

SPIN-LABELED HEMOGLOBIN*

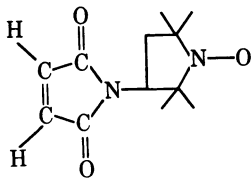
BY JAN C. A. BOEYENS† AND HARDEN M. MCCONNELL

STAUFFER LABORATORY FOR PHYSICAL CHEMISTRY, STANFORD, CALIFORNIA

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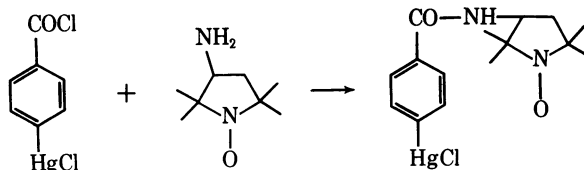
In the present paper we describe the results of a preliminary study of the paramagnetic resonance spectrum of spin-labeled horse hemoglobin in solution. Our results provide strong evidence that on oxygenation the β chains undergo a significant structural change near the "reactive" sulfhydryl group ($\beta 93$), a change not yet resolved by X-ray diffraction.¹ We also find features in the paramagnetic resonance spectra of nitroxide-maleimide spin-labeled hemoglobin that appear to be closely related to observations by Benesch and Benesch² (BB) on the inhibition of the Bohr effect by N-ethyl maleimide (NEM).

Materials and Methods.—Horse hemoglobin was purchased from Calbiochem, 2 \times crystallized, lot 62248. Fresh hemoglobin was obtained from defibrinated horse blood (W. T. Bennett Ranch Laboratory) according to the method described by Benesch and Benesch.³ Deoxygenation of the hemoglobin was effected by passing high-purity nitrogen over the stirred solution. Since the addition of a small excess of sodium dithionite yielded the same results, this more convenient and more effective method was used preferentially whenever feasible.⁴ The preparation of the nitroxide-maleimide I (N-(1-oxyl-2,2,5,5-tetramethyl-pyrrolidiny)-maleimide) is described by Griffith and McConnell.⁵



I

Preparation of a mercurial spin label (II): One gm of sodium p-chloromercuribenzoate (PCMB) was converted to the acid chloride by refluxing with thionyl chloride for 30 min.⁶ The excess thionyl chloride was removed by washing with chloroform. The crude p-chloromercuribenzoyl chloride was dissolved in pyridine, 0.5 gm of 2,2,5,5-tetramethyl-3-carbamidopyrrolidine⁷ was added, and the mixture was refluxed for 10 min. The fine precipitate of NaCl was centrifuged off and pyridine removed by rotary evaporation. The yellow residue was washed with 0.3 M HCl, then with water, and finally with a small quantity of methanol. A colorless paramagnetic substance soluble in acetone remained. All attempts to crystallize the material failed. Because of difficulties in the purification, no chemical analysis was made, but the successful spin-labeling of sulhydryl proteins with the product indicated that the reaction proceeded as below.



II

The paramagnetism of an aqueous solution of (II) fades slowly on exposure to light and disappears very rapidly on ultraviolet irradiation.

All reactions with hemoglobin were carried out at 0°C in phosphate buffer at pH 6.8. Solid (I) or (II) was dissolved in the hemoglobin solutions by stirring and the excess removed by dialysis at 0°C. To monitor spectral changes due to reactions occurring immediately after mixing, it was necessary to use the undialyzed solutions.

Results.—The paramagnetic resonance spectrum of hemoglobin labeled with (II) corresponds to incomplete or weak immobilization of the spin with respect to the protein⁸ and is similar to that shown in Figure 1*a*. In the presence of excess of (II) a strongly immobilized component, similar to that in Figure 1*b*, is observed. This corresponds to hydrophobic bonding of (II) to some unidentified region of the hemoglobin molecule; this strongly immobilized spin can be removed by dialysis, in contrast to the signal arising from the weakly immobilized spin which almost certainly arises from attachment of (II) to the reactive —SH at β 93. The weakly immobilized signal is essentially the same for oxygenated and deoxygenated molecules.

Quite different spectra are obtained if hemoglobin in the oxygenated (HbO₂) and deoxygenated (Hb) states are labeled with (I), as illustrated in Figures 1*a* and *b*. As will be seen below, the origin of this difference is quite complex and is evidently related to earlier observations of BB on the reaction of NEM with human hemoglobin. A summary of our experimental results and our interpretations are given below.

(1) In aqueous solution (I) is hydrolyzed within 5 min and subsequently does not react with hemoglobin.

(2) (I) reacts with ϵ -NH₂ groups in Hb and HbO₂ to give EPR signals corresponding to weakly immobilized spins.

(3) (I) reacts slowly⁹ with β 93 —SH in Hb and much more rapidly in HbO₂, presumably involving a direct addition of —SH to the carbon-carbon double bond of (I), and in both cases gives a weakly immobilized spin signal as in Figure 1*a*.

(4) After (I) has reacted with —SH on β 93 in Hb, it undergoes a subsequent reaction—presumably opening of the ring by hydrolysis—but the label remains attached, and the spectrum essentially unchanged and unaffected by subsequent oxygenation of the molecule. Reaction time for this hydrolysis is about 30 min.

(5) After (I) has reacted with —SH on β 93 in HbO₂, it undergoes a subsequent reaction to produce a strongly immobilized signal (Fig. 1*b*), with a reaction time of a few minutes. This immobilization is not affected by subsequent deoxygenation. (Methemoglobin gives a similar spectrum.)

(6) If Hb labeled with (I) on —SH at β 93 is oxygenated before the hydrolysis indicated in (4) reaches completion, then reaction (5) occurs and some strongly immobilized spin is obtained.

(7) Observations similar to those given in (3), (4), and (5) above were also obtained in 3 M NaCl solutions where there is a symmetrical dissociation of hemoglobin into $\alpha\beta$ chains.¹⁰

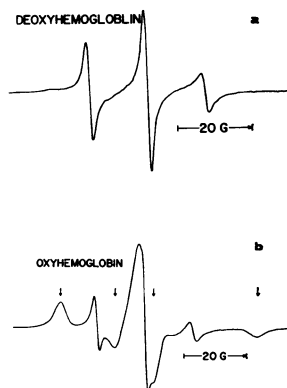


FIG. 1.—Paramagnetic resonance spectrum of horse (a) deoxyhemoglobin, and (b) oxyhemoglobin spin-labeled with the nitroxide-maleimide I. Broad signals identified by arrows in (b) are due to the nitroxide-maleimide I strongly immobilized at the SH group (β 93).

(8) Carboxypeptidase A, which splits the β chain between $\beta 144$ and $\beta 145$,¹¹ converts the strongly immobilized spin attached to the —SH on $\beta 93$ to a weakly immobilized spin.

Our conclusion that the strongly immobilized spin signal comes from attachment of (I) to —SH on $\beta 93$ is based on our observation that a strongly immobilized spin signal is never obtained when HbO₂ is treated with PCMB (or with NEM) prior to reaction with (I). The crystallographic study¹² of horse HbO₂ showed conclusively that PCMB reacts specifically with the —SH on $\beta 93$. The ultimate reaction product of spin label (I) with Hb is seen to be different from the reaction product with HbO₂, since the latter gives rise to a strongly immobilized spin and the former to a more mobile spin, even after subsequent oxygenation. We consider it likely that the two reaction products differ in covalent bond structure—i.e., in the two cases hydrolysis may take place at different C—N bonds on the ring attached to —SH. Observations (3) and (7) above show that the β chains must have different conformations in the vicinity of $\beta 93$ in horse Hb and HbO₂.

It is of interest to compare the foregoing results with studies of BB and others on the reaction of human hemoglobin with NEM.^{2, 13, 14} To do so it is necessary to bear in mind that the maleimide in (I) is hydrolyzed very rapidly in water [cf. (1) above] compared to NEM which at pH 7, in the absence of catalysts, is stable in water nearly indefinitely on our time scale. This difference in reactivity is presumably due to steric strain introduced in the maleimide ring by the tetramethylpyrrolidine group. Just as in (3) above, Riggs¹³ finds that HbO₂ reacts much faster than Hb with NEM. Similarly, BB find a subsequent reaction for NEM attached to HbO₂ with a reaction time of half an hour [cf. (5) above]. BB do not find an hydrolysis reaction analogous to (4) above, and this we attribute simply to the relatively more rapid hydrolysis of (I). BB followed the reaction of NEM with hemoglobin by its modification of the Bohr effect. Indeed there is a striking parallel between the modification of the Bohr effect due to the secondary reaction of NEM attached to HbO₂ and the strong immobilization of spin label (I) due to the secondary reaction of I attached to HbO₂ [not the simple hydrolysis of (I) attached to Hb]. BB attribute the secondary reaction of NEM attached to HbO₂ to a catalyzed opening of the imide ring, followed by the formation of a hydrogen bridge with the nitrogen of an imidazole. Precisely the same mechanism could very well lead to the immobilization of the spin label reported here.¹⁵ Acylation of imidazole associated with the opening of the imide ring is an alternative possibility. (One could refer to this as an "accidental" strong immobilization in contrast to "intrinsic" strong immobilization of spin-labeled substrates¹⁶ at active sites in enzymes, as well as spin labels held by hydrophobic bonding to proteins and nucleic acids.¹⁷)

Imidazole rings that look like good candidates for this interaction are histidine $\beta 97$ (Benesch and Benesch¹⁸) and, we suggest, histidine $\beta 146$. In the latter case the loss of strongly immobilized spin and the modification of the Bohr effect¹⁹ after digestion of carboxypeptidase A are readily explained, since this enzyme removes $\beta 146$ from hemoglobin. Our choice of $\beta 146$ was suggested by examination of drawings of the structure of horse HbO₂ given by Perutz.^{20, 21} The evidently significant change in structure of the β chain in the vicinity of $\beta 93$ doubtless has its origin in the proximity of the heme group, and oxygen-associated conformational changes within this group and/or the iron coordinated histidine ($\beta 92$).

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† On leave from the National Physical Research Laboratory of the Council for Scientific and Industrial Research, Pretoria, South Africa.

¹ Nobbs, C. L., H. C. Watson, and J. C. Kendrew, *Nature*, **209**, 339 (1966).

² Benesch, R., and R. E. Benesch, *J. Biol. Chem.*, **236**, 405 (1961).

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⁴ Hamilton, C. L., and H. M. McConnell (unpublished results) demonstrated that the EPR spectrum of a nitroxide radical cannot be observed in the presence of dithionite, but it reappears on removal of the latter by filtering through a Sephadex column, or by dialysis, if the nitroxide is attached to a protein.

⁵ Griffith, O. H., and H. M. McConnell, these PROCEEDINGS, **55**, 8 (1966).

⁶ Whitmore, F. C., and G. E. Woodward, *J. Am. Chem. Soc.*, **48**, 535 (1926).

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⁸ The effect on the nitroxide EPR spectrum of the immobilization of spins, relative to a slowly tumbling protein molecule, is discussed in reference 5.

⁹ Hydrolysis of unreacted (I) is more rapid than the reaction with —SH in Hb. Complete blocking of the available —SH groups can only be achieved by adding a large excess of (I) in small quantities, at intervals of 15 min. Blocking is complete if no strongly immobilized spin signal is observed on addition of more (I) after oxygenation of the hemoglobin.

¹⁰ Antonini, E., J. Wyman, E. Bucci, C. Fronticelli, and A. Rossi-Fanelli, *J. Mol. Biol.*, **4**, 368 (1962).

¹¹ Antonini, E., J. Wyman, R. Zito, A. Rossi-Fanelli, and A. Caputo, *J. Biol. Chem.*, **236**, Pc 60 (1961).

¹² Cullis, A. F., H. Muirhead, M. F. Perutz, and M. G. Rossmann, *Proc. Roy. Soc. (London)*, **A265**, 161 (1962).

¹³ Riggs, A., *J. Biol. Chem.*, **236**, 1948 (1961).

¹⁴ Guidotti, G., and W. Konigsberg, *J. Biol. Chem.*, **239**, 1474 (1964).

¹⁵ Guidotti and Konigsberg¹⁴ cannot reconcile the results of their study of the NEM-HbO₂ system with hydrolysis of the succinimide ring. In view of the uncatalyzed hydrolysis of the nitroxide maleimide in aqueous solution observed here, their objections do not seem to apply in the present case.

¹⁶ Berliner, L. J., and H. M. McConnell, these PROCEEDINGS, **55**, 708 (1966).

¹⁷ Ohnishi, S., and H. M. McConnell, *J. Am. Chem. Soc.*, **87**, 2293 (1965).

¹⁸ Benesch, R., and R. E. Benesch, *J. Mol. Biol.*, **6**, 498 (1963).

¹⁹ Zito, R., E. Antonini, and J. Wyman, *J. Biol. Chem.*, **239**, 1804 (1964).

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²¹ Perutz, M. F., *J. Mol. Biol.*, **13**, 646 (1965).