Electron Paramagnetic Resonance Studies of Spin-Labeled Hemoglobins. II. Roles of Subunit Interactions and of Intermediate Structures in the Cooperative Oxygenation of Hemoglobin and the Results on Hemoglobin Yakima, Hemoglobin J Capetown, and Carboxypeptidases A and B Treated Hemoglobin A*

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ABSTRACT: The spin-label technique has been used to study two genetically and two enzymatically modified human hemoglobins: Hb Yakima, Hb J Capetown, carboxypeptidases A and B treated Hb A. The results are in agreement with our recent work that the presence or absence of a set of isosbestic points in the electron paramagnetic resonance spectra of hemoglobins labeled with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide as a function of O_2 saturation is a good indication on the nature of subunit interactions in hemoglobins during oxygenation and suggests that the intermediate structures detected by the spin labeling are likely to be associated with the cooperative oxygenation process. The latter conclusion is also in agreement with the early work of McConnell and his coworkers. The spin-labeled hemoglobins that we have studied can be divided into three major categories, namely fully cooperative (such as Hb A and Hb F), intermediately cooperative (such as Hb J Capetown), and noncooperative (such as Hb Yakima and Hb Chesapeake). All the fully cooperative hemoglobins that we have studied lack a set of isosbestic points in their respective electron paramagnetic resonance spectra on CO saturation indicating the presence of intermediate structures and normal α_1–β_2 subunit interactions. Those hemoglobins (such as Hb J Capetown, Hb Chesapeake, Hb Yakima, and carboxypeptidase A treated Hb A) with intermediately cooperative or noncooperative ligand binding give a sharp set of isosbestic points as a function of CO saturation suggesting that there may be only two conformations (CO and deoxy forms) and that there are altered α_1–β_2 subunit interactions in these hemoglobins. These results as well as their implications to the nature of cooperative oxygen binding to hemoglobin are discussed with reference to Perutz's model of hemoglobin, Moffat's X-ray analysis of spin-labeled Hb A and our nuclear magnetic resonance studies on hemoglobins.

The spin-label technique has recently been applied to study the nature of cooperative ligand binding in hemoglobin (Ogawa and McConnell, 1967; Ogawa *et al.*, 1968; McConnell *et al.*, 1968; Ho *et al.*, 1970a). Based on the electron paramagnetic resonance spectra of Hb A[1] and horse Hb labeled with *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrolidinyl)-iodoacetamide and *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide, Ogawa *et al.* (1967, 1968) demonstrated that the second spin label was extremely sensitive to conformational changes that take place during oxygenation. They suggested that the lack of isosbestic points in the electron paramagnetic resonance spectra might be related to the cooperative binding of oxygen.

In previous studies, we tried to determine if the changes in the electron paramagnetic resonance spectra of spin label II[1] produced by oxygen binding and the intermediate structures between the oxy and deoxy conformations detected by spin labeling are in fact related to cooperative oxygenation of hemoglobin. Our approach has been to choose genetic variants of hemoglobin and chemically modified hemoglobins which have full, reduced, or missing cooperativity and to relate their spin-label spectra to the presence or absence of cooperativity. A preliminary report of our studies on hemoglobins A, F, Zürich, and Chesapeake has been published (Ho *et al.*, 1970a). Hemoglobins A and F are fully cooperative

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966) are: Hb A, human adult hemoglobin; Hb F, human fetal hemoglobin; α and β chains, the polypeptide chains of Hb A; CPA, carboxypeptidase A; CPB, carboxypeptidase B; spin label I, *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrolidinyl)iodoacetamide; spin label II, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide.

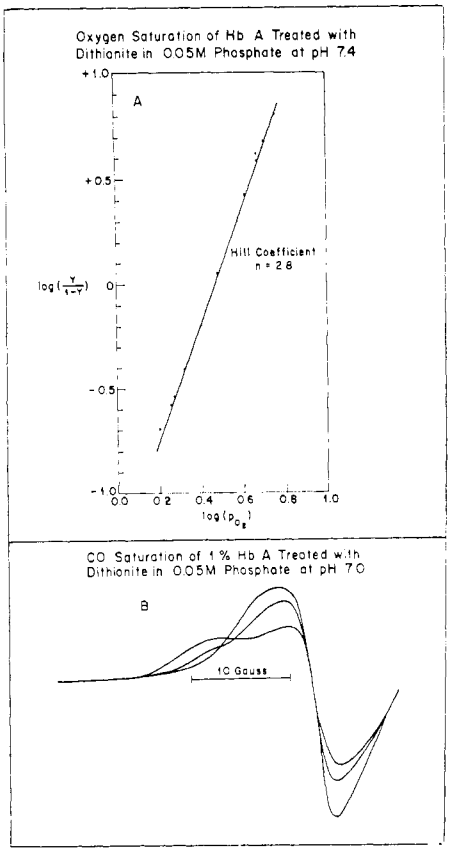

FIGURE 1: (A) Hill plot of the oxygen equilibrium of dithionite-treated HbA at 0.1% and at 23°. The partial pressure of oxygen (P_{O_2}) is in mm of mercury. (B) electron paramagnetic resonance spectra of dithionite treated Hb A labeled with spin label II (see text) at 23°.

with Hill coefficients equal to 2.9 (Antonini *et al.*, 1964; Wyman, 1964). Hb Zürich, which has a Hill coefficient of at least 1.8, is significantly cooperative (Winterhalter *et al.*, 1969), whereas Hb Chesapeake with a Hill coefficient of 1.3 is essentially noncooperative (Nagel *et al.*, 1967). Isosbestic points were not observed in the electron paramagnetic resonance spectra of hemoglobins A, F, and Zürich on CO saturation, but were present in the spectra of Hb Chesapeake (Ho *et al.*, 1970a). Since it was possible that spin label II might not detect intermediate conformations which are peculiar to Hb Chesapeake, we have extended our studies to two other genetically and two enzymatically modified hemoglobins: Hb Yakima, Hb J Capetown, carboxypeptidases A and B treated Hb A. Hb Yakima [β99(G1) Asp→His] (Jones *et al.*, 1967) and CPA-treated Hb A are noncooperative, both have Hill coefficients of approximately 1.0 (Novy *et al.*, 1967; Zito *et al.*, 1964). Hb J Capetown [α92(FG4) Arg→Gln] (Botha *et al.*, 1966) has a Hill coefficient of approximately 2.2 for purified hemoglobin (S. Charache, unpublished results) and of 1.9 for the hemolysate of Hb A and Hb J Capetown (Lines and McIntosh, 1967). Carboxypeptidase A removes the last two amino acid residues, histidine β-146(HC3) and tyrosine β-145(HC2) (Zito *et al.*, 1964), of the β chain. The loss of cooperativity is probably due to the removal of tyrosine at β-145 which causes perturbations in structure throughout the β chain (Perutz *et al.*, 1969; Moffat, 1970; Moffat *et al.*, 1970). Finally, carboxypeptidase B, which removes the carboxy-terminal arginine α-141(HC3) of the α chain of Hb, decreases the Bohr effect but does not affect the cooperative oxygenation process (Zito *et al.*, 1964).

The X-ray analysis of Perutz and coworkers (Perutz, 1969) indicates that both amino acid residues at β-99 and α-92 are located at the α_1–β_2 subunit contacts. Since all known hemoglobin variants which have amino acid substitutions at the α_1–β_2 contacts exhibit diminished cooperativity (Perutz and Lehmann, 1968; Imanura *et al.*, 1969), it is important to establish if the presence of isosbestic points in the electron paramagnetic resonance spectra of the spin-labeled hemoglobins on CO or O_2 saturation can be correlated with altered interactions at the α_1–β_2 subunit interface.

Experimental Section

Materials. Human Hb A was prepared from fresh red blood cells by lysis with either toluene or water. Digestions with carboxypeptidases A and B (Worthington, diisopropylfluorophosphate treated) were carried out according to Antonini *et al.* (1961). After suitable periods of time during the reaction, aliquots were drawn and precipitated with cold trichloroacetic acid. The supernatant was extracted five times with ether and analyzed on silica gel F-254 thin-layer chromatography plates[2] (E. Merck AG, Darmstadt). Purified amino acids (Eastman Kodak) were used as markers for the identification of the digested amino acids. The digested hemoglobins were separated from CPA or CPB on Bio-Gel G-100 equilibrated with 0.05 M sodium phosphate at pH 7.0. Hb J Capetown was isolated from the blood heterozygotes and was purified by the same procedure as that for Hb Chesapeake (Ho *et al.*, 1970a), namely chromatography on DEAE-Sephadex (Huisman and Dozy, 1965). This blood sample was kindly provided to us by Dr. Trefor Jenkins of the South African Institute for Medical Research (Jenkins *et al.*, 1968) and contained no detectable amount of methemoglobin. Hb Yakima was isolated from the blood sample of a heterozygote and was purified according to the procedure of Jones *et al.* (1967). Since there was a significant amount of methemoglobin present (~10%), HbCO Yakima was treated with sodium dithionite (Manox British Brand, Hardman & Holden Ltd.) (2 moles of dithionite:1 mole of methemoglobin) according to the procedure of Geraci *et al.* (1969). The dithionite was removed by Sephadex G-25 column equilibrated against distilled water. All solutions were flushed with CO and kept in a CO atomosphere. HbCO A, which had autooxidized, was treated in a similar manner as a control. It gave essentially the same Hill coefficient and electron paramagnetic resonance spectra as the untreated hemoglobin as can be seen in Figure 1.

The procedure for introducing the spin label II (Synvar Associates) to hemoglobin was the same as described by Ogawa *et al.* (1968). Complete reaction of spin label II with the two reactive SH groups of cysteines at β-93 was determined by the spectrophotometric titration method described by Benesch and Benesch (1962). In the present study,

[2] We have found only two spots in the thin-layer chromatography plate which corresponded to histidine and tyrosine in the supernatant of Hb A digested with CPA and only one spot which corresponded to arginine in the supernatant of Hb A digested with CPB.

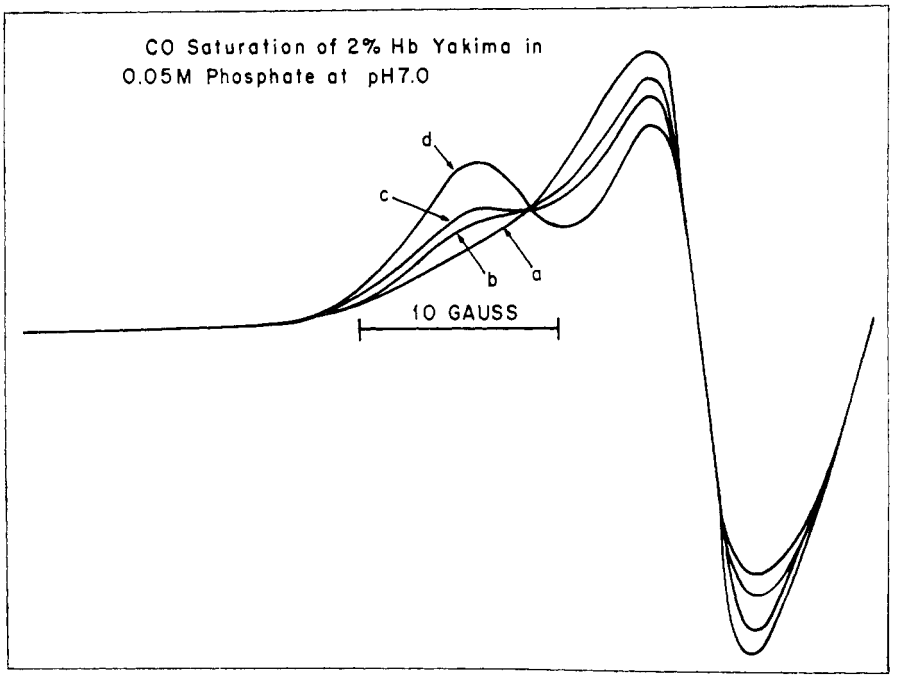

FIGURE 2: Electron paramagnetic resonance spectra of Hb Yakima labeled with spin label II (see text) as a function of CO saturation at 23°: (a, b, and c) intermediate CO saturation; (d) fully CO form.

HbCO was used instead of HbO_2. Deoxyhemoglobin was prepared by first passing oxygen to replace CO and then flushing with oxygen-free nitrogen to remove oxygen. As judged by the visible spectrum, the deoxyhemoglobin samples were better than 90% in the deoxy form and contained less than 2% methemoglobin. All studies were carried out in 0.05 M sodium phosphate at pH 7.0. pH readings were obtained directly from a Radiometer pH meter Model 26 in conjunction with a Beckman Model 39030 frit junction combination electrode. All the reagents used were the best commercially available and used without further purification.

Methods. A Bruker B-ER418s electron paramagnetic resonance spectrometer operating at the X-band frequency (*ca* 9.5 kMHz) was used to obtain the electron paramagnetic resonance spectra. The ambient temperature inside the microwave cavity was 23°. Standard electron paramagnetic resonance quartz aqueous solution flat cells were used for the electron paramagnetic resonance and optical measurements. The cell holder of a Cary 14 spectrophotometer was modified so that all optical measurements could be taken in the quartz cell under the same conditions as the electron paramagnetic resonance studies.

The oxygen saturation of Hb A treated with dithionite was measured spectrophotometrically by essentially the method of Benesch and Benesch (1965). Deoxyhemoglobin was prepared in the same manner as that used in the electron paramagnetic resonance study described above.

Results

Figure 1A shows a Hill plot of Hb A (which had been treated with dithionite to remove methemoglobin) as a function of oxygen saturation in 0.05 M phosphate at pH 7.4 and at 23°. The Hill coefficient for this dithionite treated Hb A is 2.8 which is within experimental error of the published values (Antonini *et al.*, 1964) and our unpublished data for untreated Hb A. This serves as a control sample for Hb Yakima which had been treated with dithionite to remove methemoglobin. Figure 1B also gives the sensitive portion of the electron paramagnetic resonance spectra of Hb A as a function of CO saturation. The spin-label spectra are very similar to those of Ogawa *et al.* (1968) and of Ho *et al.* (1970a). An analysis of the spectral features of Hb A as a function of oxygen saturation was presented by Ogawa *et al.* (1968).

Figures 2–5 give the sensitive portion of the electron paramagnetic resonance spectra of the spin-labeled Hb Yakima, Hb J Capetown, and CPA- and CPB-treated Hb A. CPA-treated Hb A, Hb J Capetown, and Hb Yakima all give a sharp set of isosbestic points in their respective electron paramagnetic resonance spectra as a function of CO saturation similar to that for Hb Chesapeake (Ho *et al.*, 1970a). In the case of CPB-treated Hb A, there is no set of isosbestic points in its electron paramagnetic resonance spectra similar to those for Hb A, Hb F, and Hb Zürich (Ho *et al.*, 1970a). Since several of the modified hemoglobins, such as CPA-treated Hb A, have unusually high oxygen or carbon monoxide affinity, we were unable to obtain completely deoxygenated hemoglobin without the formation of methemoglobin or denaturation. This experimental difficulty does not affect the interpretation of our spin-labeled experiments because the deviations from isosbestic points occur at high O_2 or CO saturation rather than at low saturations (H. M. McConnell, personal communication).

Discussion

McConnell and his coworkers (Ogawa *et al.*, 1968) found deviations from isosbesty in the electron paramagnetic resonance spectra of Hb A labeled with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide. They suggested that the spin label II could sense conformational changes in the

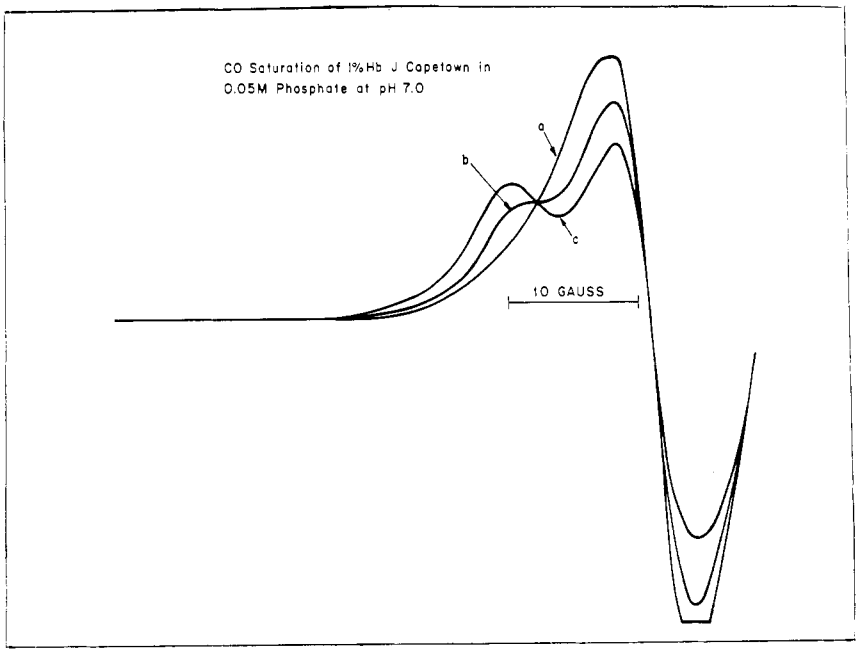

FIGURE 3: Electron paramagnetic resonance spectra of Hb J Capetown labeled with II (see text) as a function of CO saturation at 23°: (a) deoxy; (b) intermediate CO saturation; (c) fully CO form.

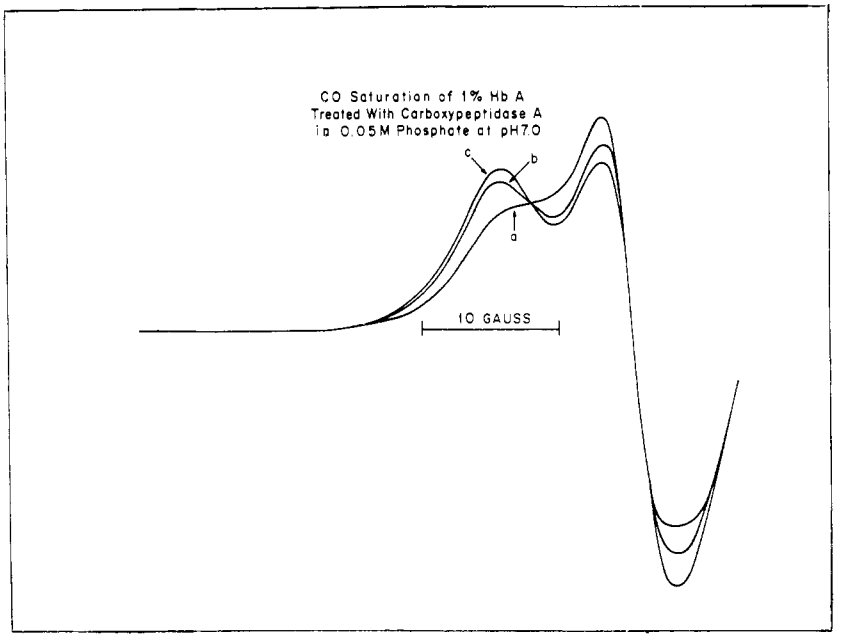

FIGURE 4: Electron paramagnetic resonance spectra of CPA-treated Hb A labeled with spin label II (see text) as a function of CO saturation at 23°: (a and b) intermediate CO saturation; (c) fully CO form.

region of α_1–β_2 subunit contacts and that the intermediate structures detected by spin labeling might be related to the mechanism of cooperativity. Our results (Table I) confirm and extend their work.

Hemoglobins A, F, and CPB-treated Hb A which are fully cooperative lack sets of isosbestic points in their respective electron paramagnetic resonance spectra as a function of CO saturation. Hemoglobins Chesapeake, Yakima, and CPA-treated Hb A which are noncooperative give sharp sets of isosbestic points (Figure 2 and 4). It is tempting to go one step further and generalize that the presence of isosbestic points (*i.e.*, the absence of intermediate structures) in the electron paramagnetic resonance spectra of spin-labeled hemoglobins on CO saturation is correlated with a loss of cooperativity. However, the results of Hb Zürich and Hb J Capetown suggest that this generalization may not be true. Hb Zürich has a Hill coefficient of at least 1.8 (Winterhalter *et al.*, 1969) and lacks a set of isosbestic points in its electron paramagnetic resonance spectra as a function of CO saturation (Ho *et al.*, 1970a). Hb J Capetown has a Hill coefficient of approximately 2.2 and has a set of isosbestic points in its electron paramagnetic resonance spectra on CO saturation (Figure 3). The Hill coefficient for Hb Zürich may not be as good an empirical measure of cooperativity as that in other hemoglobins for the following two reasons. First, since the amino acid substitution in Hb Zürich is at β-63, *i.e.*, the distal histidine is replaced by an arginine (Muller and Kingma, 1961), the heme environment in the

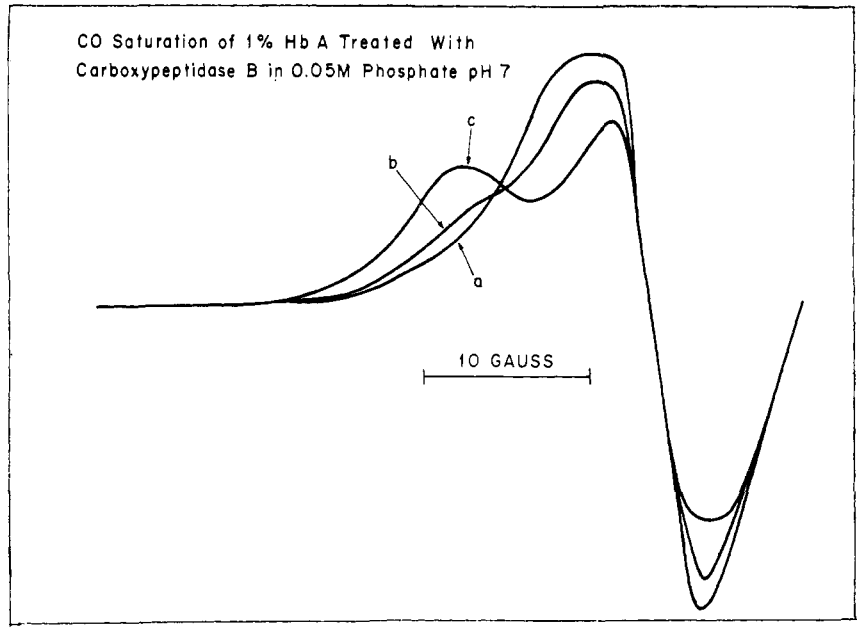

FIGURE 5: Electron paramagnetic resonance spectra of CPB-treated Hb A labeled with spin label II (see text) as a function of CO saturation at 23°: (a) deoxy; (b) intermediate CO saturation; (c) fully CO form.

abnormal β chain is quite different from that in the α chain (Perutz and Lehmann, 1968; Davis *et al.*, 1969; Ho *et al.*, 1970a; Davis *et al.*, 1970). If the α and β chains in this abnormal hemoglobin have different affinities for oxygen, the cooperativity as measured by the Hill coefficient would be underestimated. Secondly, since Hb Zürich is known to be extremely sensitive to autoxidation (Perutz and Lehmann, 1968), the Hill coefficient determined by Winterhalter *et al.* (1969) may have been underestimated due to the formation of methemoglobin (Darling and Roughton, 1942). Thus, the presence of a set of isosbestic points in the respective spectra of the spin-labeled hemoglobins Chesapeake, Yakima, J Capetown, and CPA-treated Hb A may be accounted for if (i) the spin label II is insensitive to the intermediate structures that are present in these four modified hemoglobins; or (ii) there are only two conformations, *i.e.*, oxy and deoxy forms, in these four hemoglobins; or (iii) Hb J Capetown is a special case or it is not cooperative enough to produce the intermediate structures. Unfortunately, we do not have unambiguous evidence to resolve these possibilities. It should be noted that even for those fully cooperative hemoglobins, the deviations from isosbesty are small, suggesting that these intermediate forms detected by the spin label II are present in low concentration. If the spin label II is insensitive to the intermediate structures, the presence of isosbesty in these four hemoglobins might be caused by structural modifications in the α_1–β_2 subunit interface which in turn fail to influence the spin label.

Our spin-label studies indicate that hemoglobins, such as Hb Yakima, Hb J Capetown, Hb Chesapeake, and CPA-treated Hb A, which have modifications in the subunit contacts yield results significantly different from those having normal subunit contacts, such as Hb A, Hb F, Hb Zürich, and CPB-treated Hb A. Hb Yakima, Hb Chesapeake, and Hb J Capetown are especially significant since they have single amino acid substitutions in the α_1–β_2 contacts. The substitution in Hb Yakima is at β-99(G1) Arg→His (Jones *et al.*, 1967). The substitution in Hb Chesapeake is at α-92(FG4) Arg→Leu (Clegg *et al.*, 1966) and that in Hb J Capetown is also at α-92(FG4) Arg→Gln (Botha *et al.*, 1966). The nuclear magnetic resonance results of Ho and coworkers (Ho *et al.*, 1970b; Davis *et al.*, 1970; unpublished results on Hb J. Capetown) along with Perutz's model of hemoglobin (Perutz, 1969) suggest that the single amino acid substitutions in Hb Chesapeake and Hb J Capetown not only could cause altered interactions among the several amino acid residues located in the α_1–β_2 contact region but also could cause perturbations to the heme groups as shown in the differences in the heme proton resonances among deoxyhemoglobins A, Chesapeake,

TABLE I:[a] Relations between the Presence of Isosbesty in the Electron Paramagnetic Resonance Spectra on CO Saturation and Subunit Interactions.

Hemoglobin	Isosbesty during CO Saturation	Modifications at α_1–β_2 Contacts	Hill Coeff, n
Hb A	No	None	2.9
Hb F	No	None	2.9
CPB-treated Hb A	No	None	2.7
Hb Zürich	No	None	1.8[b]
Hb J Capetown	Yes	Yes, α-92 Arg→Gln	2.2
Yb Chesapeake	Yes	Yes, α-92 Arg→Leu	1.3
Hb Yakima	Yes	Yes, α-99 Asp→His	1.1
CPA-treated Hb A	Yes	Yes	1.0

[a] See the text for detailed information on individual hemoglobins and literature references. [b] See the text for a discussion on the Hill coefficient of Hb Zürich.

and J Capetown.[3] According to Moffat's X-ray analysis of Hb A labeled with II, the four protecting methyl groups of the spin label II touch histidine at β-97(FG4), valine at β-98(FG5), and aspartic acid at β-99 (G1) and the piperidinyl ring touches leucine at β-141(H19) and alanine at β-142(H20) (Moffat, 1970). Therefore, the X-ray results of Moffat (1970) show that the spin label II could sense conformational changes that are brought about by the alterations in the α_1–β_2 subunit contacts of these Hb variants. It should be noted that the spin label attachment site is at the SH group of cysteine β-93(F9) and is in the vicinity of α_1–β_2 contacts (Moffat, 1970). Since Perutz *et al.* (1969) have pointed out that the removal of histidine at β-146(HC3) and tyrosine at β-145(HC2) by CPA causes changes throughout the β chain, the CPA-digested Hb A should be expected to have altered α_1–β_2 subunit contacts. The presence of a set of isosbestic points in the electron paramagnetic resonance spectra of CPA-treated Hb A labeled with II as a function of CO saturation is consistent with that suggestion (Figure 4).

Carboxypeptidase B treated Hb A is of interest since it has been shown that digestions with CPB cause partial inhibition of the Bohr effect without affecting the cooperative oxygenation process (Antonini *et al.*, 1961). According to Perutz *et al.* (1969), the carboxy-terminal arginine at α-141 is quite far away from the spin-label attachment site at β-93. One would expect that the CPB treatment should have little effect on the electron paramagnetic resonance spectrum of CPB-treated Hb A and that is what was observed (Figure 5).

A valid criticism can usually be made against all studies involving chemical modifications in proteins, namely does the modification so perturb the system under investigation that the results obtained are no longer relevant to the properties of the unperturbed systems? Based on entirely independent experimental evidence, both the spin labeled (perturbed) and the nuclear magnetic resonance (nonperturbed) results focus on a section of the FG region of the α and β chains of hemoglobin whose conformational changes are sensitive to the state of oxygenation of the α as well as the β chains (Ogawa *et al.*, 1968; Ho *et al.*, 1970a; Ho *et al.*, 1970b; Davis *et al.*, 1970). This region forms part of the α_1–β_2 subunit contacts, and in addition valines at FG5(α-93) and at FG5(β-98) are in contact with the heme groups in α and β chains (Perutz, 1969). According to Ho and coworkers (Ho *et al.*, 1970b; Davis *et al.*, 1970), the proton nuclear magnetic resonance spectra of Hb A and Hb Chesapeake have differences in the aromatic proton resonances which have been interpreted in terms of altered interactions among the amino acid residues at the α_1–β_2 subunit contacts; the changes in the ring-current shifted proton resonances upon carbomonoxylation or oxygenation of deoxyhemoglobin along with Perutz's model of hemoglobin (Perutz, 1969) suggest that the leucine at α-91(FG3) and the valine at α-93(FG5) as well as the valine at β-98(FG5) may be important informational links between the heme groups and the subunit interface at α_1–β_2 in the cooperative ligand binding process; and the proton nuclear magnetic resonance spectra of the heme groups in the deoxyhemoglobin Chesapeake suggest that the altered functional properties of this mutant hemoglobin involve not only changes in the α_1–β_2 subunit contacts but also changes which affect the heme group in the α chain. According to Moffat (1970), the distortions induced by the presence of the spin label II are numerous and extensive, with the largest concentration in the environment of the heme groups of both the α and β chains, the F-helix of the β chain, and the FG region of the α_1–β_2 subunit interface. In fact, one of the protecting methyl groups of the spin label II touches the valine at β-98(FG5) (Moffat, 1970). In spite of the extensive structural perturbations produced by the spin label II, the cooperative oxygen binding to hemoglobin is not markedly affected by the spin label (the Hill coefficient, $n = 2.3$ instead of $n = 2.9$ for unlabeled Hb) (Ogawa *et al.*, 1967, 1968). Hence, one may conclude with some confidence that the spectral changes observed in the spin-labeled hemoglobins are, in fact, related to the cooperative oxygenation process in the corresponding unlabled proteins. Furthermore, our spin label and nuclear magnetic resonance results provide strong evidence that the structural changes observed in single crystals of hemoglobin in going from deoxy to oxy form are closely related to those observed in aqueous solution.

In conclusion, we have confirmed the interpretations of McConnell and coworkers as well as ours that the intermediate structures detected by spin label II in the fully cooperative hemoglobins, such as Hb A, Hb F, and CPB-treated Hb A, are likely to be associated with the cooperative oxygenation process. The three noncooperative hemoglobins, Hb Chesapeake, Hb Yakima, and CPA-treated Hb A, which have modifications in the α_1–β_2 subunit contact region, give no evidence for the existence of intermediate structures in the oxygenation process. Hb J Capetown, a fairly cooperative hemoglobin, also gives a sharp set of isosbestic points suggesting that either the spin label II has been influenced by an amino acid substitution in the α_1–β_2 contacts so that it may no longer be sensitive to the intermediate structures during oxygenation or there are only two conformations in this Hb variant. Our spin label results support Perutz's model of hemoglobin as well as our nuclear magnetic resonance results that normal interactions among the amino acid residues located at the α_1–β_2 subunit contacts are fundamentally important to the function of hemoglobin as an oxygen carrier. It should be mentioned that the presence or absence of isosbestic points in the electron paramagnetic resonance spectra of hemoglobin labeled with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide as a function of O_2 or CO saturation is a good diagnostic tool on the nature of the subunit interactions and may also be a good indicator of the cooperativity in an unknown hemoglobin. In a strict sense, the presence of intermediate structures means that at intermediate stages of oxygenation there are some hemoglobin molecules in solution which have a structure that differs from either the oxy or deoxy conformation in the vicinity of the labeled cysteine β-93. However, our results suggest that the six-membered nitroxyliodoacetamide spin label is very sensitive to the

[3] This interpretation differs from that of Shulman and his coworkers on their studies of the mixed-state hemoglobins and Hb J Capetown. Shulman *et al.* (1969) found no effects of subunit interaction on the heme proton nuclear magnetic resonance spectrum of a mixed state Hb [(α chains in deoxy form) (β chains in met form)]. Ogawa *et al.* (1970) could detect no difference between the heme proton nuclear magnetic resonance spectra of deoxyhemoglobins A and J Capetown. They concluded that alterations at the subunit interface do not affect the heme groups. However, our proton nuclear magnetic resonance results on deoxyhemoglobins A, Chesapeake, and J Capetown clearly indicate that a modification at the α_1–β_2 subunit interface does perturb the heme environment (Davis *et al.*, 1970; D. G. Davis, N. H. Mock, T. R. Lindstrom, S. Charache, and C. Ho, unpublished results on Hb J Capetown).

α_1–β_2 subunit interactions. Hence, the absence of a set of isosbestic points in those fully cooperative hemoglobins suggests that there may be an intermediate quaternary conformation during the oxygenation process.

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