A Spin-Label Study of Hemoglobin–Haptoglobin Complexes†

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ABSTRACT: The reaction of haptoglobin with hemoglobin spin-labeled at cysteine-β93 with *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (IAM-5) or *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (IAM-6) was studied. Starch gel electrophoresis demonstrated the presence of both the hemoglobin–haptoglobin (Hb·Hp) complex and the intermediate complex. The electron spin resonance (esr) spectrum of the complex indicated that the environment of β93 changed on complex formation. In agreement with previous work using IAM-6 the spectral change observed on complex formation was similar to that observed on removal of oxygen. However using IAM-5 the Hb·Hp complex had an esr spectrum intermediate to those of oxy- and deoxyhemoglobin. Thus, it appears that the environments sensed by the two spin labels are different, and therefore they respond differently to conformational changes induced at position β93 by complex formation. The binding of modified haptoglobins to spin-labeled hemoglobin was studied in order to determine some of the structural requirements for binding.

Haptoglobins bind hemoglobin specifically to form a stoichiometric and largely undissociable complex (Chiancone *et al.*, 1968). In humans there are three haptoglobin phenotypes, Hp 1-1, Hp 2-1, and Hp 2-2,[1] each consisting of heavy and light polypeptide chains linked by disulfide bonds (Smithies *et al.*, 1966; Hamaguchi, 1969). The heavy chains are believed to be involved in the binding of hemoglobin and are similar in all three genetic types (Gordon *et al.*, 1968). Several similarities have been noted between haptoglobins and antibodies (Black and Dixon, 1968; Malchy and Dixon, 1970). Two complexes between hemoglobin and haptoglobin have been observed—the hemoglobin–haptoglobin complex consisting of one molecule each of hemoglobin and haptoglobin, and the hemoglobin–haptoglobin intermediate, in which only an $\alpha\beta$ hemoglobin dimer is attached to a haptoglobin molecule (Hamaguchi, 1969). Several studies (Hamaguchi, 1969; Nagel and Gibson, 1967; Malchy and Dixon, 1970; Bunn, 1969) have indicated that Hp does not combine with the intact Hb tetramer but does combine with two $\alpha\beta$ Hb dimers at two distinct sites.

The spin-labeling technique has been used to study oxygen-induced conformation changes in hemoglobin (Ogawa and McConnell, 1967; Ogawa *et al.*, 1968) by attaching substituted iodoacetamides at cysteine-93 of the β chains. These substituted iodoacetamides contain a nitroxide group with the nitrogen atom in either a five- or six-membered ring (IAM-5 and IAM-6, see Figure 2). It has been shown that when IAM-5 (Ogawa and McConnell, 1967) or IAM-6 (McConnell and Hamilton, 1968; Moffat, 1971) is attached to hemoglobin at the sulfhydryl of cysteine-β93 it can have two different spatial orientations relative to the hemoglobin molecule. When the electron spin resonance (esr) spectrum of the attached IAM-5 was examined as a function of oxygenation of the hemoglobin it could be seen that the relative proportions of the spin label in the two different orientations changed considerably (Ogawa and McConnell, 1967). With hemoglobin containing IAM-6 (Ogawa *et al.*, 1968), the spectrum changed to a greater extent, and it was suggested that the spin label had a third orientation relative to the hemoglobin.

More recently using X-ray crystallographic techniques Moffat (1971) concluded that attachment of IAM-6 to β93 had changed the conformation of the Hb. This was implied by earlier work which showed that spin-labeled Hb had a much higher oxygen affinity than unlabeled Hb, although both hemoglobins retained about the same degree of cooperativity (Ogawa and McConnell, 1967).

Makinen and Kon (1971) have studied the esr spectrum of IAM-6 (attached to hemoglobin position β93) in the hemoglobin–haptoglobin complex. They concluded that the spin-label spectrum of the oxy- or deoxyHb·Hp complex was very similar to that of spin-labeled deoxyHb.

We have studied the effect of adding various haptoglobins to hemoglobin spin labeled with IAM-5. The effect produced on the spin-label spectrum by the addition of unmodified haptoglobin was in the same direction as that produced by deoxygenation, but quantitatively quite different. In addition we have studied the effect of some modified haptoglobins on the spectrum of spin-labeled hemoglobin in order to determine possible structural requirements for binding. A preliminary account of some of this work has appeared elsewhere (Smith, 1972).

Materials and Methods

Human and horse hemoglobin were prepared by lysing red blood cells with an equal volume of water. The membranes were removed by centrifugation, and the Hb solution was dialyzed against water. Hb was spin labeled with IAM-5 as described by Ogawa *et al.* (1968). IAM-6 was a product of Syva Associates, Palo Alto, Calif. Excess spin label was

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[1] Nonstandard abbreviations used are: Hp, haptoglobin; Hb, hemoglobin; IAM-5, *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide; IAM-6, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide; MetHb, methemoglobin; MetHbCN, methemoglobin cyanide.

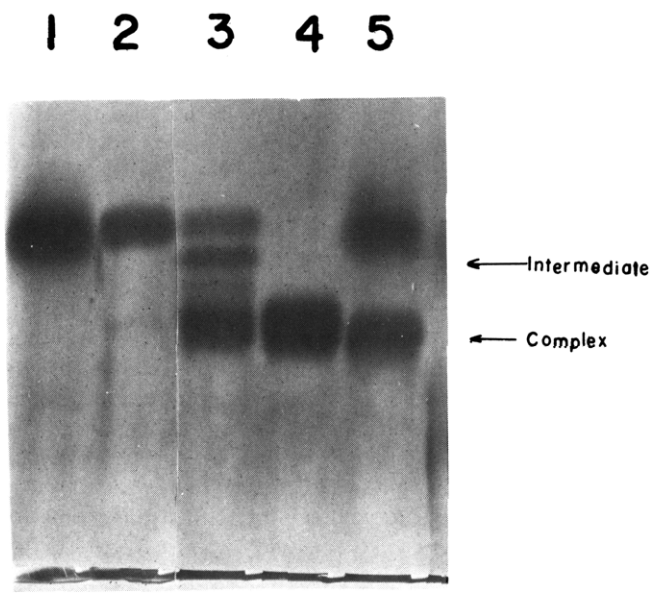

FIGURE 1: Starch electrophoresis of mixtures of spin-labeled hemoglobin and haptoglobin. Molar ratio of haptoglobin to hemyglobin: slot 1, 0; slot 2, ∞; slot 3, 2:1; slot 4, 1:1; slot 5, 0.5:1.

removed by chromatography on Sephadex G-25 in 0.05 M phosphate (pH 7) or by extensive dialysis against phosphate buffer.

Methemoglobin was prepared by adding a small amount of potassium ferricyanide to a solution of labeled hemoglobin and removing the excess reagent by dialysis at 4° against 0.05 M phosphate buffer (pH 7.2). Cyanomethemoglobin was prepared by dialyzing labeled methemoglobin against the same buffer containing a small amount of potassium cyanide.

Haptoglobin was prepared according to the standard methods (Ofosu *et al.*, 1971; Black *et al.*, 1970). Plasmin-digested haptoglobin, fragments P_1 and P_2, and glycosidase-treated haptoglobin were prepared as described by Ofosu (1971). The concentrations of hemoglobins and haptoglobins were determined by assuming $E^{1\%}_{540\,nm} = 8.4$ for hemoglobin and $E^{1\%}_{280\,nm} = 12.0$ for haptoglobin (Jayle and Moretti, 1962).

Starch gel electrophoresis was performed as described by Smithies (1959). Esr spectra were recorded at 21° with a Varian E-3 or E-9 spectrometer using a flat aqueous sample cell. Spectra were run at a series of concentrations of hemoglobin to ensure that the spectral changes observed on complex formation were independent of absolute concentration.

Results and Discussion

Figure 1 shows starch gel electrophoretic analyses of the mixtures of spin-labeled Hb and Hp. It can be seen that when equimolar amounts of Hp and Hb are mixed the complex is formed, and no unreacted Hb or Hp is present. In the presence of excess Hp, the Hb·Hp intermediate is also formed. Thus, by the criterion of starch gel electrophoresis the reaction of spin-labeled hemoglobin with haptoglobin is similar to the reaction of unmodified hemoglobin with haptoglobin.

Figure 2a is the esr spectrum of hemoglobin labeled with IAM-5. Components S and W are interpreted to represent two states of the spin label relative to the Hb, *i.e.*, a strongly immobilized position and a weakly immobilized position. When Hp is added to the solution of spin-labeled hemoglobin, a significant change in the spectrum occurs. This change is the same when Hp 1-1 or Hp 2-1 is added, and cannot be produced by the addition of albumin or γ-globulin. The spectrum of the spin-labeled Hb·Hp complex, when compared to that of Hb, shows an increased intensity in the region between the peaks of the weakly and strongly immobilized components of the Hb spectrum (Figure 2). Thus, the conformational change occurring on complex formation results in a decrease in the populations of strongly and weakly immobilized spin-label states and the appearance of a third state of intermediate mobility. The spectrum of the complex is somewhat similar to the spectrum observed for hemoglobin (spin labeled with IAM-6) in 2 M sodium phosphate (pH 5.1) (Deal *et al.*, 1971).

Using IAM-6 Makinen and Kon (1971) obtained an esr spectrum for the Hp·Hb complex which was similar to that obtained on deoxygenation of Hb. This result, together with the work of Malchy and Dixon (1969), which showed that the β93 SH of Hb was less reactive in the Hb·Hp complex, suggested that the environment around β93 in the Hb·Hp complex was similar to that of β93 in deoxyHb. Our esr spectra of the complex do not confirm this conclusion. The spectrum of deoxyHb spin labeled with IAM-5 (Ogawa and McConnell, 1967) consists of only a component of intermediate mobility, which is considerably more mobile than IAM-5 in the Hb·Hp complex. In this context we have measured the esr spectra of the Hb·Hp complex containing IAM-6 (Makinen and Kon, 1971) and of deoxyhemoglobin containing IAM-5 (Ogawa and McConnell, 1967); our spectra were similar to those previously reported. Thus, while on formation of a Hb·Hp complex labeled at Hb β93 with IAM-6 the esr spectrum resembles that of deoxyhemoglobin, on formation of a complex labeled at the same position with IAM-5 the esr spectrum resembles that of neither oxyhemoglobin nor deoxyhemoglobin. One can conclude from studies with both spin labels that the conformational change occurring at β93 of hemoglobin on complex formation is such that populations of weakly and strongly immobilized spin labels are decreased to populate a state of intermediate mobility. One cannot conclude that the conformation around β93 in the complex is the same as that in deoxyhemoglobin.

Addition of Hp to MetHb or MetHbCN, spin labeled at β93 with IAM-5, resulted in spectral changes qualitatively similar to those found with oxyhemoglobin. In addition the esr spectra, and spectral changes induced by haptoglobin addition, were essentially the same for horse and human hemoglobin.

The esr spectra of spin-labeled hemoglobins must be interpreted carefully in view of the recent X-ray data of Moffat (1971). He has observed extensive distortions in the positions of some amino acids after spin labeling. The areas particularly affected are the environment of the heme groups, the F helix of the β chain, and the FG region of the $\alpha_1\beta_2$ interface. Thus, since it has also been demonstrated that IAM-5 and IAM-6 sample slightly different regions of the hemoglobin structure (Ogawa *et al.*, 1968), the structural perturbations caused by each may well be different. It is not surprising, therefore, that they respond somewhat differently to formation of the Hb·Hp complex. While their esr spectra are valuable for studying properties of the complex formed by one species of spin-labeled hemoglobin and various types of haptoglobin (*vide infra*) the data indicate that one must proceed with caution in using conformational data from the spin-labeled complex to discuss the properties of the region around β93 in the unlabeled complex. This study does show that it is advantageous to use more than one spin label when

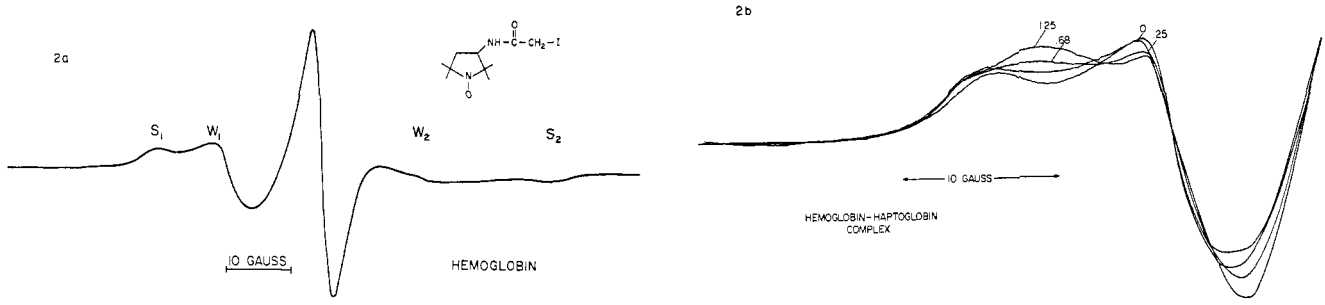

FIGURE 2: (a) The esr spectrum of spin-labeled hemoglobin at pH 7.2, 1% in 0.05 M phosphate buffer. Spectral components due to strongly and weakly immobilized spin labels are indicated by S and W, respectively. (b) Superpositions of the low field portions of esr spectra due to spin-labeled hemoglobin complexed with human haptoglobin 1-1. The numbers represent the molar ratios of haptoglobin to hemoglobin.

intending to use data from the modified protein to draw conclusions about the unmodified protein, as was done in studies of the Hb·HbO_2 equilibrium (Ogawa and McConnell, 1967; Ogawa *et al.*, 1968).

We have also studied the effects of some modified haptoglobins on hemoglobin labeled with IAM-5. The modified haptoglobins were produced by removing carbohydrates, by reduction and alkylation, and by plasmin digestion. Other techniques have indicated that removal of carbohydrates from haptoglobin does not affect its binding to hemoglobin while reduction and alkylation destroy binding ability (Ofosu, 1971). Plasmin digestion splits haptoglobin into two fragments, P_1 and P_2, which can be separated. While neither P_1 nor P_2 can bind hemoglobin separately, an equimolar mixture of P_1 and P_2 does (Ofosu and Connell, 1971).

Figure 3 shows a comparison of the esr spectra of spin-labeled oxyhemoglobin and spin-labeled oxyhemoglobin plus plasmin-digested haptoglobin (molar ratio Hp:Hb = 0.7). The spectral changes induced by plasmin-digested haptoglobin are essentially identical to those induced by native haptoglobin, while fragments P_1 or P_2 produced no spectral changes. A recombined mixture of P_1 and P_2 produced the same spectral change as plasmin-digested haptoglobin. Haptoglobin treated with sialidase, or a mixture of sialidase, β-galactosidase, and β-*N*-acetylglucosaminidase, produced spectral changes similar to those due to normal haptoglobin binding, while reduced and alkylated haptoglobin produced no changes. Thus, the modified haptoglobins judged by electrophoresis and ultracentrifugation analysis (Ofosu and Connell, 1971) to be capable of binding hemoglobin all produce essentially the same esr spectrum for spin-labeled hemoglobin as does unmodified haptoglobin. This implies that the mechanism of binding and the type of complex are similar in each case.

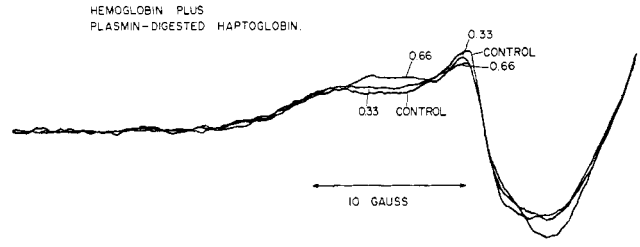

FIGURE 3: Superpositions of the low-field portion of the esr spectra of spin-labeled hemoglobin, and spin-labeled hemoglobin plus fragments P_1 and P_2. The molar ratio of P_1 or P_2 to hemoglobin is indicated in the figure.

Conclusion

On binding haptoglobin a conformational change occurs at position β93 in spin-labeled hemoglobin resulting in a state of intermediate mobility for the spin label. The environment around the spin label at β93 in the complex of Hp and Hb spin labeled with IAM-5 is not identical with that in the corresponding spin-labeled deoxyHb, although earlier work with IAM-6 (Makinen and Kon, 1971) has shown that the esr spectra of deoxyhemoglobin and the Hb·Hp complex are similar. Because of the extensive conformational changes around β93 in spin-labeled hemoglobin, and the differences between spectra of Hb·Hp complexes in which the Hb was labeled with IAM-5 or IAM-6, one must be very careful in reaching conclusions about the environment of β93 in the unlabeled Hb·Hp complex. It is possible to conclude that the environment around β93 of Hb in the Hp·Hb complex changes so as to allow a third state of intermediate mobility for the spin label. Studies using plasmin- or sialidase-digested haptoglobin demonstrated the formation of a complex. In addition, fragments P_1 or P_2 from the plasmin digest produced no spectral change while an equimolar mixture of the two produced a definite spectral change. Reduced and alkylated haptoglobin did not affect the esr spectrum of spin-labeled hemoglobin.

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Interaction between Sodium Dodecyl Sulfate and Ferricytochrome *c*†

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ABSTRACT: The interaction of sodium dodecyl sulfate with ferricytochrome *c* from horse heart was studied at pH 7.35. The interaction appears to occur in two phases. The first of these is suggested to involve binding of the dodecyl sulfate anions to the cationic sites of the protein causing an unfolding of the protein, loss of its absorption band at 695 nm, and hyperchromic blue shift of its Soret peak to approximately 408 nm. The second phase is suggested to involve binding of the hydrophobic portions of the dodecyl sulfate to the hydrophobic amino acid residues of the protein and to be associated with a partial loss of the initial hyperchromicity of the Soret peak and a further shifting to approximately 406 nm.

Detergents have a variety of uses in biochemical laboratories. Two examples are the use of sodium dodecyl sulfate to solubilize mitochondrial proteins (see Morton, 1955, for review) and gel electrophoresis of sodium dodecyl sulfate–protein complexes to determine molecular weights (Shapiro *et al.*, 1967; Weber and Osborn, 1969). It has long been known, however, that detergents can denature proteins and considerable effort has been made by numerous investigators to elucidate this type of denaturation. Much of this effort has been directed toward explaining the denaturation of bovine serum albumin in terms of protein–ion interactions (see Putnam, 1948; Foster, 1960; Steinhardt and Reynolds, 1969; Tanford, 1968, 1970, for reviews). We thus thought it appropriate to study the interaction between the anionic detergent dodecyl sulfate and the mitochondrial protein cytochrome *c* since these two substances often encounter each other during biochemical manipulations and little has been done toward explaining the effects of this detergent on cytochrome *c* in terms of protein–ion interactions. This paper reports some of our observations and it is hoped they will be of interest to those involved in fractionating mitochondrial proteins or studying detergent–protein interactions.

The fact that dodecyl sulfate affects cytochrome *c* has been known since 1940 when Keilin and Hartree reported that it altered the visible absorption bands of this protein in both of its oxidation states (Keilin and Hartree, 1940). Further studies of this observation have been made since then (Rabinovitz and Boyer, 1950; Tsushima and Miyajima, 1956), but to date these spectral alterations have not been related to the interaction that occurs between these substances. Accordingly, we reexamined the spectral changes caused by adding dodecyl sulfate to ferricytochrome *c* and determined the extent of interaction between these two substances so that a correlation between these could be made and hopefully provide further information on how cytochrome *c* is affected by this detergent.

Experimental Section

Dodecyl Sulfate Samples. Sodium dodecyl sulfate was obtained from two sources: Matheson Coleman & Bell (Norwood, Ohio) and the Mann Research Laboratories (New York, N. Y.).

The detergent from the first source was found to contain dodecyl sulfate and tetradecyl sulfate, in a ratio of approximately 2:1 by weight, plus small amounts of unesterified alcohols and inorganic matter. The purity of this detergent could be improved by extraction of the unesterified alcohols with ether, extraction of the detergent from the inorganic matter by use of hot *n*-butyl alcohol, and precipitation from this solvent by adding ether.

The detergent supplied by Mann was a special grade recommended by Dr. J. Steinhardt of Georgetown University for use in equilibrium dialysis (personal communication). It was used without further purification.

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