PHOSPHATE-DEPENDENT SPECTROSCOPIC CHANGES IN LIGANDED HEMOGLOBIN

Mary L. Adams and Todd M. Schuster
Biological Sciences Group, University of Connecticut,
Storrs, Connecticut, 06268

Received April 8,1974

SUMMARY: The addition of organic phosphates (inositol hexaphosphate, IHP; diphosphoglycerate, DPG; adenosine triphosphate, ATP) or orthophosphate to fully liganded human hemoglobin produces ultraviolet and visible spectroscopic changes which are very similar to those observed when isolated unliganded alpha and beta subunits combine to form hemoglobin (Brunori, et al. 1968). This characteristic difference spectrum is associated with changes in ligand binding cooperativity and affinity and appears to reflect changes in quaternary and tertiary structure of the protein.

Although there are reports that organic phosphates bind to fully liganded hemoglobin (1-7) current theories of the mechanism of action of these effector substances emphasize mainly the importance of their binding to unliganded hemoglobin. We have observed spectroscopic changes when organic phosphates are added to solutions of fully liganded DPG-free human hemoglobin which may reflect mechanistically relevant conformational changes in the protein. Figure 1 shows the IHP induced difference spectrum of oxyhemoglobin (HbO_2) in the visible and ultraviolet spectral regions. The maximum difference, at 584.5 nm, corresponds to about a 9% increase in extinction of HbO_2. In the visible region the difference maxima occur at 584.5, 574, 552, and 532 nm with isosbestic points at 578.5, 563, 546, and 513 nm. In the ultraviolet region difference maxima occur at 338, 302, 291, 287, 283, and 279 nm with isosbestic points at 348.6, 309.6, 297, 289, 284 and 282 nm. In the Soret region (not shown in Figure 1) there are difference maxima at 431 and 404 nm with isosbestic points at 413 and 386 nm. Qualitatively similar difference spectra are obtained with carbonmonoxyhemoglobin, the main difference being that the magnitude of the $\Delta\epsilon(\lambda)$ values are about half those observed with HbO_2 and the λ max are shifted. Addition of IHP to unliganded hemoglobin (Hb) does not produce a difference spectrum. Control experiments have shown that these difference spectra are not due to

minor component hemoglobins or to the presence or formation of methemoglobin or to a change in ligand saturation or to changes in the tetramer-dimer equilibrium. Carbamylation with KNCO of the four N-terminal amino groups of HbO_2 eliminates the IHP induced difference spectrum (L. L. Chung and T. M. Schuster, unpublished results). Feline (domestic cat) HbO_2-B which is naturally acetylated at the beta chain N-termini (8) shows no IHP induced difference spectrum whereas cat HbO_2-A which is not acetylated yields an IHP difference spectra similar in shape but smaller in amplitude to that seen with human HbO_2 (Fig. 1). These results are consistent with the interpretation

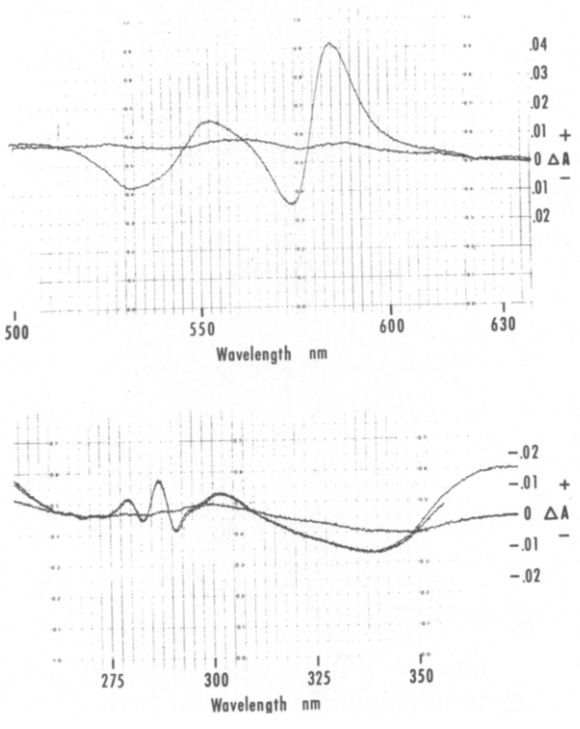

Figure 1. IHP difference spectra of human oxyhemoglobin (HbO_2). Hemoglobin A_0 was purified by the method of Williams and Tsay (17). Conditions: 47 μM heme concentration, 5.6°C, 0.1 M N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.0, 1 atm. O_2. Sample cell: 2 mM IHP. All spectra were recorded on a Cary 15 spectrophotometer in 10 mm cuvettes. Baseline was obtained with HbO_2 (no IHP) in both cuvettes. Similar results were obtained with HbO_2 in 0.1 M Bis-tris-HCl (2,2-bis[hydroxymethyl]-2, 2', 2"-nitriloethanol).

that the binding site for IHP in liganded hemoglobin is the same as that in Hb. This Hb site has been shown to be the same one to which DPG binds (9,10).

Figure 2 presents the results of an IHP titration of HbO_2. The well

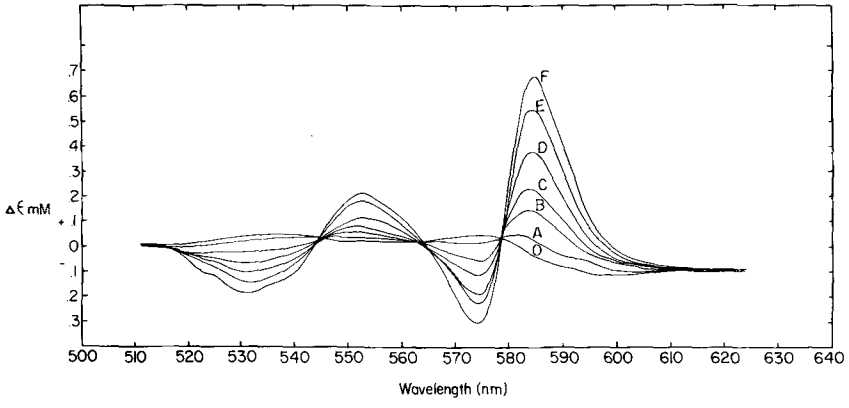

Figure 2. Spectrophotometric titration of HbO_2 with IHP. Total concentration of IHP in sample cell: O, no IHP; A, 0.997 μM; B, 2.97 μM; C, 4.92 μM; D, 7.79 μM; E, 11.54 μM; F, 2 mM. Further addition of IHP causes no change. Equivalent volumes of buffer were added to the reference cuvette. Conditions: heme concentration approximately 60 μM, 6°C, 0.1 M HEPES, pH 7.0, 1 atm. O_2, 10 mm cuvettes.

defined isosbestic points suggest that IHP binds preferentially to one form of hemoglobin yielding two spectroscopically distinguishable forms. The binding of IHP appears to be non-cooperative at all wavelengths where there is a maximum $\pm\Delta\mathcal{E}$. Previous studies have shown that there is one strong binding site on HbO_2 for IHP at 0.1 M ionic strength (4). Our results agree with this.

Figure 3 shows the spectroscopic changes observed when other organic phosphates are added to HbO_2. All of these phosphate compounds are known to affect the ligand binding properties of hemoglobin. It can be seen that at equivalent phosphate concentrations (1 mM) there are different magnitudes of $\Delta\mathcal{E}(\lambda)$ although the band shapes and isosbestic points are the same. We have not performed direct binding studies but the differences in $\Delta\mathcal{E}$ observed with

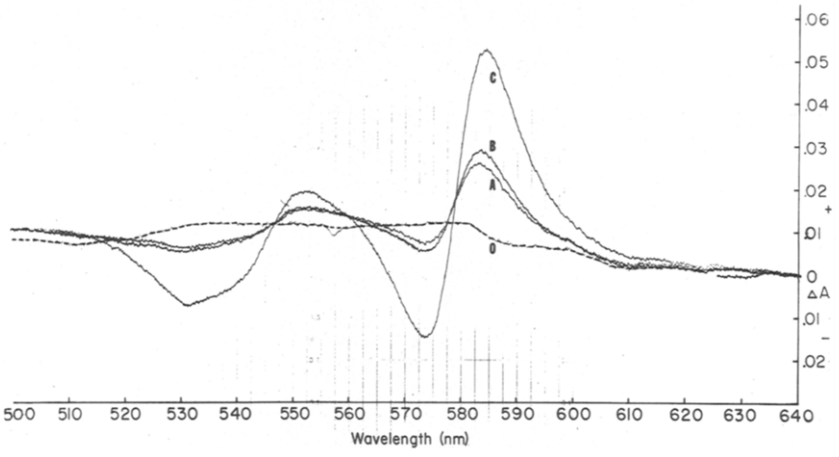

Figure 3. Organic phosphate induced difference spectra. Conditions: Oxyhemoglobin A_o, 68.2 μM heme, 5.6°C, 0.1 M HEPES, pH 7.0, 1 atm. O_2. Sample cell: A, 1 mM DPG (pentacyclohexylammonium salt); B, 1 mM ATP; C, 1 mM IHP; O, HbO_2, no phosphates. Reference cell for all: HbO_2, no phosphates.

different phosphates parallel approximately the published binding constants to Hb.

Since IHP binds strongly to the crystallographically defined "deoxy form" of hemoglobin which is fully in the so-called "unliganded T-form" (11,12) we conclude from these results, 1) that in HbO_2 IHP may bind preferentially to an "oxy T-form" thus shifting an R-T conformational equilibrium in HbO_2, and 2) that there are characteristic visible and ultraviolet spectroscopic differences between R and T quaternary forms.

Support for these interpretations comes from the following independent experimental results. 1) The cooperativity of O_2 binding to Hb is reduced in the presence of IHP at pH 7.0, 0.1 ionic strength (13). 2) The cooperativity of O_2 binding to cat hemoglobin is pH dependent, the Hill coefficient, n, being about 2.1 at pH 6.5 and about 3.0 at pH 8.0 (14). Figure 4 shows a pH difference spectrum of phosphate free cat HbO_2. This difference spectrum is very similar to the IHP induced difference spectrum of human HbO_2 (compare Fig. 1). In the same pH range the Hill coefficient, n, is constant in human

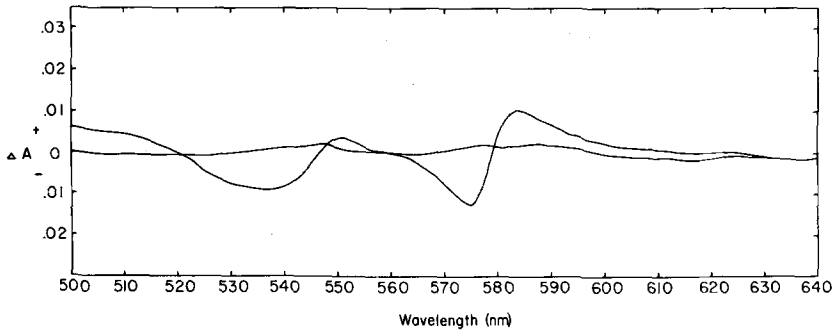

Figure 4. Cat hemoglobin pH difference spectrum. DPG-free cat hemoglobin, containing both types A and B. Conditions: Hemoglobin concentration 76.6 μM heme, 7°C. Sample cell: HbO_2 in 0.05 M HEPES, pH 6.5. Reference cell: HbO_2 in 0.05 M HEPES, pH 8.2. Baseline: respective buffers.

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hemglobin, which does not exhibit a pH difference spectrum. This result with cat hemoglobin demonstrates a pH dependent equilibrium between two fully liganded forms which exhibit a difference spectrum very similar to that produced by organic phosphates. Therefore, conditions which decrese cooperativity, i.e. decrease in pH of cat Hb or addition of IHP to human Hb, lead to similar spectroscopic changes in these fully liganded hemoglobins. 3) It is generally agreed that the functional properties and conformations of the isolated liganded alpha and beta subunits do not change greatly when they combine to form the fully liganded tetramer. That is, they are in the so-called "r-form" as monomers and in the tetramer. However, when the unliganded subunits combine there is a change in the absorption spectrum as they convert to the unliganded tetramer (15) which is in the "T-form". Figure 5 shows this difference spectrum, obtained kinetically at several selected wavelengths, by Brunori, et al. (15), and the IHP induced difference spectrum of HbO_2. The similarity suggests closely related structural changes in the region of the chromophore. 4) Perutz has shown by crystallographic methods that the mutant hemoglobin Kempsey is in the quaternary R-form when unliganded but switches to the quaternary T-form upon binding IHP. There results an IHP difference spectrum with hemoglobin Kempsey which is virtually identical

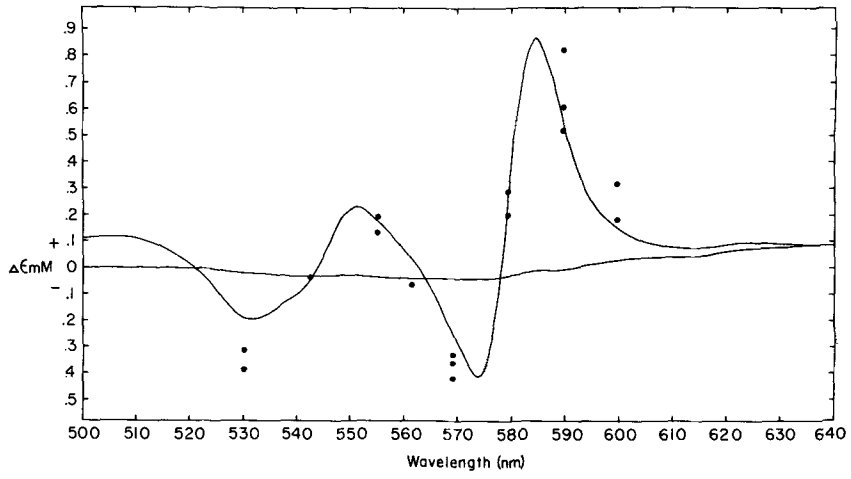

Figure 5. IHP difference spectrum of human HbO_2 and the difference spectrum obtained kinetically by Brunori et al. (15). Points represent the difference between deoxyhemoglobin and the isolated deoxy α and β chains in 0.1 M phosphate buffer, pH 6.9 - 7.0, 20°C. Conditions for IHP difference spectrum: 64.4 μM heme concentration, 2.8°C, 0.1 M HEPES, pH 7.0, 1 atm. O_2. Sample cell IHP concentration: 2 mM. Baseline: H_2O vs. H_2O.

with that obtained by combining unliganded alpha and beta subunits (M. Perutz, personal communication).

The fact that such similar spectroscopic changes are obtained in unliganded and liganded hemoglobin suggests that the structural alterations at or near the heme pocket are similar. The extent to which each subunit type contributes to the observed spectroscopic changes is not known.

Since the isosbestic points of these difference spectra are not the same as those for Hb to HbO_2 changes, and IHP binds more strongly to Hb than HbO_2, it is not expected that O_2 binding data evaluated from visible absorption spectra in the presence of phosphates will give exactly the same saturation isotherms at all wavelengths. The O_2 saturation values evaluated at 577 and 541 nm, the wavelengths usually used, though not the same should be in close agreement with each other whereas saturation values obtained at 555 nm should differ significantly from those obtained at 577 and 541 nm.

In a recent study of the influence of DPG on the Bohr effect in human

hemoglobin Kilmartin (16) has demonstrated a differential pH dependence for DPG binding to deoxy and oxyhemoglobin which is postulated to result from there being different binding sites. Our results suggest that organic phosphates bind to the same central cavity site (9) in both forms of hemoglobin. This would mean that the two spectroscopic forms of oxyhemoglobin reported here would correspond to an equilibrium between quaternary forms having an "open" and "closed" central cavity. An understanding of the mechanistic implications of this suggestion must await crystallographic analysis of organic phosphate-bound oxyhemoglobin.

Acknowledgement: This investigation was supported by research grant GM-18472 from the National Institutes of Health, USPHS.

References

1. Chanutin, A., and Hermann, E. (1969) Arch. Biochem. Biophys. 131, 180-184.
2. Lugue, J., Diederich, D., and Grisolia, S. (1969) Biochem. Biophys. Res. Comm. 36, 1019-1023.
3. Garby, L., Gerber, G., and deVerdier, C. (1969) Eur. J. Biochem. 10, 110-115.
4. Gray, R. D., and Gibson, Q. H. (1971) J. Biol. Chem. 246, 7168-7174.
5. Hedlund, B., Danielson, C., and Lovrien, R. (1972) Biochem. 11, 4660-4668.
6. Berger, H., Janig, G. R., Gerber, G., Ruckpaul, K., and Rapoport, S. M. (1973) Eur. J. Biochem. 38, 553-562.
7. deBruin, S. H., Janssen, L. H. M., and van Os, G. A. J. (1973) Biochem. Biophys. Res. Comm. 55, 193-199.
8. Taketa, F., Mauk, A. G., and Lessard, J.L. (1971) J. Biol. Chem. 246, 4471-4476.
9. Arnone, A. (1972) Nature 237, 146-149.
10. Arnone, A. Presentation at the EMBO Workshop, "The Structure and Function of Hemoglobin.", London. February, 1973.
11. Perutz, M. F. (1970) Nature 228, 726-734.
12. Arnone, A. (1974) Nature 247, 143-145.
13. Bunn, H. F., and Guidotti, G. (1972) J. Biol. Chem. 247, 2345-2350.
14. Hamilton, M. N., and Edelstein, S. J. (1972) Science 178, 1104-1106.
15. Brunori, M., Antonini, E., Wyman, J., and Anderson, S.R. (1968) J. Mol. Biol. 34, 357-359.
16. Kilmartin, J. V. (1974) FEBS Letters 38, 147-148.
17. Williams, R. C., Jr. and Tsay, K-Y. (1973) Anal. Biochem. 54, 137-145.