Interaction of Zinc with Hemoglobin: Binding of Zinc and the Oxygen Affinity†

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ABSTRACT: Stripped human hemoglobin was shown to have a high apparent zinc association constant of 1.3×10^7 M^{-1} with a stoichiometry of one zinc for every two hemes. The saturation of this site produces a dramatic 3.7-fold increase in the oxygen affinity. The effect of zinc on the oxygen affinity is interrelated with the interaction of 2,3-diphosphoglyceric acid (2,3-DPG) and hemoglobin. Thus, a smaller zinc effect is observed in the presence of added 2,3-DPG. Information about the location of the zinc-binding site responsible for the increased oxygen affinity has been obtained by comparing the binding of zinc to various hemoglobins. Blocking the β93 sulfhydryl group decreases the apparent zinc association constant by an order of magnitude. The substitution of histidine-β143 in hemoglobin Abruzzo [β143 (H21) His→Arg] and hemoglobin Little Rock [β143 (H21) His→Gln] decreases the apparent zinc association constant by two orders of magnitude. The substitution of histidine-β143 by other amino acids and the reaction of the β93 sulfhydryl group are known to produce dramatic increases in the oxygen affinity. The binding of zinc to one or both of these amino acids can, therefore, explain the zinc-induced increase in the oxygen affinity.

It has previously been reported that zinc produces a small increase in the oxygen affinity of whole blood (Oelshlegel et al., 1973) and unpurified hemoglobin (Oelshlegel et al., 1974). Such a mechanism which can play a role in regulating the oxygen affinity of hemoglobin may be of importance in understanding the proper functioning of hemoglobin within the erythrocyte.

In order to try to explain the effect of zinc on hemoglobin, we have studied the interaction of zinc with stripped human hemoglobin where all the low-molecular-weight components of the erythrocyte have been removed (Berman et al., 1971; Berger et al., 1973). The direct interaction of zinc with stripped hemoglobin is shown to produce a much more dramatic increase in the oxygen affinity than that previously reported for unpurified hemoglobin. This discrepancy is at least partially related to the coupling of the effects of different low-molecular-weight substances which can alter the oxygen affinity. The increase in the oxygen affinity is shown to correlate with the binding of two zinc atoms per tetramer. Studies under various conditions and with various modified hemoglobins suggest a possible binding site for zinc as well as a possible explanation for the increased oxygen affinity.

Experimental Section

Animal blood was obtained from Bioquest. Normal human blood was obtained from the participants of the longitudinal program at the Gerontology Research Center of the National Institute on Aging. Blood from a donor heterozygous for hemoglobin Little Rock was a gift from F. Padilla of Little Rock Veterans Hospital. Hemoglobin Little Rock was purified from this blood sample by the procedure of Bromberg et al. (1973). Blood from a donor homozygous for hemoglobin F was a gift from S. Charache of Johns Hopkins University. Purified hemoglobin Abruzzo was a gift from J. Bonaventura of Duke University, Beaufort, and purified hemoglobin A_2 was a gift from R. Lumry of the University of Minnesota.

BME[1] was a gift from S. Simon of the State University of New York, Stony Brook. All other chemicals were reagent grade. Hemoglobin was prepared from whole blood by procedures previously described (Rifkind, 1972; 1974; Rifkind et al., 1976). Low-molecular-weight components were removed by gel filtration on Sephadex G-25 eluted at 4 °C with 0.01 M Trizma–0.1 M NaCl, pH 7.4, buffer (Berman et al., 1971). To be certain of the complete removal of the low-molecular-weight ionic material, isoionic hemoglobin was then prepared by using Bio-Rad AG 501-X8 (D) mixed-bed resin (Berger et al., 1973).

The oxygenation of hemoglobin was performed using the method of Keyes et al. (1967). NEM–hemoglobin and BME–hemoglobin were prepared by incubating the hemoglobin overnight with an excess of reagent. The unreacted reagent was removed by gel filtration. 4,4′-Dithiodipyridine was then used to check for unreacted sulfhydryl groups (Ampulski et al., 1969). In both cases >98% of the free SH groups was blocked.

The binding of zinc to hemoglobin was performed by equilibrium dialysis according to procedures similar to those previously described (Rifkind, 1974). Hemoglobin solutions of the proper concentration were placed inside small dialysis bags and the necessary concentrations of zinc and buffer were placed on the outside.

For all experiments with modified or abnormal hemoglobins a large enough vessel was used so that more than one dialysis bag could be placed in the same vessel. By this procedure, binding to these hemoglobins was always performed with a dialysis bag containing normal adult human hemoglobin in the same vessel. A direct comparison of zinc binding was thus made between both hemoglobins at equilibrium with the identical free zinc concentration, pH, buffer concentration, oxygen pressure, etc. The reported differences in the association constants are therefore valid, even though corrections were not made for the binding of zinc to salt, buffer, and hydroxyl ions.

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[1] Abbreviations used are: BME, bis(*n*-maleimidomethyl) ether; NEM, *N*-ethylmaleimide; 2,3-DPG, 2,3-diphosphoglyceric acid; ATP, adenosine triphosphate; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol.

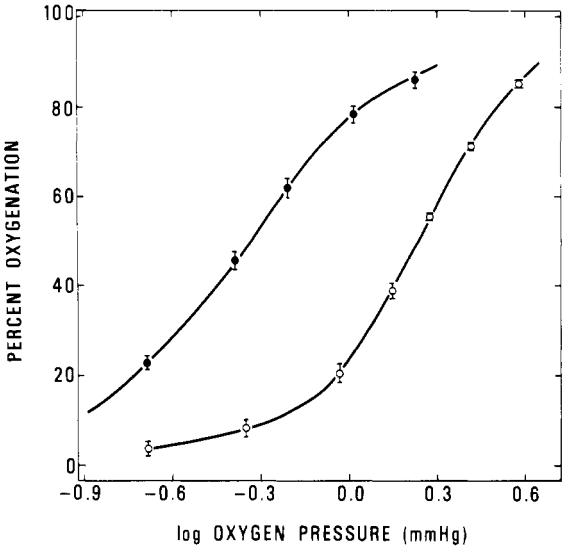

FIGURE 1: The effect of zinc on the oxygenation of 8.6×10^{-5} M stripped human hemoglobin in 0.02 M Bistris, pH 7.4, at 25 °C: (○) no zinc added, $P_{50} = 1.71$; (●) zinc/heme molar ratio = 0.52, $P_{50} = 0.46$.

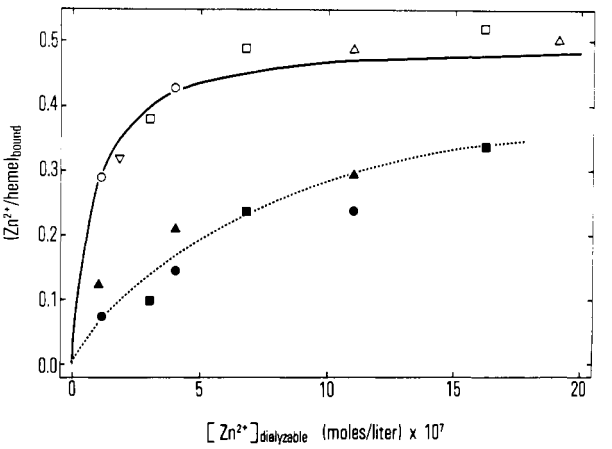

FIGURE 2: The binding of zinc to 1×10^{-4} M human hemoglobin (open symbols) and the effect of blocking the β93 sulfhydryl groups (solid symbols). The lines are calculated assuming two identical noninteracting zinc-binding sites per tetramer: (—) $K_{app} = 1.3 \times 10^7$ M^{-1}; (···) $K_{app} = 1.3 \times 10^6$ M^{-1}. (○, △, ▽) Three different experiments in 0.01 M Trizma, pH 7.2, at 2 °C; (□) 0.01 M Trizma–0.1 M NaCl, pH 7.2, at 2 °C; (●) the sulfhydryl groups are blocked by NEM. The experimental conditions are 0.01 M Trizma, pH 7.2, at 2 °C; (■) the sulfhydryl groups are blocked by NEM. The experimental conditions are 0.01 M Trizma–0.1 M NaCl, pH 7.2, at 2 °C; (▲) the sulfhydryl groups are blocked by BME. The experimental conditions are 0.01 M Trizma, pH 7.2, at 2 °C.

A magnetic stirrer was placed inside the dialysis vessel which was covered with parafilm and placed on a stirrer for 3–4 days in a 2 °C cold room during which time equilibrium was reached. Oxygen diffusion through the parafilm assures that the hemoglobin samples will remain oxygenated during this extended period of time (Ueda and Tyuma, 1971; Lo and Schimmel, 1969).

At equilibrium the concentration of zinc inside and outside the dialysis bags was determined with a Perkin-Elmer Model 306 atomic absorption spectrophotometer. The difference between the inside and outside concentrations measured the concentration of zinc bound to hemoglobin. The outside concentration or the concentration of dialyzable zinc was used as the free concentration without correcting for hydroxylated zinc or the binding of zinc to salt or buffer. The association constants must therefore be considered apparent constants with all the intrinsic constants actually larger. The different buffer and salt conditions used were seen to have no significant effect on the apparent association constant of adult human hemoglobin (see below). In addition, visible spectra from 650 to 470 nm were recorded after removing the hemoglobin from the dialysis bags in order to check for possible deoxygenation, denaturation, or oxidation. No indication of any deoxygenation was observed visually before opening the dialysis bags or by spectra run immediately after removing the hemoglobin from the bags. Denaturation due to zinc precipitation was occasionally found at the relatively high zinc concentrations. Binding constants were not calculated for the denatured material. The only appreciable oxidation occurred when the pH of the outside buffer was lowered below pH 6 (see below).

Results and Discussion

The Effect of Binding Zinc on Oxygenation. The oxygen affinity of hemoglobin is known to be affected by the interaction of various small molecules which bind to hemoglobin (Rifkind, 1973). Thus, the oxygen affinity of human hemoglobin within the erythrocyte is much lower than that of hemoglobin which is stripped of all the organic phosphates such as 2,3-DPG and ATP (Benesch and Benesch, 1967; Chanutin and Curnish, 1967; Berger et al., 1973).

Figure 1 shows that the oxygen affinity of stripped adult human hemoglobin is increased by a factor of 3.7 ($\Delta \log P_{50} = 0.57$) at a zinc/heme molar ratio of 0.52. The binding of zinc to human hemoglobin A (Figure 2, open symbols) shows that this increase in the oxygen affinity coincides with the binding of zinc to a site with a stoichiometry of one zinc for every two hemes, and a high apparent association constant of 1.3×10^7 M^{-1} for oxyhemoglobin. Under the conditions of our oxygenation experiments (Figure 1), the free zinc concentration is very low and zinc is bound to ~96% of the oxyhemoglobin high-affinity sites.

The amount of zinc bound to deoxyhemoglobin can be predicted by thermodynamic considerations. Linked functions (Wyman, 1948) require that the increased oxygen affinity in the presence of zinc be linked to an increase in the association constant of zinc in the presence of oxygen; i.e., deoxyhemoglobin has a lower association constant. The quantitative relationship between the intrinsic zinc association constants for oxyhemoglobin ($K_{Zn,O}$) and deoxyhemoglobin ($K_{Zn,D}$) and the change in oxygen affinity produced by zinc for a stoichiometry of one zinc for every two hemes (Bare et al., 1974) is given by the expression:

$$\frac{K_{Zn,O}}{K_{Zn,D}} = \frac{(P_{50})_0^2}{(P_{50})_{Zn\to\infty}^2} \quad (1)$$

where $(P_{50})_0$ = the oxygen pressure necessary to produce 50% oxygenation in the absence of zinc and $(P_{50})_{Zn\to\infty}$ = the oxygen pressure necessary to produce 50% oxygenation at a concentration of zinc, which saturates the binding site. Unfortunately, concentrations of zinc much higher than that corresponding to a zinc/heme molar ratio of 0.5 precipitate oxyhemoglobin and cannot be used for oxygenation experiments. Because of the high zinc association constant, the P_{50} in the presence of zinc shown in Figure 1 approximates $(P_{50})_{Zn\to\infty}$ but is probably too high. Therefore, the ratio $K_{Zn,O}/K_{Zn,D} \geq 13.8$.

These results on the effect of zinc on the oxygen affinity of human hemoglobin are much more dramatic than the previously reported 1.1-fold increase ($\Delta \log P_{50} = 0.04$) for whole blood and 1.3-fold increase ($\Delta \log P_{50} = 0.11$) for hemolyzed cells (Oelshlegel et al., 1973, 1974).

TABLE I: The Effect of 2,3-DPG on the Change in P_{50} Produced by Zinc.[a]

	No DPG	5×10^{-5} M DPG	1×10^{-3} M DPG
$(P_{50})_0$; no Zn	1.71	6.67	6.67
$(P_{50})_{Zn}$; 4.5×10^{-5} M Zn	0.46	1.88	3.62
$(P_{50})_0/(P_{50})_{Zn}$	3.7	3.5	1.8
Δ Log P_{50}	0.57	0.54	0.26

[a] Experimental conditions are the same as those of Figure 1.

The difference in the oxygenation properties of hemolyzed cells and stripped hemoglobin is primarily due to the interaction of 2,3-DPG with hemoglobin (Berger et al., 1973; Benesch and Benesch, 1967; Chanutin and Curnish, 1967). The greater effect of zinc on stripped hemoglobin is, therefore, perhaps due to the absence of 2,3-DPG. Table I shows that the addition of 2,3-DPG does appreciably decrease the effect of zinc on the oxygen affinity of stripped human hemoglobin. At a concentration of 1×10^{-3} M 2,3-DPG, the effect of zinc on stripped hemoglobin (Δ log P_{50}) is 2.3 times greater than on hemolyzed cells, instead of the fivefold difference in the absence of 2,3-DPG. The remaining difference between our results and those of Oelshlegel et al. (1974) can be due to the much higher hemoglobin concentrations used in their experiments, and (or) another substance in hemolyzed cells, which further inhibits the increase in the oxygen affinity due to zinc.

Binding studies indicate that 1×10^{-3} M 2,3-DPG decreases the apparent association constant of oxyhemoglobin for zinc (K_{app}) by a factor of 6. Under the conditions of our oxygenation experiments (Table I), zinc is still bound to the large majority of the oxyhemoglobin high-affinity zinc-binding sites; i.e., 90% of the sites are filled in the presence of 1×10^{-3} M 2,3-DPG as compared to 96% in the absence of any 2,3-DPG. This finding agrees with the results of Oelshlegel et al. (1974), which suggest that, even under their experimental conditions, most of the zinc was associated with hemoglobin.

In order to explain these results, it is necessary to consider possible effects due to both the binding of 2,3-DPG to hemoglobin and the binding of zinc to 2,3-DPG.

The binding of zinc to 2,3-DPG would lower the free zinc concentration and increase the dialyzable zinc concentration. Such an effect would decrease K_{app} without altering the intrinsic zinc association constants $K_{Zn,O}$ and $K_{Zn,D}$ or the contribution to Δ log P_{50} from the binding of zinc to hemoglobin (eq 1). Since the oxygenation of hemoglobin is linked to both the binding of 2,3-DPG and zinc, eq 1 is only valid when the free concentration of 2,3-DPG is the same at $(P_{50})_0$ and $(P_{50})_{Zn\to\infty}$. If zinc also binds to 2,3-DPG, the free concentration of 2,3-DPG will decrease and the observed $(P_{50})_0/(P_{50})_{Zn\to\infty}$ will be greater than $(K_{Zn,O}/K_{Zn,D})^{1/2}$.

In order for the binding of zinc to 2,3-DPG to explain the entire DPG effect (Table I), it is necessary that $(P_{50})_{Zn\to\infty}$, in the presence of 1×10^{-3} M 2,3-DPG, be much lower than $(P_{50})_{Zn=4.5\times10^{-5}M}$. However, this hypothesis also predicts that 2,3-DPG will have the same effect on the binding of zinc to oxyhemoglobin and deoxyhemoglobin. On the basis of eq 1 and the oxyhemoglobin binding data, it is possible to show that, even for deoxyhemoglobin, 68% of the high-affinity zinc-binding sites will be saturated at 1×10^{-3} M 2,3-DPG and 4.5×10^{-5} M zinc. Since the effect on the oxygenation is linked to the binding of zinc to hemoglobin (eq 1), a value of $(P_{50})_0/(P_{50})_{Zn=4.5\times10^{-5}M} \gg 1.8$ would be expected in the presence of 1×10^{-3} M 2,3-DPG. Furthermore, the binding of zinc to 2,3-DPG would contribute even less at the high hemoglobin concentrations used by Oelshlegel et al. (1973, 1974), where the observed value of $(P_{50})_0/(P_{50})_{Zn}$ is even lower.

Therefore, at least part of the 2,3-DPG effect must be related to the competition between zinc and 2,3-DPG for hemoglobin. Since the binding of both zinc and 2,3-DPG is linked to the oxygenation, it is reasonable to expect that 2,3-DPG may alter the zinc binding to deoxyhemoglobin and oxyhemoglobin to a different extent. This phenomenon would change $K_{Zn,O}/K_{Zn,D}$ and thereby $(P_{50})_0/(P_{50})_{Zn\to\infty}$.

The Binding of Zinc to Various Hemoglobins. Proteins generally have a large number of amino acids which can potentially be involved in the binding of metal ions. It, nevertheless, seemed possible to determine at least the region of the hemoglobin molecule where zinc binds. This expectation was based on the knowledge of the entire amino acid sequence of many normal hemoglobins (Dayhoff, 1969), the ability to chemically modify hemoglobin at certain specific sites, and the availability of many abnormal hemoglobins with single amino acid substitutions (Dayhoff, 1969).

The reaction of the cysteine-β93 residue with sulfhydryl reagents (Taylor et al., 1966; Moffat et al., 1971; Rifkind, 1972) is one of the most extensively studied modifications of hemoglobin. The sulfhydryl group on this residue is the only reactive sulfhydryl on hemoglobin and can be stoichiometrically blocked by many reagents. This group was of particular interest because of the large increase in the oxygen affinity produced by blocking this group (Taylor et al., 1966; Moffat et al., 1971). Furthermore, evidence has been presented both for the binding of Cu(II) in the region of this sulfhydryl and a similarity between the binding of Cu(II) and Zn(II) to hemoglobin (Rifkind et al., 1976).

Figure 2 shows that blocking the β93 sulfhydryl group by NEM produces an order of magnitude decrease in the apparent zinc association constant for oxyhemoglobin (K_{app}). These results are consistent with preliminary x-ray diffraction studies (Moffat and Berg, private communication) which show a change in the region of the cysteine-β93 residue when zinc is incorporated into horse methemoglobin crystals. Both of these findings suggest that the β93 sulfhydryl group is perhaps part of the zinc-binding site. However, it is also necessary to consider the possibility whereby the β93 sulfhydryl group and the region of the molecule where zinc binds are conformationally linked in such a manner that reaction of the β93 sulfhydryl group alters the conformation of the zinc-binding site so as to reduce the affinity of hemoglobin for zinc. An indirect effect of the sulfhydryl group is supported by the finding that changes in the region of the sulfhydryl, similar to those found in the x rays of zinc hemoglobin, have been found with other hemoglobin derivatives which do not involve a direct interaction with the sulfhydryl (Moffat et al., 1974; Deatherage et al., 1976).

The relatively high affinity of hemoglobin for zinc even after NEM is reacted with the β93 sulfhydryl groups ($K_{app} = 1.3 \times 10^6$ M^{-1}) explains why Oelshiegel et al. (1974) were unable to observe an effect of NEM on the binding of zinc to hemoglobin when the zinc concentration was adequate to saturate the high-affinity binding site. This high affinity also implies that cysteine-β93 cannot be the only ligand involved in the binding of zinc to hemoglobin. Such a prediction is consistent with the observation that the high-affinity binding of metal ions like zinc to proteins generally involves the simultaneous coordination with several ligands brought together by the tertiary folding of the protein (Breslow, 1973).

An investigation of the effect of pH on the apparent binding constant can be used to suggest what types of ligands are involved in the binding of metal ions to proteins. Many potential

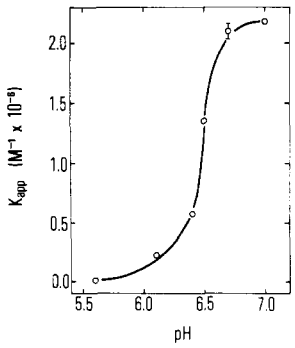

FIGURE 3: The effect of pH on the human hemoglobin apparent zinc association constant (K_{app}). The heme concentration was 1×10^{-4} M and the temperature was 2 °C. $ZnCl_2$ corresponding to a Zn/heme molar ratio of 0.25 was added to enough Bistris for a final buffer concentration of 0.02 M. The pH was adjusted initially and checked after equilibrium was reached.

ligands involve an ionizable group. Since the metal ions and protons are competing for the same group, K_{app} will depend on the pH (Breslow 1973)

$$K_{app} = \frac{K_m}{1 + K_h[H^+]} \quad (2)$$

where K_m is the pH-independent metal-ion association constant and K_h is the proton association constant of the ionizable group. At pHs well above the pK_a, $K_h[H^+] \ll 1$, and K_{app} is independent of pH. However, at pHs in the region of and below the pK_a, K_{app} is a function of pH with $K_{app} = K_m/2$ when $K_h[H^+] = 1$, and the pH is equal to the pK_a of this group. This expression is valid even if $K_m \gg K_h$ and the protons do not significantly displace the metal ions from the ligand.

Figure 3 shows the effect of pH on K_{app} for the binding of zinc to hemoglobin.[2] The observation that K_{app} becomes independent of pH in the region of pH 7 indicates that the group responsible for the effect shown in Figure 3 is nearly completely deprotonated at this pH. The 50% decrease in K_{app} at about pH 6.5 suggests a pK_a ~6.5 for the group. Histidines, which generally have a pK_a on proteins in the range of 6.4–7.2, are the only titratable groups with a pK_a in this region (Breslow, 1973). Therefore, these results suggest that a histidine is involved in the binding of zinc to hemoglobin. This conclusion is also consistent with the observation that histidines generally have been implicated in the binding of zinc to proteins.

Unfortunately, hemoglobin has a large number of histidines spread out all over the molecule. There are ten on each α chain and nine on each β chain. In order to determine which histidine(s) is (are) involved, it is necessary to either specifically modify individual histidines or to compare hemoglobins with known differences in their histidine content.

The results on the effect of blocking cysteine-β93 (Figure 2) suggested the possibility of histidine-β97 (FG-4) which is located in the same region of the molecule. This possibility was, however, ruled out by comparing the binding of zinc to hemoglobin reacted with NEM and BME (Figure 2). While NEM blocks the sulfhydryl, BME makes a cross-link between cysteine-β93 and histidine-β97 (Moffat et al., 1971). Since both of these modified hemoglobins have essentially the same affinity for zinc, histidine-β97 does not seem to be involved in the binding of zinc to hemoglobin.

[2] The difference between the K_{app} for human hemoglobin A at pH 7 shown in Figure 3 and the value of 1.3×10^7 M^{-1} determined from the data of Figure 2 can be attributed to the different buffer system. The buffer system was changed because Trizma is an ineffective buffer at low pHs.

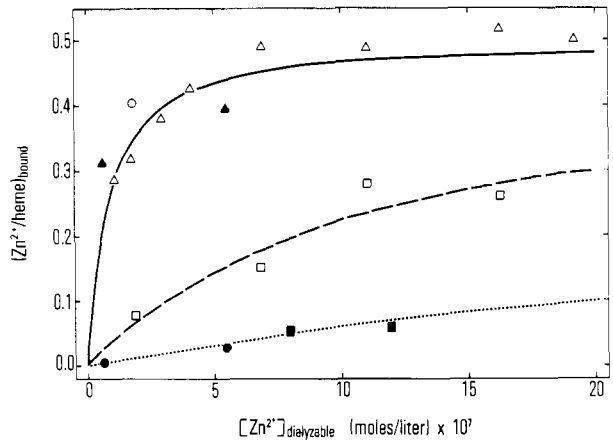

FIGURE 4: The binding of zinc to various human hemoglobins. The effect of altering certain histidine residues. The lines are calculated assuming two identical noninteracting zinc-binding sites: (—) $K_{app} = 1.3 \times 10^7$ M^{-1}; (- - -) $K_{app} = 8 \times 10^5$ M^{-1}; (· · ·) $K_{app} = 1.3 \times 10^5$ M^{-1}. (△) The human hemoglobin data shown in Figure 2 (see legend for experimental conditions); (▲) human hemoglobin A in 0.05 M Trizma, pH 7.3, at 2 °C; (○) human hemoglobin A_2 in 0.01 M Trizma, pH 7.2, at 2 °C; (□) human hemoglobin F in 0.01 M Trizma, pH 7.2, at 2 °C; (●) purified hemoglobin Abruzzo [β143 (H21) His→Arg] in 0.05 M Trizma, pH 7.3, at 2 °C; (■) purified hemoglobin Little Rock [β143 (H21) His→Gln] in 0.05 M cacodylate, pH 7.2, at 2 °C.

No significant differences were found in the binding of zinc to human, horse, bovine, sheep, and rabbit hemoglobins. These results suggest that the histidine(s) involved in the binding of zinc is (are) present in all of these hemoglobins.

We also compared the binding of zinc to adult human hemoglobin, which contains 80–90% hemoglobin A, with that of pure hemoglobin F and hemoglobin A_2 (Figure 4). In hemoglobin F, the β chains are replaced by γ chains in which histidine-β116 is replaced by isoleucine and histidine-β143 is replaced by serine. In hemoglobin A_2, the β chains are replaced by δ chains in which histidine-β116 is replaced by arginine and histidine-β117 is replaced by asparagine. Human hemoglobin F has an appreciably lower apparent zinc association constant ($K_{app} = 8 \times 10^5$ M^{-1}) than adult human hemoglobin, while hemoglobin A_2 is very similar to adult human hemoglobin. These results suggest that histidine-β143, which is the only histidine absent in hemoglobin F but present in both hemoglobin A and A_2, as well as the other animal hemoglobins studied, is perhaps involved in the binding of zinc.

Unfortunately, there is a large difference in the amino acid sequence of the β and the γ chains (there are 38 amino acid substitutions in the γ chains) and hemoglobin F is very different from hemoglobin A in many of its properties. Some of these properties are: the rates of alkaline denaturation (Huehns et al., 1962), the reactivity of the β93 sulfhydryl group (McDonald and Noble, 1974), the oxygen affinity of stripped hemoglobin and the decrease in oxygen affinity produced by organic phosphates (Tyuma and Shimizu, 1969; 1970), and the relative stability of the two quarternary conformations (Wind et al., 1976). The zinc-binding site may, therefore, be completely different in both hemoglobins, and the decrease in the zinc association constant of hemoglobin F (Figure 4) cannot necessarily be attributed to the replacement of histidine-β143 by serine, but only suggests a possible involvement of this histidine residue.

To determine whether histidine-β143 is actually involved, we studied the binding of zinc to two abnormal hemoglobins where the only amino acid alteration involves histidine-β143, i.e., hemoglobin Abruzzo [β143 (H21) His → Arg] (Bonaventura et al., 1975) and hemoglobin Little Rock [β143 (H21)

His → Gln] (Bromberg et al., 1973). The apparent zinc association constant (1.3×10^5 M^{-1}) for purified hemoglobins Abruzzo and Little Rock is two orders of magnitude lower than for hemoglobin A (Figure 4). Histidine-β143 is, therefore, probably involved in the interaction of zinc with hemoglobin.

The Location of the Zinc-Binding Site and a Possible Explanation of the Increased Oxygen Affinity. Our results suggest a possible involvement of both cysteine-β93 and histidine-β143 in the interaction of zinc with hemoglobin. However, inspection of a model of hemoglobin places these two side chains >10 Å apart with histidine-β143 facing into the internal cavity between the β chains, while cysteine-β93 is closer to the heme pocket and the $\alpha_1\beta_2$ interface.

Zinc cannot chelate across such a large distance. However, it is possible that the strong binding of zinc provides the necessary free energy to produce the small conformational change necessary to bring both residues into closer proximity.

Alternatively, zinc might bind to only one of these residues and the observed effects involving the other residue would be mediated through conformational changes linked to the binding of zinc. Consistent with this possibility is the effect of 2,3-DPG on the reaction of 4,4′-dipyridine disulfide (4PDS) with cysteine-β93 (Taketa and Morell, 1969). 2,3-DPG binds to hemoglobin at the entrance to the central cavity between the two β chains (Arnone, 1972). The binding of 2,3-DPG involves a number of basic groups including histidine-β143, but does not directly involve cysteine-β93. The decreased rate for the reaction involving cysteine-β93, therefore, indicates a conformational change extending to the cysteine residue.

The much greater decrease in K_{app} found when substituting histidine-β143 (Figure 4) than when substituting cysteine-β93 (Figure 2) suggests that, if only one of these two amino acids is directly involved, it is probably histidine-β143. This conclusion is also supported by the similar dramatic effect of amino acid substitution in hemoglobin Little Rock and hemoglobin Abruzzo on K_{app} of zinc binding. Both arginine and glutamine have a much lower metal-ion affinity than histidine. However, arginine and glutamine have different charges at neutral pH and are structurally quite different. It is, therefore, expected that the replacement of histidine in these two hemoglobins would have quite different effects on the possible binding of zinc to cysteine-β93, although the effect on the possible binding to β143 would be quite similar.

Since all sulfhydryl reagents have been found to produce a large increase in the oxygen affinity (Taylor et al., 1966; Moffat et al., 1971), binding of zinc involving cysteine-β93 would explain the dramatic increase in the oxygen affinity (Figure 1). However, binding of zinc involving β143 and not β93 can also explain the effect of zinc on the oxygenation of hemoglobin. All the abnormal hemoglobins involving the single substitution of histidine-β143 with another amino acid have also been found to produce very significant increases in the oxygen affinity. This behavior has been found for hemoglobin Little Rock, hemoglobin Abruzzo, and hemoglobin Syracuse [β143 His → Pro] (Jensen et al., 1975).

It has been suggested (Perutz, 1973) that the increased oxygen affinity for hemoglobin Little Rock and perhaps also hemoglobin Abruzzo (Bonaventura et al., 1975) may be due to the formation of a hydrogen bond with the side chains of asparagine H17 (139) of the opposite β chain which stabilizes the liganded conformation. In normal hemoglobin, histidine-β143 is surrounded by water and no hydrogen bonding is observed. However, it is feasible that zinc may coordinate with the histidine H21 (143) of one β chain and asparagine H17 (139) of the second β chain. Such an interaction can take place in the liganded conformation but it is unlikely in the unliganded conformation where the distance between these two groups is considerably greater. Such an interaction would therefore increase the oxygen affinity.

The relationship between the effects of zinc and 2,3-DPG (Table I) can now be understood in terms of the involvement of histidine-β143 in the binding of both 2,3-DPG and zinc. Since there is overlap between the binding site of zinc and 2,3-DPG, competition between the binding of zinc and 2,3-DPG is expected, and the effect of zinc on the oxygenation (Table I) will decrease in the presence of a large excess of 2,3-DPG.

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A Raman Spectroscopic Investigation of the Disulfide Conformation in Oxytocin and Lysine Vasopressin†

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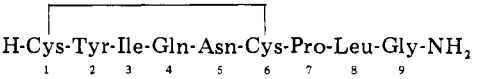
ABSTRACT: The conformation of the CCSSCC moiety in oxytocin and lysine vasopressin is investigated using laser Raman spectroscopy. The Raman spectra of solutions of these hormones in water and in dimethyl sulfoxide show an intense band at 508 cm^{-1} which is assigned to the S-S stretching mode. The presence of shoulders on this band between 490 and 525 cm^{-1} shows that there is an equilibrium among several conformations for the disulfide unit of these hormones in solution. Most of the CS-SC dihedral angles are within 30° of ±90°, but some of the molecules have CS-SC dihedral angles strained away from this value by more than 30°. The previously published circular dichroism spectra of these hormones are reinterpreted, and it is shown that the circular dichroism spectra indicate the presence of more than one conformation for the disulfide unit, in agreement with the Raman results.

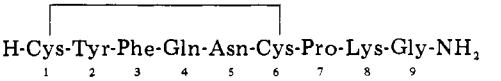
Recently, Van Wart and Scheraga (1976a, b, 1977) and Van Wart et al. (1976a, b) have shown that Raman spectroscopy can be useful for investigating the conformations of disulfides. In this paper, the results of a Raman spectroscopic investigation of the disulfide moiety in oxytocin and lysine vasopressin are reported, and the conformational implications of the Raman spectra, based on the analysis of Van Wart and Scheraga (1976a, b, 1977) and Van Wart et al. (1976a, b), are discussed. The conformations of oxytocin and lysine vasopressin have been investigated previously using NMR[1] (Von Dreele et al., 1971, 1972a; Brewster and Hruby, 1973; Brewster et al., 1973; Glickson et al., 1976; Meraldi et al., 1977), CD (Beychok and Breslow, 1968; Urry et al., 1968, Frič et al., 1975), and conformational energy calculations (Kotelchuck et al., 1972; Brewster et al., 1973). The NMR studies have yielded considerable information about the conformation of these molecules in water and in dimethyl sulfoxide, but this technique has not been useful for studying the conformation around the disulfide bond. CD has been used previously to study the disulfide conformation in these hormones (Beychok and Breslow, 1968; Urry et al., 1968; Frič et al., 1975), but the bands due to the disulfide chromophore are partially obscured by the CD of tyrosine (Frič et al., 1975), making band assignments difficult. In this paper, we present a new interpretation of the CD of these hormones which is consistent with our Raman results and with recent theoretical studies of the optical activity of disulfides (Linderberg and Michl, 1970).

Materials and Methods

Oxytocin



and lysine vasopressin



were generously synthesized, assayed, and supplied by Drs. Roderich Walter and Clark Smith, and were used as received. The oxytocin possessed 495 ± 25 units of avian vasodepressor activity per mg, and the lysine vasopressin had 275 ± 15 units of rat pressor activity per mg. These values may be compared with 507 ± 15 units/mg of avian vasodepressor activity for oxytocin (Chan and du Vigneaud, 1962) and 275–300 units/mg of rat pressor activity for lysine vasopressin (Meienhofer and du Vigneaud, 1960). Solutions were prepared by dissolving the hormones in dilute aqueous acetic acid or in dimethyl sulfoxide, with concentrations ranging between 7 and 10% (w/v). The pH of the aqueous solutions was adjusted to a value between 4 and 4.5 with acetic acid. After the Raman spectra

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; CD, circular dichroism.