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REACTIVITIES OF THE SULPHYDRYL GROUPS OF DOG HEMOGLOBIN

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Summary

Dog hemoglobin has four sulphydryl groups at positions α^{111} (G18) and β^{93} (F9), all of which are titratable with mercurials. Only two of these, however, react with non-mercurial sulphydryl reagents. Kinetic results indicate that the reacting site might be the β^{93} (F9).

An examination of the environment of the α^{111} (G18) shows that this sulphydryl must be unreactive towards non-mercurials because of the presence near it of several interacting groups. These are the carboxyl group of Glu 27α, which is only 4.5 Å away; the carbonyl of Val 107α; and the hydroxyl of Tyr 24α. There is also a strong interaction with the carboxyl of Glu 116α which, though 12 Å away, is separated from the α^{111} (G18) not by water but by protein, a low dielectric constant medium. All these interactions would considerably raise the pK of the Cys 111α thiol. Therefore reaction with non-mercurial sulphydryl reagents via nucleophilic attack by the thiol anion becomes impossible.

The effect of inositol hexaphosphate on the kinetics of the sulphydryl group reaction was investigated. Inositol hexaphosphate slows down the reaction by a factor of three for a 10 M excess of inositol hexaphosphate per hemoglobin tetramer and makes about 25% of the sulphydryl contents of the β^{93} and α^{111} sites unavailable for reaction by any sulphydryl reagent.

Introduction

Much interest has been focussed on the reactive sulphydryl groups of hemoglobin, in view of changes in their reactivity with change in state of ligation of the heme iron atoms. In deoxyhemoglobin, their reactivity is considerably diminished because of steric hindrance by tyrosine 145β which becomes fixed in a pocket between helices F and H of the β chains [1]. The structural changes

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accompanying oxygenation result in the breaking of salt bridges; this sets the carboxyl termini of the β chains free and so tyrosine 145β is no longer fixed in its pocket. As a result, reaction with the β^{93} sulphydryls is no longer sterically hindered and therefore the reactivity increases [1].

Recently, Perutz and coworkers [2] observed a similar decrease in reactivity of the β^{93} sulphydryl groups of methemoglobin in the presence of inositol hexaphosphate. In support of their spectral and magnetic susceptibility data which indicated a transformation by inositol hexaphosphate of methemoglobin from the R to the T quaternary structure, they drew attention to this decrease in sulphydryl group reactivity and concluded that the salt bridges formed with Asp (β^{94}) by the carboxyl terminal histidines of the β chains must be formed in the presence of inositol hexaphosphate [2].

In this paper we investigate the reactivities of the sulphydryl groups of dog hemoglobin *. Dog hemoglobin has four reactive sulphydryl groups [3] located at positions α^{111} (G18) and β^{93} (F9) [3,4]. These sulphydryls are titratable with mercurials [5,6]. We show here that only two of these sulphydryls react with sulphydryl reagents other than mercurials and that the unreactive centre is the α^{111} (G18). By considering the environment of the two sets of sulphydryl groups within the molecule, we show why the α^{111} (G18) reacts only with mercurials.

Materials and Methods

Iodoacetamide for biochemical work was purchased from British Drug Houses. Sephadex G50 and carboxymethyl cellulose were products of Pharmacia Fine Chemicals, Sweden. Human and dog blood were obtained from the local blood bank and veterinary clinic, respectively. All chemicals used were of reagent grade.

Hemoglobin was prepared by using a mixture of ice-cold water and ether for lysing the cells which had previously been washed with isotonic saline. The hemoglobin was dialysed at 5°C against at least three changes of a 1 mM NaCl solution, pH 6.5—7.5, with small amounts of NaH_2PO_4 and Na_2HPO_4 solutions.

Iodoacetamide-modified dog hemoglobin was prepared by the procedure of Taylor et al. [7]. Determination of sulphydryl group content was according to the method of Boyer [8]. The kinetics of the reaction of iodoacetamide with hemoglobin was followed by withdrawing aliquots at different time intervals from a mixture containing hemoglobin and a 20-fold molar (per tetramer) excess of iodoacetamide in phosphate buffer, pH 7.6, ionic strength 0.05 M. Each aliquot was quickly passed through a column of Sephadex G50 equilibrated with phosphate buffer, pH 7.6. The effluent was titrated according to the method of Boyer for determining sulphydryl group content [8].

Starch gel electrophoresis was carried out using Tris/EDTA/barbitone buffer, pH 8.3. Polyacrylamide gels were prepared from Cyanogum 41, supplied by BDH, using Tris-HCl buffer, pH 8.9; the electrode buffer was composed of Tris-glycine.

* Hemoglobin here refers to oxyhemoglobin.

Gradient elution chromatography on carboxymethyl cellulose was carried out between pH 6—8.

Oxygen binding studies were performed with an arrangement that allowed continuous simultaneous observation of absorption changes as well as changes in oxygen partial pressure within the optical cell. An oxygen electrode (Radiomer Type E5046) was fitted to the top of an optical cell of 40-mm path length by an arrangement that made the cell water-tight. The optical cell was connected by polythene tubing, through a liquid pump, to an out-side thermostated reservoir of hemoglobin. Hemoglobin was pumped into the optical cell until it was full. Continuous flow of hemoglobin between the optical cell and the outside reservoir was ensured with the liquid pump. The optical cell was fitted into a Zeiss PMQ II spectrophotometer. Absorbance as a function of oxygen partial pressure was determined at 650 nm. Oxygen partial pressure was monitored on a Radiometer pH meter 27 combined with a gas monitor. Deoxygenation was effected by continuous flushing with nitrogen, previously freed of oxygen traces, through the out-side reservoir of hemoglobin.

Results

Boyer titration of unmodified dog hemoglobin gave a sulphydryl content of 4 per tetramer; dog hemoglobin modified with iodoacetamide gave 2 free sulphydryl groups per tetramer. Table I gives the result of a kinetic experiment, from which it is seen that two sulphydryl groups have reacted after 20 min. There is no change in this number even after 2 h. Similar results were obtained with *N*-ethylmaleimide and mercaptoethanol.

Electrophoresis and chromatography of the iodoacetamide-treated dog hemoglobin indicated that there was only one species present. This was confirmed by the oxygen binding reaction. Fig. 1 shows the Hill plots for the oxygen binding and Table II the binding parameters of iodoacetamide and unmodified dog hemoglobins. The two plots are well displaced on the log PO_2

TABLE I

NUMBER OF FREE SULPHYDRYL GROUPS PER TETRAMER AS A FUNCTION OF TIME FOR DOG HEMOGLOBIN REACTING WITH IODOACETAMIDE

Conditions were the same as those in Fig. 2.

Time (min)	Free SH groups
0	3.89
10	2.63
20	2.00
30	2.20
40	2.10
50	1.98
60	2.25
70	2.40
80	2.10
90	2.30
120	2.09

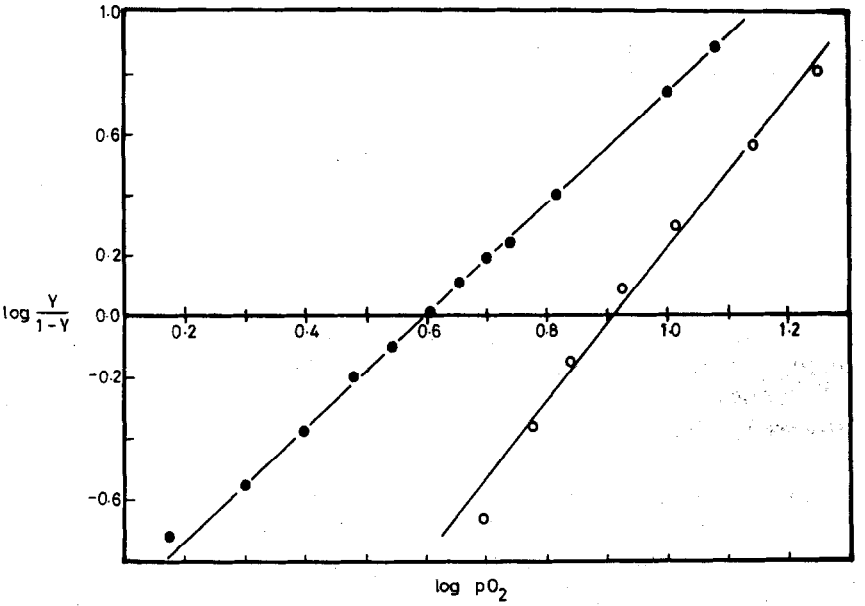


Fig. 1. Hill Plots, log Y/1 — Y versus log pO_2, for oxygen binding to stripped dog hemoglobin modified by iodoacetamide and, for comparison, the corresponding plot for unmodified dog hemoglobin. Conditions: Bistris-HCl buffer pH 6.60, ionic strength 0.10 M, 23°C. Hemoglobin concentration, 254 μM heme; λ-650 nm. ○, dog hemoglobin; ●, modified dog hemoglobin.

axis. Clearly, the modified sample contains only one pure homogeneous species. If there were two species in the modified sample, there would have been a biphasic Hill plot [9].

To have an indication which of the sulphydryl groups is reacting, the kinetics of iodoacetamide binding to dog and human hemoglobins under identical experimental conditions were compared. In Fig. 2 the pseudo first-order rate plots for both hemoglobins are shown. It is seen that the apparent second-order rate constants are the same; also, the plots pass through the origin and they are both monophasic. This indicates that only one site is reacting in dog hemoglobin and that it may be the β^{93} (F9) *.

In Fig. 3 we compare the kinetics in the presence and absence of inositol hexaphosphate. It is seen that the organic phosphate slows down the reaction of the β^{93} sulphydryls of both hemoglobins by a factor of about 3. This is similar to the observations of Perutz et al. [2] and indicates further that it may be the β^{93} (F9) of dog that is reacting. It should be noticed that although the plots go through the origin in the absence of the organic phosphate, they cut the ordinate axis when it is present. This means either that inositol hexaphosphate causes some of the sulphydryl groups to react so fast that their reaction cannot be monitored at the given iodoacetamide concentration or that the organic phosphate makes some of the sulphydryls inaccessible for reaction.

To distinguish between these two possibilities, we carried out the reaction at

* In similar experiments with guinea pig hemoglobin, we detected two kinetic phases. Guinea pig hemoglobin, like dog hemoglobin, has four reactive sulphydryl groups.

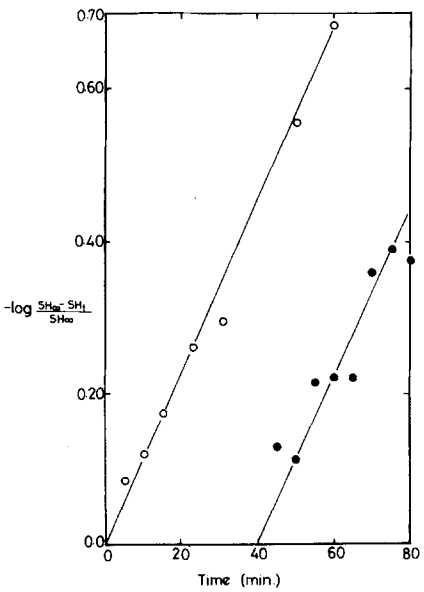
TABLE II

OXYGEN BINDING PARAMETERS FOR STRIPPED DOG HEMOGLOBIN AND STRIPPED DOG HEMOGLOBIN MODIFIED BY IODOACETAMIDE

Conditions were the same as those in Fig. 1.

Hemoglobin	log P_{50}	Hill coefficient
Normal dog	0.91	2.5
Iodoacetamide-treated dog	0.595	1.8

lower iodoacetamide concentrations; only one kinetic phase was observed. We also determined (by Boyer titration [8]) the sulphydryl contents of native dog and human hemoglobins in the presence of a 10-fold molar excess (per tetramer) of organic phosphate. The values obtained are 2.9 and 1.465 per tetramer, respectively. From the intercepts of the rate plots in Fig. 3, the calculated values of the sulphydryl content at zero time are 3.5 and 1.6 for dog and human hemoglobins, respectively. These results clearly indicate that inositol hexaphosphate makes some of the sulphydryl groups inaccessible to reaction. It should be noted that under the conditions of this experiment, i.e. pH 7.6, inositol hexaphosphate has no effect on the sulphydryl content of



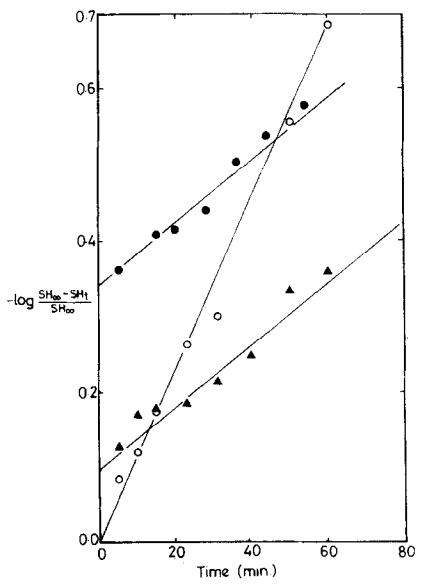


Fig. 2. Pseudo first-order rate plots for the reaction of iodoacetamide with dog and human hemoglobins. The plot for dog hemoglobin has been displaced 40 min along the time axis for clarity. Conditions: phosphate buffer, pH 7.6, ionic strength 0.05 M, 20° C; hemoglobin concentration, 1 mM heme; iodoacetamide concentration, 5 mM. ○, human hemoglobin; ●, dog hemoglobin.

Fig. 3. Pseudo first-order rate plots for the reaction of iodoacetamide with dog and human hemoglobins; effect of inositol hexaphosphate. Conditions as in Fig. 2. ○, human hemoglobin, no inositol hexaphosphate; ●, dog hemoglobin plus 10 M excess (per tetramer) inositol hexaphosphate; ▲, human hemoglobin plus 10 M excess (per tetramer) inositol hexaphosphate. The dog hemoglobin plot has been displaced upwards by 0.2 units for clarity.

methemoglobin; inositol hexaphosphate is known to bind only weakly to methemoglobin at pH 7.6 [2].

Discussion

(a) Inaccessibility of the α^{111} site of dog hemoglobin to non-mercurials

Our results indicate that reaction of dog hemoglobin with iodoacetamide and other non-mercurial sulphydryl reagents results in attack only at the β^{93} (F9) site and that the α^{111} (G18) site is not attacked by these reagents. Both sites are, however, accessible to mercurials. A similar result was obtained by Bucci et al. [10] who observed that *p*-hydroxymercuribenzoic acid at the β^{93} position can be replaced by cysteine, *N*-ethylmalemide, cystamine or iodoacetamide; none of these non-mercurials, however, reacts at the β^{112} or α^{104} site which are in the subunit contact region [11].

Our results, however, differ from those of these authors in one important aspect. Chiancone et al. [12] have observed that the reaction of *p*-hydroxymercuribenzoic acid with the α^{104} and β^{112} sulphydryls has a half-life of several hours. We, on the other hand, have observed that the reaction of the α^{111} of dog with *p*-hydroxymercuribenzoic acid is so fast that it cannot be followed by static spectrophotometry. This is also obvious from the fact that it can be titrated by the method of Boyer [8]. Therefore, the inaccessibility of the dog α^{111} sulphydryl to non-mercurials cannot be because it is 'masked'.

We may now examine the environment of α^{111} (G18). There are three groups that are close enough to the α^{111} (G18) to interact with it and raise the thiol p*K*. These are the carboxyl of α^{27} (Glu) which is only 4.5 Å away, the carbonyl of α^{107} (Val) and the hydroxyl of α^{24} (Tyr) (Perutz, M.F., personal communication). A further contribution to a p*K* rise of the α^{111} (Cys) may come from α^{116} (Glu) which is at the GH corner and located 12 Å away. At first sight this distance may appear large; however, a closer look shows that the α^{116} (GH4) residue is separated from the α^{111} (G18) residue not by water but by protein, comprising the peptide backbone of both the G helix and the GH corner as well as the hydrophobic side groups in between them. Consequently, the effective dielectric constant of the medium (protein) between the α^{116} (Glu) and α^{111} (Cys) residues is not 80 (the value for water), but 4, the value suggested for the internal dielectric constant of a protein [13]. Thus the electrostatic effect of the α^{116} (Glu) residue on the p*K* of the α^{111} (Cys) thiol would be greater than would be suggested by their distance of separation.

In contrast to the interactions suffered by the thiol group of α^{111} (G18), the thiol of β^{93} (F9) is relatively free of interactions. The nearest residue that could raise its p*K* is β^{94} (Asp), located 6 Å away. Even then, there is a lysine residue at β^{95}, 7.5 Å from β^{94} (Asp), that could reduce the effective electrostatic contribution of β^{94} (Asp) at the β^{93} position. Calculation shows that the combined electrostatic effects of α^{27} (Glu) and α^{116} (Glu) at the α^{111} site is seven times greater than that of β^{94} (Asp) at the β^{93} site: a force of 3717 $N \cdot mol^{-1}$ compared to 536 $N \cdot mol^{-1}$. When the additional interactions of α^{107} (Val) and α^{24} (Tyr) with α^{111} (Cys) are taken into consideration, it is readily appreciated that the p*K* of this thiol would be considerably higher than that of β^{93} (F9).

We are led to conclude that at the β^{93} position the sulphydryl group may ionize whereas, at the α^{111} position, formation of the thiolate anion is not feasible, i.e. the pK of this cysteine is raised. The extent of this rise in pK is seen from the fact that, even at pH 9.2, we find that the α^{111} sulphydryls do not react with non-mercurials. Our conclusion regarding a possible rise in pK of the α^{111} sulphydryls due to the nearness of interacting neighbouring residues is supported by the results of Benesch and Benesch [14]. They found that, for a series of cysteine derivatives in which the carboxyl group has been modified, the pK values of the sulphydryl group are lower than in the parent compound. This is also the case when the distance of the carboxyl group from the -SH group is increased, e.g. in cysteine peptides [14]. The rise in pK in going from L-cysteine ethyl ester to L-cysteine is 1.08 [14]. Our results at pH 9.2 where non-mercurials still do not interact with the α^{111} -SH groups indicates that the rise in pK of this residue is greater than 1.6.

The inability of the non-mercurial sulphydryl reagents to react at the α^{111} (G18) site of dog is not surprising, because it is known that cysteine reacts with iodoacetamide via nucleophilic attack by the thiolate anion. We have found that at pH 6.0 the β^{93} sulphydryl group does not react with iodoacetamide; this confirms that reaction is via the thiolate anion. The inability of non-mercurials to react at the α^{104} and β^{112} sites [11] is therefore due to two reasons:

(1) These groups cannot exist in the anionic form at the hydrophobic subunit interface;

(2) Reaction at this interface is sterically hindered. Steric hindrance is implied by the slow reaction of *p*-hydroxymercuribenzoic acid with the α^{104} and β^{112}, because PMB is known to react fast with the neutral and anionic forms of -SH groups [2].

(b) Effect of inositol hexaphosphate on sulphydryl groups

Inositol hexaphosphate and hemoglobin form a series of compounds in rapid equilibrium according to the equation,

$$\text{Hb} + n \text{ inositol hexaphosphate} \rightleftharpoons \text{Hb (inositol hexaphosphate)}\ i \qquad (1)$$

where $i = 1,n$. Under our experimental conditions i is not greater than unity (cf. Gray and Gibson [15]). Our system therefore simplifies to

$$\text{Hb} + \text{inositol hexaphosphate} \rightleftharpoons \text{Hb inositol hexaphosphate} \qquad (2)$$

This system has a dissociation constant of 1 μM at pH 7.0 at room temperature [15]. We have measured the dissociation constant at pH 7.6, and obtained a value of 34 μM. Calculation from our data shows that the fraction of uncomplexed hemoglobin is only 0.3%. Effectively, therefore, the iodoacetamide reaction with this system is essentially a reaction with the species Hb inositol hexaphosphate.

We may now ask how inositol hexaphosphate could slow down the iodoacetamide reaction. Since the above equilibration process is over within the dead time of the stopped flow apparatus [16], its forward and reverse rate constants must be very high compared to the rate constant of the iodoacetamide reaction, i.e. under our experimental conditions, the equilibration rate is several orders of magnitude faster than the rate of the iodoacetamide reaction. Therefore, the equilibration process and the iodoacetamide reaction are

kinetically uncoupled. The rate of the iodoacetamide reaction is therefore completely independent of the equilibration process; it could depend on the equilibration process only if it is faster or, at the least, of the same order of magnitude as the equilibration rate.

From the above argument, we conclude that the lowering by inositol hexaphosphate of the iodoacetamide reaction rate must be due to the intrinsic nature of the species with which it is reacting, namely Hb inositol hexaphosphate. In this species 25% of the β^{93}-SH groups of dog and human hemoglobins are inaccessible to iodoacetamide under our experimental conditions (see Results). Also 25% of the β^{93}-SH groups of human hemoglobin is inaccessible to mercurials. We have shown (Fig. 2) that the β^{93}-SH groups of both hemoglobins are similar in their reactivity and in the effect of inositol hexaphosphate on their reactivity (Fig. 3). For this reason, we expect that the fraction of the β^{93} sulphydryls made inaccessible to mercurial, should be the same in both hemoglobins, namely 25%. Since in the presence of inositol hexaphosphate only 2.9 -SH groups are available to mercurial in dog hemoglobin, we conclude that inositol hexaphosphate also makes 25% of the α^{111} (G18) inaccessible to mercurial; therefore inositol hexaphosphate perturbs both α and β chains of dog hemoglobin.

The binding site of inositol hexaphosphate to hemoglobin is still unknown; that of deoxyhemoglobin has been determined by X-ray crystallography. [17]. The quaternary structure change that accompanies oxygenation of deoxyhemoglobin separates the positively charged terminal groups of the β chains to which the organic phosphate binds, as well as narrows the β_1—β_2 interchain distance. It is therefore unlikely that the organic phosphate binds at the same site in hemoglobin. However, our results show that both hemoglobin chains are perturbed by inositol hexaphosphate binding. This may result in tertiary structure changes that partially 'mask' the β^{93} and α^{111} sites.

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