CIRCULAR DICHROISM AND RAMAN STUDIES OF THE ALLOSTERIC TRANSITION IN METHEMOGLOBIN

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SUMMARY. Circular Dichroism and resonant Raman spectra have been measured for methemoglobin with and without inositol hexaphosphate (IHP) between pH 5 and pH 9. The transition induced by IHP is accompanied by large changes in the rotational strength of the Soret band. Without IHP, the rotational strength of methemoglobin euqals that of oxyhemoglobin; with IHP it equals that of deoxyhemoglobin. The rotational strength of the 260 nm band shows no change with IHP; the Raman bands change in a manner indicative of a shift in spin state equilibrium. The most direct explanation of these results is that IHP changes quaternary structure with few tertiary changes. It is proposed that the Soret rotational strength is a quantitative marker for quaternary structure.

INTRODUCTION. A promising approach to the study of cooperativity in hemoglobin was opened by the demonstration that, upon addition of inositol hexaphosphate (IHP), methemoglobin could exhibit a transition similar to, if not identical with that observed in ferrous hemoglobin upon deoxygenation. (1,2) Subsequently, questions have been raised as to the degree to which the methemoglobin plus IHP system resembles the ferrous deoxy structure. (3,4) While awaiting the x-ray crystallography which will prove definitive, one can turn to optical probes of structure such as circular dichroism and resonant Raman scattering for useful insights.

Empirical studies of the Soret circular dichroism show a sensitivity to events of a quaternary nature, including chain separation (5,6), C-terminal digestion (6) or cooperativity-lowering mutation (7). Calculations by Hsu and Woody account for the Soret circular dichroism as the result of coupled oscil-

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lator interaction between heme transitions and those of aromatic amino acids. In their calculation (8) considerable rotational strength arises from interactions across the $\alpha_1\beta_2$ interface, and provides an intrinsically quaternary probe. Less well characterized are the 285 nm band and the 260 nm band. As is now well established, the 285 nm band is a quaternary structure monitor. (9,10,11) The 260 nm bnad is almost complimentary to the 285 nm band in its persistant response to heme ligation even when quaternary structure is frozen. (9,10) Resonance Raman scattering monitors the heme plane geometry. A number of bands have been assigned as spin state or oxidation markers (12,13) and normal coordinate analysis indicates that deoxygenation is accompanied by a disruption of the porphyrin pi-system, as would occur on doming of the heme. (14) Raman spectra should thus be sensitive to other distortions, such as a twisting of the pyrroles. Shifts in band positions indicate force constant shifts in some cases, from which a measure might be had of cooperative energy stored at the heme.

MATERIALS AND METHODS. Hemoglobin was prepared from whole human blood by a modification (13) of the procedure of Drabkin (15). Methemoglobin derivatives were prepared by addition of one-fold excess potassium ferricyanide followed after one half hour by dialysis against 0.1 N NaCl overnight at 4°C with four changes of water. Organic phosphates were removed by passage through Sephadex G-25 Fine first at 0.02 Tris buffer, pH 7.5, 0.1 N NaCl, and again at 0.002 M Tris buffer, pH 7.5, 0.1 N NaCl. The methemoglobin was stored cold and used within two weeks.

Buffers used in the circular dichroism runs were: 0.02 M PIPES (Calbiochem) for pH 5.0 to pH 7.0 and 0.01 M Tris (Sigma) for pH 7.5 to pH 9.0. Salt was 0.1 M NaCl. IHP concentrations were approximately fourfold excess per tetramer; the IHP (Sigma) as Na^+ salt was neutralized to pH 7.0 and added dropwise from a concentrated solution. Hemoglobin concentration was 80 μM (heme); cell path length was 1 mm. Spectra were recorded on a system described previously (16,17). Data was recorded on charts, as well as automatically digitized every 5 nm. (18) Rotational strengths were computed by numerically integrating the digitized spectra according to

$$R = A \int [\theta] \lambda \, d(1/\lambda)$$

where [θ] is the ellipticity, measured in deg-cm²/dmole, R is the rotational strength, measured in Debye-Bohr Magnetons (DBM), λ is the wavelength, and A is a proportionality constant equal to 0.75×10^{-4} DBM/(deg-cm²-dmole^{-1}). (19)

All Raman spectra were taken in 0.05 M PIPES buffer at pH 6.5, 0.1 N NaCl. IHP was prepared and added as above. Fluoride and cyanide derivatives were prepared using a 20 fold and 2 fold excess of the respective salts. Raman spectra were obtained as described elsewhere (12), using the 457.9 nm and 514.5 nm lines of an argon ion laser.

RESULTS. By monitoring the 285 nm CD band it was confirmed that IHP had in-

duced the same transition as seen by Perutz (2) as seen in Fig. 1. It is interesting to note that no substantial transition is seen at low pH, though some admixture of T state cannot be ruled out.

As seen in Fig. 2, the Soret band changed markedly upon addition of IHP, well above the sloping changes seen with varying pH. The magnitude of the changes seen equal those which occur in the deoxygenation of ferrous hemoglobin. The changes are most pronounced in the negative band on the low wavelength side of the Soret band, as shown in Fig. 3. It is of particular note that this negative band grows steadily as pH is lowered (and the high spin component increased) but shrinks when IHP is present as pH is lowered. In contrast to these changes, the rotational strength of the 260 nm band remains unperturbed (Fig. 4) with the possible exception of changes in its fine structure.

Changes in the Raman spectra are limited to shifts in relative intensity consistent with a small shift in the spin equilibrium toward high-spin heme. Raman frequencies which are sensitive to spin state have been identified at 1640, 1590 and 1500 cm^{-1} for low spin ferric hemes, and at 1607,

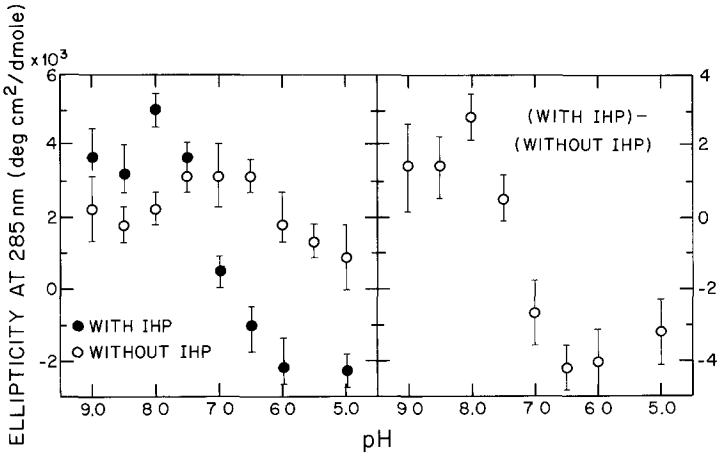

Fig. 1. Ellipticity at 285 nm versus pH. The left panel shows methemoglobin without IHP (o) and with IHP (●). The decrease in ellipticity at low pH may indicate a small admixture of T state. The right panel shows the difference in ellipticities, versus pH.

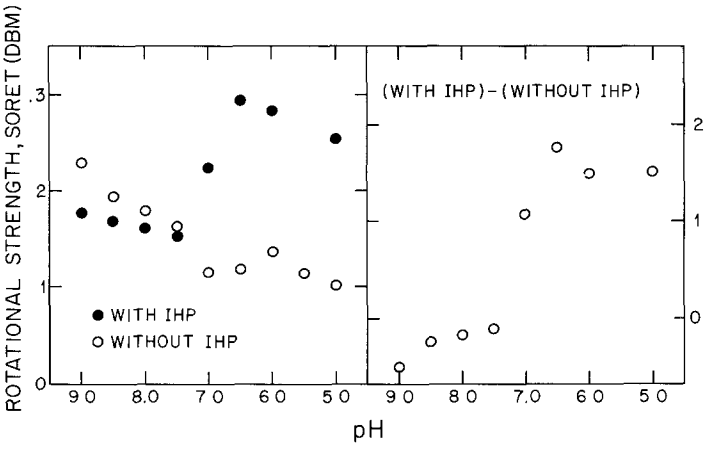

Fig. 2. Soret rotational strength versus pH. Rotational strength was computed by numerical integration of the region 380 nm to 430 nm. The left panel shows the results without (o) and with (•) IHP; the right panel shows the differences. The net change in rotational strength is .15 DBM. The data of Nagai (22) were analyzed and gave .17 DBM for oxyhemoglobin and methemoglobin, and .33 DBM for deoxyhemoglobin. Thus the difference in the ferrous RT transition is .15 DBM as well.

1555 and 1478 cm^{-1} for high spin ferric hemes. Aquo-methemoglobin shows both sets of bands, though the latter are stronger, reflecting a preponderance of the high spin form. Addition of IHP lowers the intensity of the low-spin bands still further relative to the high-spin bands. However, shifts in high-spin frequencies, which might indicate further porphyrin doming or other heme distortion, are not observed.

DISCUSSION. The large changes in the Soret rotational strength due to the addition of IHP are consistent with changes expected from the calculations of Hsu and Woody as well as experimental data cited in the introduction. Other sources of the changes we observe appear unlikely, though they cannot be categorically disregarded. These alternate explanations are (1) changes in the oscillator strength, (2) changes in heme geometry, and (3) changes in tertiary structure.

(1) The total oscillator strength was found to change by about 10%, while the strength of the CD changes by about 100%. Since rotational strength

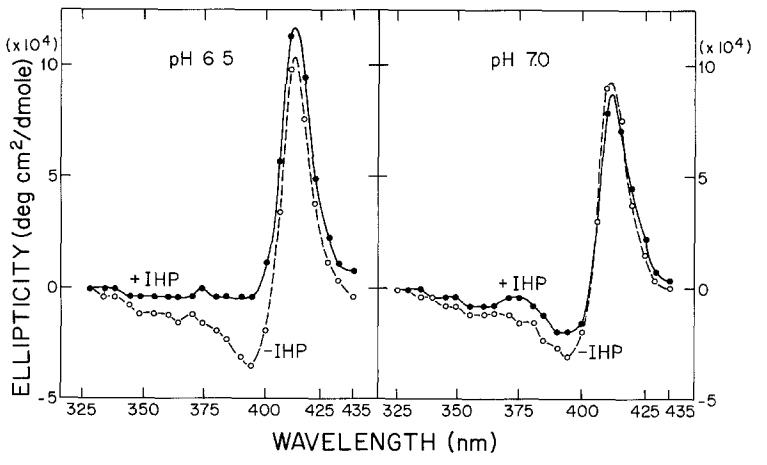

Fig. 3. Soret ellipticity versus wavelength. In lowering pH, the negative peak becomes more negative; on addition of IHP it is diminished. The circles indicate digitization points.

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is proportional to oscillator strength, the only way to assert that the 10% absorption change has such a large effect is via a subunit inequivalence, for Hsu and Woody find the Soret rotational strength to be composed of large contributions of opposite signs for the α and β chains respectively. Since the oscillator strength is observed to increase, an increase in rotational strength due to absorption effects must come from a rotational strength of positive sign. This would require the increased absorption to reside on the α chains. This seems unlikely in that (a) IHP binds between the β chains, (20) and (b) IHP alters the rate of azide binding to the β chains in methemoglobin, but leaves the α chains unchanged. (3)

(2) The changes in rotational strength might be induced by changes in the heme geometry if such changes tilted the pyrroles to give the heme intrinsic chirality. Such a change ought to alter the Raman spectra. The changes that are seen with IHP are only intensity changes, and these are consistent with a small change in spin state. To see the effect of such a change in spin state on the CD, we turn to Fig. 2 and the plot of rotational strength versus pH. The small change in spin state is analogous to a small change in pH, and

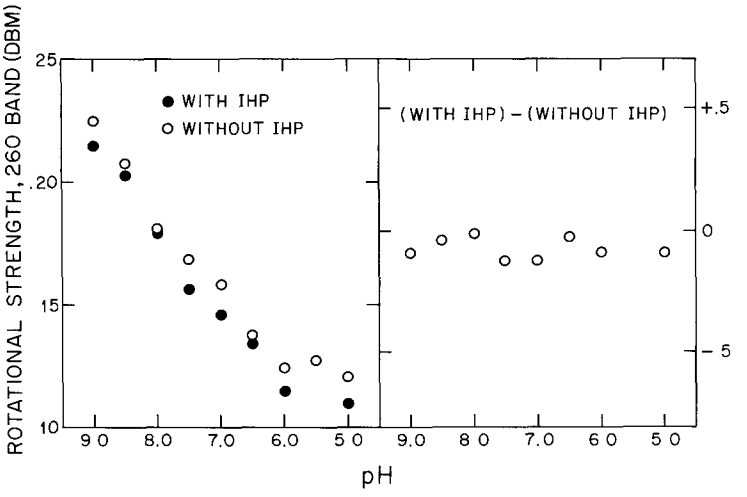

Fig. 4. Rotational strength of the 260 nm band versus pH. Rotational strength was computed by numerical integration from 250 nm to 280 nm. There is no noticable effect of IHP other than a slight dilution of the sample by the IHP.

is clearly incapable of producing a change in rotational strength of the size observed upon addition of IHP.

(3) Tertiary effects are the most ill-defined category of changes, and consequently can only be treated in a general way. The 260 nm CD band responds to tertiary changes associated with ligation, and in this experiment does not change upon addition of IHP. If tertiary effects are the source of the change in Soret rotational strength, they must somehow differ from the ligation-related events to which the 260 nm band responds.

It thus seems likely that the Soret rotational changes are quaternary in nature and this has two important consequences. First, this assignment provides a valuable probe for quaternary structure, since the Soret is a larger and more thoroughly understood band than the 285 nm band. Since methemoglobin, oxyhemoglobin and carboxyhemoglobin (21) all have the same rotational strength, and since deoxyhemoglobin and methemoglobin + IHP also have the same strength, the Soret rotational strength seems a quantitative marker for quaternary structure. In contrast, the 285 nm band shows its characteristic signature only in

the change of quaternary structure and the change in CD spectra, not in the spectra themselves. Secondly, these results imply that quaternary structure has changed with little change in tertiary structure or heme. This might account for the unusual behavior of methemoglobin + IHP distinguishing it from the deoxyhemoglobin (T) structure.

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