NON-LINEAR RELATION BETWEEN OXYGEN-BINDING AND STRUCTURAL CHANGES OF HEMOGLOBIN

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Summary: Intermediary structures between oxy- and deoxy-hemoglobins in solution were studied by the heme-spin-label method at room temperature. The optical and oxygen binding properties of the spin-labeled hemoglobin, in which one of the propionic acid groups of the heme was spin-labeled with 2,2,5,5-tetramethyl-3-amino-pyrrolidine-1-oxyl, were identical to those of native hemoglobin. The spin-labeled hemoglobin exhibited an electron paramagnetic resonance spectrum of a moderately immobilized label. Upon deoxygenation, the resonance amplitude decreased to less than a half of the original intensity, and there was a small change in the line-shape. The fractions of the EPR changes were found to be non-proportional to the optical changes, indicating a non-linear relation between oxygen binding and structural changes of hemoglobin.

INTRODUCTION

A difficulty in the study of the molecular mechanism of cooperative oxygen binding to hemoglobin has been the lack of an appropriate method to detect the conformational properties of the intermediates between oxy- and deoxy-hemoglobins in solution. In an attempt to study the structures of dissolved hemoglobins, we have been using the heme-spin-label method in which the prosthetic group of a hemoprotein is directly spin-labeled with stable nitroxide free radicals (1,2). Preliminary experiments with this method showed that the electron paramagnetic resonance spectra of spin-labels provide significant information not only about the protein conformation in the vicinity of the label, but also about the spin-state of the heme-iron (2,3). Recently, we have succeeded in the preparation of spin-labeled oxy-hemoglobin in which a nitroxide spin-label was covalently attached to the 6 or 7 propionic acid group of the heme (4). The spin-labeled hemoglobin showed optical and oxygen binding properties indistinguishable from those of native hemoglobin (3,4) and therefore it may be used for studying the molecular mechanism of oxygen binding to native hemoglobin. The present communication

deals with the results obtained with this spin-labeled hemoglobin on the relation between oxygen-binding and the conformational changes of the hemoglobin molecule.

MATERIALS AND METHODS

The spin-labeled ferrihemoglobin containing mono-spin-labeled protoheme was prepared as described elsewhere (3,4). The oxy-form was obtained by the enzymatic reduction with a ferredoxin reductase system, which reduces the heme-iron selectively without destroying the attached spin-label (4). The spin-labeled hemoglobin was then purified by a heat treatment followed by a column chromatography on carboxymethyl cellulose. The hemoglobin containing one of the two isomeric forms of the spin-labeled hemes is removed during the purification (3). The oxygen equilibrium curves of the purified hemoglobin were determined with an automatic apparatus similar to that reported by Imai et al (5), modified for small scale measurements. The apparatus requires a sample of 1.5 ml for the single measurement. Simultaneous measurements of optical and EPR spectra during deoxygenation process were carried out by a circulating method (3). A solution of the spin-labeled hemoglobin was circulated through an EPR sample tube (1 mm, i.d.), an optical flow cell and a gas exchange chamber, from which oxygen was slowly removed or added from the sample by passing appropriate gases over the surface.

RESULTS

The mono-spin-labeled oxy-hemoglobin exhibits absorption peaks at 415, 542 and 577 nm, and the deoxy-form at 430 and 556 nm in 0.1 M potassium phosphate buffer, pH 7.0. These absorption positions as well as the millimolar absorption coefficients were indistinguishable from those of native hemoglobin. The oxygen equilibrium curves of the spin-labeled hemoglobin were also normal between pH 6-9.5. The n-values were 2.6-2.8. As shown by x-ray crystallography, the propionic acid groups where a spin-label was attached are extended to the outside of the heme-crevice (6). This intramolecular orientation of the heme might be

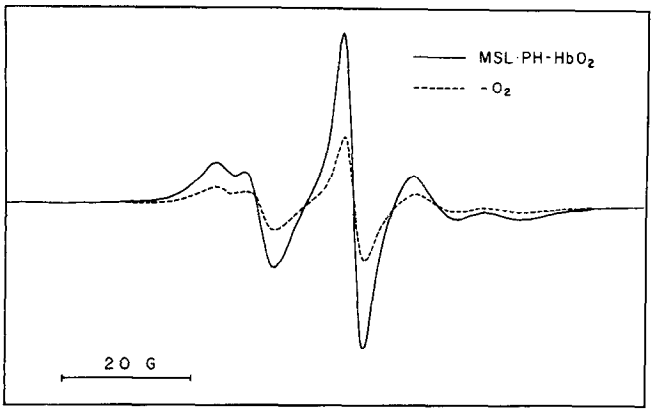

Fig. 1.

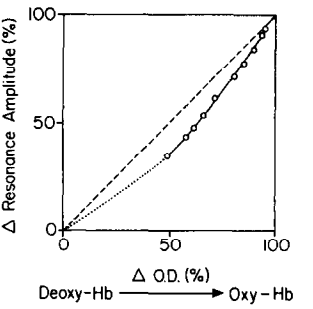

Fig. 2.

Figure 1. Electron paramagnetic resonance spectra of spin-labeled oxy- and deoxy-hemoglobins in 0.1 M potassium phosphate buffer, pH 7.0 at 20°. Microwave power: 20mW, modulation amplitude: 0.5 gauss.

Figure 2. The relation between fractional EPR and optical changes during deoxygenation of spin-labeled oxyhemoglobin.

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the reason why chemical modification of these groups has little effect on the oxygen binding properties of hemoglobin (7).

The EPR spectra of the spin-labeled oxy- and deoxy-hemoglobin are compared in Fig. 1. Although the EPR line-shape was not greatly changed upon deoxygenation the resonance amplitude of the spin-labeled oxy-hemoglobin was markedly decreased. This decrease in the resonance amplitude is due partly to the spin-state change from low-spin oxy-ferroheme to the high-spin ferroheme, and partly to the conformational changes of the protein molecule (3). Spin-labels attached to macromolecules have been shown to interact magnetically with other paramagnetic components located close to the spin-label (2,8,9). A very interesting result is obtained when we plot the fractional change of the central resonance amplitude against the fractioanl optical change. As shown in Fig. 2, the result clearly deviates from the diagonal straight line, indicating that the spin-state and/or the conformational changes of hemoglobin are not proportional to the total oxygen binding.

It should also be pointed out that the EPR spectra of both the oxy- and deoxy-hemoglobins showed mixtures of two different spectra reflecting two different environments for the spin-label. This splitting was missing in the EPR spectra of the acid-met-form of the spin-labeled myoglobin (2) and hemoglobin(4 McConnell et al. (10) observed similar splitting in the EPR spectra of the hemoglobin spin-labeled at the β-93 cysteines and they proposed that it was due to isomeric states of the label relative to protein. The complete interpretation of the splitting in the heme-spin-labeled hemoglobin will require experiments with α or β spin-labeled hemoglobin in which the α or β subunits are selectively spin-labeled, and such experiments are now in progress.

DISCUSSION

X-ray crystallography by Perutz and coworkers (6) has shown that there is a conformational difference between oxy- and deoxy-hemoglobins, suggesting that a conformational change will occur upon oxygen binding to hemoglobin. Such a ligand-linked conformational change has been believed to cause the cooperative nature of oxygen binding to hemoglobin. In order to study the relationship between the conformational change and the fractioanl saturation with ligands, the parameters which will reflect protein conformation, such as EPR change in the spin-label attached to β-93 cysteine residues (11), release of Bohr protons (12), change in the reactivity of the β-93 sulfhydryl groups (13) and change in the circular dichroism in the near ultraviolet region (14), have been measured as a function of the fractional saturation with oxygen or carbon monoxide. The results obtained by these methods showed a linear relationship between the conformational parameters and the fractional saturation with ligands. Therefore, it was considered that the conformational properties of the hemoglobin chains depend on the presence of the ligand on that chain, independently of the state of the others (15).

The present result, however, clearly shows that the fractional EPR change during deoxygenation is not linearly related to the fractional saturation with oxygen. Since the EPR spectrum of the spin-label attached to heme group reflects

sensitively the protein conformation in the vicinity of the label as well as the spin-state of the heme-iron, the above result obtained by the heme-spin-label method provides important evidence that the structural change in the heme and its surroundings is not proportioanlly related to the fractions of oxygen binding to hemoglobin. The result is at least contradictory to the induced-fit model of Koshland et al. (16). A model should include the non-linear conformatioanl change of the protein.

Non-equivalent interactions between α and β subunits due to oxygen binding have been demonstrated by several different techniques using valency hybrid hemoglobins (17-20). All these results showed that oxygen binding to one kind of subunit alters the structure of the other subunits, and that the nature of the interactions are different for the α and β subunits. Although these results obtained form hybrid hemoglobins containing ferric heme cannot be directly applied to ferrohemoglobin, the non-equivalent interactions between different subunits may also occur in ferrohemoglobin. The non-linear relationship between the fractional EPR changes and the fractional optical changes can probably be attributed to this non-equivalent interaction between the hemoglobin subunits. Although further interpretation of the deviation must await similar experiments carried out with half-spin-labeled hemoglobins in which the heme groups of either α or β subunits are selectively spin-labeled, the present result clearly reveals the non-linear relation between oxygen binding and the structural change of hemoglobin.

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