NON-LINEAR RELATIONSHIPS BETWEEN OXYGEN SATURATION AND MAGNITUDE OF FINE STRUCTURE OF ULTRAVIOLET OXY VERSUS DEOXY DIFFERENCE SPECTRUM IN HUMAN HEMOGLOBIN

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Received December 22, 1972; revised January 8, 1973

SUMMARY: Ultraviolet difference spectra of fully oxygenated hemoglobin vs. successively deoxygenated or reoxygenated hemoglobin were determined in the absence and presence of organic phosphates. Magnitude of fine structure in the difference spectrum around 290 nm, which is considered to be a partial reflection of oxygenation-induced changes in quaternary conformation of hemoglobin, was not linearly related to fractional oxygen saturation of hemoglobin of the reference cell. The non-linear feature was influenced by the organic phosphates as predicted by the allosteric model of Monod et al. The present study suggests that the ultraviolet oxy vs. deoxy difference spectrum measurements provide a useful way to examine the validity of the model.

In order to clarify the mechanism of allosteric effects in hemoglobin, it is indispensable to investigate the conformational and functional properties of hemoglobin molecules in partially ligated states. For this purpose, relationships between fractional saturation with ligands and the extent of protein conformation changes during the ligation have been studied by differential titration experiments (1), circular dichroism measurements in a near ultraviolet region (2), and electron paramagnetic resonance measurements using spin-labels attached to specific sites of protein (3), heme side chains (4), or triphosphates (5).

In the previous study (6), it was suggested that magnitude of fine structure of oxy vs. deoxy difference spectrum around 290 nm is a partial reflection of the extent of oxygenation-induced changes in quaternary conformation of hemoglobin since (a) the spectral fine structure is tentatively attributed

to perturbation of C3β tryptophan residues at the $\alpha_1\beta_2$ contact which undergoes conformational changes essential to cooperative oxygen binding (7) and (b) the magnitude of the fine structure is closely related to the cooperativity of oxygen binding (6). In this study, the relationships between oxygen saturation and the magnitude of the fine structure were examined.

Human adult hemoglobin prepared from fresh blood was stripped of phosphates as described by Benesch et al. (8). Ultraviolet difference spectra of fully oxygenated hemoglobin vs. successively deoxygenated or reoxygenated hemoglobin were recorded on Cary 118C spectrophotometer. Thunberg-type anaerobic cuvettes (light path, 10 mm) were used for sample and reference cells, the latter of which was equipped with a magnetic stirrer. Deoxygenation and reoxygenation of hemoglobin solution in the reference cell were performed by flushing pure argon gas and oxygen gas, respectively, with gentle stirring of the solution. At several stages during the deoxygenation and reoxygenation, both the stirrer and the gas flow were stopped and a difference spectrum was recorded. The difference spectrum did not change at all during the recording. The fine structure around 290 nm which was five times enlarged was also recorded at every stage. Band width of incident light was about 1.5 nm around 290 nm. Temperature in the cell compartment was about 25°.

As shown in the previous study (6) and in Figure 1, the difference spectra exhibited a narrow banded difference peak around 290 nm which was superimposed on a broader heme contribution with a peak at 275 nm. The position of a maximum at 290.5 nm and a minimum at 288 nm of the fine structure was affected by the addition of neither 2,3-diphosphoglycerate (DPG) nor inositol hexaphosphate (IHP). Magnitude of the fine structure was expressed by the difference between ΔA at 290.5 nm and ΔA at 288 nm. In order to subtract the heme contribution, the difference of ΔA was corrected for base line which was extrapolated from a linear region between 295 nm and 300 nm (7) (see Figure 1). The magnitude of the fine structure in the difference spectrum of fully oxygenated hemoglobin vs. completely deoxygenated hemoglobin was $\Delta\varepsilon = 6.1 \times 10^2$

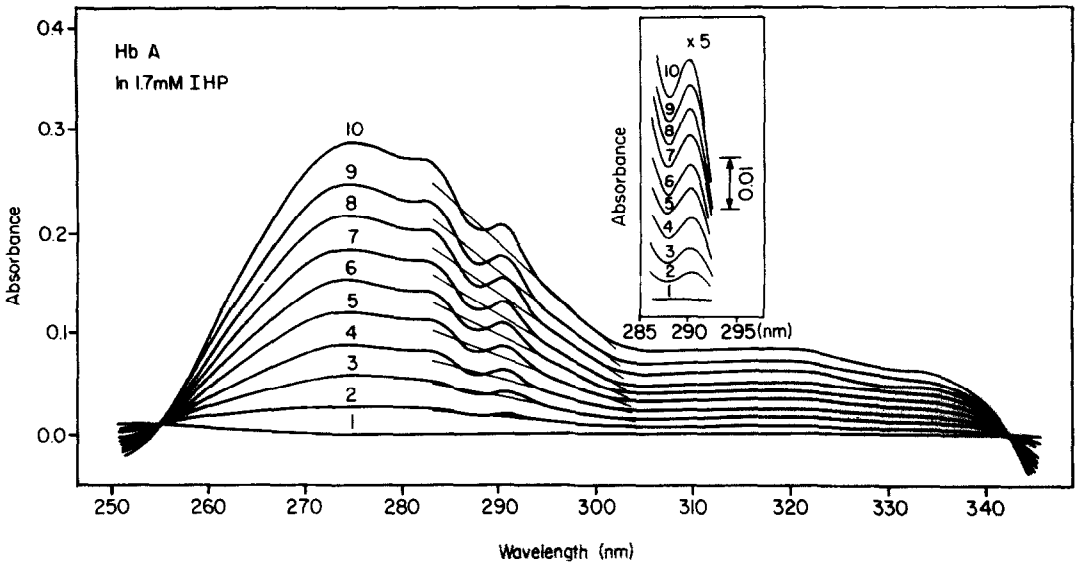

Figure 1. Ultraviolet difference spectra of fully oxygenated hemoglobin vs. successively deoxygenated hemoglobin in the presence of 1.7 mM IHP. Hemoglobin concentration, 5.8 X 10^{-5} M on heme basis; in 0.05 M bis-Tris buffer (pH 7.4). The degree of deoxygenation of hemoglobin in the reference cell changes successively from 0 to 1 as the number attached to the difference spectra increases from 1 to 10. Straight lines extrapolated from a linear region between 295 nm and 300 nm were used as base lines of the fine structure around 290 nm and were used for calculation of the oxygen saturation of the reference hemoglobin. Inset shows the difference spectra around 290 nm which were five times enlarged along the ordinate. Vertical positions of the spectra are arbitrary.

M^{-1} cm^{-1} for stripped hemoglobin. The magnitude was slightly smaller in the presence of 2 mM DPG ($\Delta\varepsilon$ = 5.8 X 10^2 M^{-1} cm^{-1}) as previously observed (6) and was still smaller in the presence of 1.7 mM IHP ($\Delta\varepsilon$ = 4.9 X 10^2 M^{-1} cm^{-1}). Fractional oxygen saturation, Y, of the hemoglobin in the reference cell was calculated from the slope of the linear region between 295 nm and 300 nm by assuming that the slope, which was exactly proportional to ΔA at 275 nm, is proportional to the degree of deoxygenation, 1 - Y, of the reference hemoglobin.

Figure 2 shows the dependence of fractional change of the magnitude of the fine structure in the difference spectrum upon 1 - Y of the reference hemoglobin. As far as examined in the presence of 2 mM DPG or 1.7 mM IHP, the

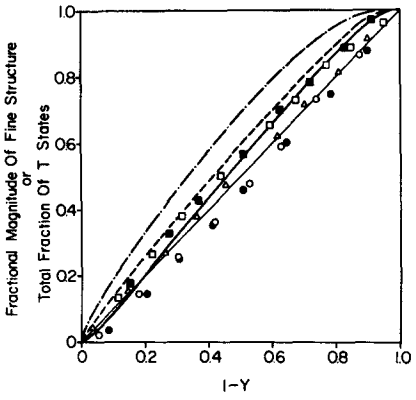

Figure 2. Dependence of fractional magnitude of the fine structure around 290 nm upon the degree of deoxygenation (1 - Y) of hemoglobin in the reference cell. Circles, stripped hemoglobin; triangles, in 2 mM DPG; squares, in 1.7 mM IHP. Open and closed symbols refer to separate experiments for different samples. Hemoglobin concentration, (6.0 ± 0.2) X 10^{-5} M on heme basis; in 0.05 M bis-Tris buffer (pH 7.4). Bold lines represent dependences of total fraction of hemoglobin molecules in the T-state upon 1 - Y. ——, stripped hemoglobin; ----, in 2 mM DPG; —·—, in 1.7 mM IHP. The values of the parameters for the model of Monod et al. used for the calculation were c = 0.0266 and L = 3.89 X 10^3 for stripped hemoglobin, c = 0.00148 and L = 2.56 X 10^8 for hemoglobin in 2 mM DPG, and c = 0.00117 and L = 6.75 X 10^9 for hemoglobin in 1.7 mM IHP. Fine straight line is a diagonal line.

dependence obtained for successive deoxygenation of the reference hemoglobin was completely reversible, that is, the dependence was reproduced in the successive reoxygenation of the reference hemoglobin. The dependence in the presence of 2 mM DPG is approximately linear whereas that in the absence of phosphates significantly deviates from the diagonal line toward the right hand side and that in the presence of 1.7 mM IHP toward the left hand side.

Since the magnitude of the fine structure in the difference spectrum is considered to be a partial reflection of oxygenation-induced quaternary conformation changes which are observed at the $\alpha_1\beta_2$ contact (6), the present results will be useful to examine the validity of the allosteric model of Monod et al (8). Total fraction of hemoglobin molecules in the T-state, which is referred to the deoxy structure defined by Perutz (9), was calculated from the parameters involved in the oxygen saturation function of the model. The values of the parameters used for stripped hemoglobin and for hemoglobin in 2

mM DPG were those reported elsewhere (10). The values of the parameters for hemoglobin in 1.7 mM IHP were estimated from previous oxygen equilibrium data (11) as described elsewhere (10). As shown in Figure 2, the calculated dependences of the total fraction of the T-state molecules upon 1 - Y predict that the allosteric transition from the R-state to the T-state is more advanced than the deoxygenation of hemes in a wide range of 1 - Y in the absence of the phosphates and in the whole range of 1 - Y in the presence of the phosphates. The dependences of the magnitude of the fine structure for stripped hemoglobin and for hemoglobin in 2 mM DPG apparently do not accord with the above prediction from the oxygen equilibrium data. It is worthy of emphasis, however, that the plot of the magnitude of the fine structure against 1 - Y is successively shifted on the addition of 2 mM DPG and 1.7 mM IHP toward the left hand side, i.e., toward the same direction as the plot of the total fraction of the T-state molecules against 1 - Y is shifted. It is probable that the magnitude of the fine structure observed in this study involves contributions of other aromatic chromophores than the C3β tryptophans. If these contributions can be subtracted, the present spectral data may accord well with the oxygen equilibrium data. Although further studies are required for confirmation, the present study suggests that the ultraviolet oxy vs. deoxy difference spectrum measurements provide a useful way to examine the validity of the model of Monod et al., especially on the allosteric equilibrium between the T and R states which is perturbed by allosteric effectors such as DPG and IHP.

This study was supported by NIH HL 14508 and AHA 71-747.

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