reactive SH groups produced changes in the oxygen affinity and in the haem-haem interactions. There is no need to assume that this effect has any special biological significance.

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