METHEMOGLOBIN IMIDAZOLE: EVIDENCE AGAINST AN IHP-INDUCED CHANGE IN QUATERNARY STRUCTURE

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Received 22 December 1979

1. Introduction

The study of quaternary structural transitions in various liganded forms of methemoglobins provides an experimental approach to the measurement of the energetics of heme–heme interaction in the absence of changes in ligation [1]. Among the diverse ligands which may be bound to the ferric iron in methemoglobins [2], those which induce a predominantly high spin electronic configuration of the metal's electrons appear to confer upon the protein a near equivalence in the equilibrium between the high oxygen affinity (R) quaternary conformation and the low affinity (T) conformation. In these derivatives, a quaternary structural transition from the R to the T conformation may be induced by addition of organic phosphates [3]. It has recently been suggested that human imidazole-methemoglobin [(metHbA(Im^-)], a low spin derivative, may also be induced to undergo a quaternary structural change by inositol hexophosphate (IHP) [3]. Since other low spin derivatives, such as cyanometHbA which is fully low spin and azidometHbA which has only a small high spin component remain locked in the R structure even in the presence of IHP, we undertook to study the properties of metHbA(Im^-) to see if this apparently unique conformational transition could be substantiated. Here we report circular dichroism, ultraviolet, CD visible absorption and resonance Raman (RR) spectra on metHbA(Im^-) in the presence and absence of IHP. We find no evidence for an organic phosphate-induced transformation of metHbA(Im^-) to the T structure, but can account for the spectral changes entirely by an IHP-induced replacement of the low spin ligand by water to form the mixed spin derivative, aquomethemoglobin [metHbA(H_2O)].

2. Materials and methods

Human adult hemoglobin was isolated and purified by standard procedures [4]. Oxidation to the ferric form was accomplished by the addition of nitrite which was subsequently removed by repeated dialysis. In the optical absorption and Raman scattering experiments, heme 55 μM and in the CD experiments, 100 μM. In all cases imidazole was 0.1 M. The Raman scattering and optical absorption measurements were made at pH 6.3–6.5 in 0.1 M bis–Tris buffer and when IHP was present, its concentration was 180 μM. The CD measurements were made in 0.01 M bis–Tris and IHP was 1.0 mM. Optical absorption measurements were typically made with matched cells of 0.1 cm or 1.0 cm pathlength. To eliminate possible spurious effects in the optical absorption measurements, a duplicate set of data were obtained in a set of sectored cells designed according to [5], all components (imidazole, IHP, buffer) were present in both sample and reference cells, either mixed with hemoglobin or placed in a separate sector, depending on the experiment. The RR measurements were made on a new instrument recently reported [6,7] in which small spectral differences can be measured accurately by simultaneous data collection from two samples and subsequent analysis on a minicomputer.

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3. Results and discussion

The ultraviolet CD spectrum of hemoglobin is characterized by two bands, one at 257 nm which is sensitive to spin state but not to quarternary structure, and another at 287 nm which is sensitive to quaternary structure but not to spin state [8]. The appearance of negative ellipticity in the 287 nm band is specifically associated with the *T* quaternary structure. In fig.1, we show the ultraviolet CD spectra of metHbA(Im^-) in the presence and absence of IHP, along with the spectra of metHbA(H_2O) under comparable conditions for comparison. As reported [8], in the presence of IHP negative ellipticity appears in metHbA(H_2O); this observation is consistent with other chemical and spectroscopic indicators of a quaternary structural transition to the *T* structure. In contrast, addition of IHP to metHbA(Im^-) produces some slight changes in the CD spectrum, but no band of negative ellipticity. Qualitatively similar changes have been seen in cyanomethemoglobin A [8]. We may conclude that the CD spectrum of metHbA(Im^-) indicates the retention of

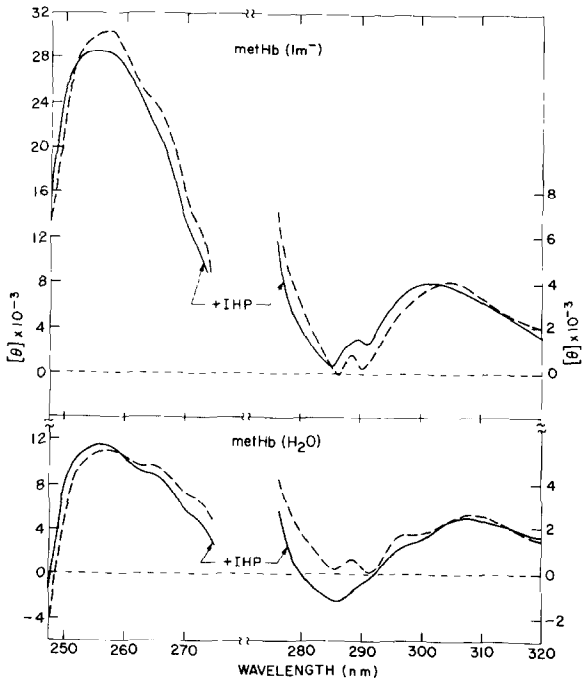

Fig.1. CD spectra (Cary 60) of metHbA(Im^-)±IHP and metHbA(H_2O)±IHP. The region of negative ellipticity at 285 nm in the metHbA(H_2O)+IHP spectrum is characteristic of the *T* structure. It is absent in the corresponding spectrum of metHbA(Im^-).

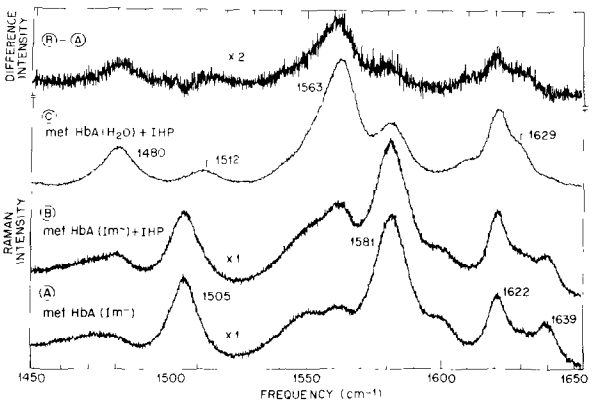

Fig.2. Raman spectra of: (A) metHbA(Im^-); (B) metHbA-(Im^-)+IHP; (C) metHbA(H_2O)+IHP; and on the top the difference spectrum of B–A. In obtaining the difference spectrum the relative intensities of A and B were adjusted so as to cancel out the spectral features of A and B which are unaffected by the addition of IHP. These spectra were obtained with 4131 Å excitation and a 2 cm^{-1} spectral slitwidth.

the *R* quaternary structure even in the presence of IHP.

To determine the origin of the changes in the optical spectrum which led to the conclusion that metHbA(Im^-) may be switched to the *T* conformation, [3], we have obtained RR spectra and optical absorption spectra. The 1450–1650 cm^{-1} region of the RR spectra of metHbA(Im^-) with and without the addition of IHP is displayed on the bottom of fig.2. On the top is a difference spectrum balanced so as to cancel out the unchanged spectral features and bring out the new lines that appear in the metHbA(Im^-)+IHP spectrum. A comparison between this difference spectrum and the spectrum of metHbA(H_2O)+IHP below it show that within experimental error, the new lines that appear in the metHbA(Im^-)+IHP are at the same frequencies and have the same relative intensities as the aquomethemoglobin.

We have also obtained data on a Raman line located at 1374.4 cm^{-1} in metHbA(Im^-). Clear shifts in frequency of this line associated with the quaternary conformational transition in several deoxyhemoglobin derivatives [7] and methemoglobins [9] have been observed recently. Addition of IHP to metHbA-(Im^-) produces no large frequency shift but does result in a broadening of the line, consistent with the sum of contributions from both metHbA(Im^-) and metHbA(H_2O) to the Raman spectrum. In view of

this data, the results from the 1450–1650 cm^{-1} region described above, and the optical absorption data described below, we conclude that the major effect of addition of the organic phosphate is the partial displacement of imidazole by water as the sixth ligand. This result is in full agreement with the imidazole binding studies [3] where hemoglobin could not be saturated in imidazole even at a 500/1 molar ratio and that the binding of imidazole was substantially reduced by addition of IHP. The high frequency value of 1374.4 for the Raman line in the 1370–1380 region in metHbA(Im^-) compared with lower frequencies of 1372 cm^{-1} in metHbA(H_2O) and of 1371.4 cm^{-1} in metHbA(F^-) would suggest that the *R* conformation is markedly more stable than the *T* structure when imidazole serves as the sixth ligand and might not be switched by addition of IHP [7].

To determine how the changes in relative H_2O/imidazole binding constants may affect the optical spectra, we recorded several visible and ultraviolet difference spectra. At the top of fig.3 are visible, Soret, and ultraviolet difference spectra of metHbA-(Im^-)±IHP and on the bottom, for comparison, are the metHbA(H_2O)±IHP difference spectra. In the middle are the difference spectra of metHbA(H_2O)+IHP versus metHbA(Im^-). The features of the visible and Soret difference spectra of metHbA(H_2O)+IHP versus metHbA(Im^-) are in qualitative agreement with the corresponding difference spectra of metHbA-(Im^-)±IHP. If it is assumed that the entire metHbA-(Im^-)±IHP difference spectrum resulted from the formation of aquometHb, the ratio of $\Delta\epsilon_{mM}$ in the visible would indicate 20% replacement of imidazole

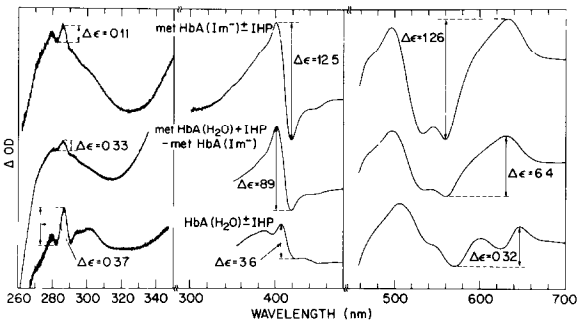

Fig.3. Ultraviolet, Soret, and visible difference spectra (Cary 118). On the top are metHbA(Im^-)±IHP spectra; in the middle are the spectra of metHbA(H_2O)+IHP–metHbA-(Im^-); and on the bottom are metHbA(H_2O)±IHP. $\Delta\epsilon$ refers to heme mM extinction differences.

by water in the metHbA(Im^-)+IHP sample; a similar computation of the ratio of the extinction differences in the Soret indicates 14% replacement of imidazole by water in the presence of IHP. Because it is known that there are visible and Soret spectral changes even in the absence of quarternary structure transformations [8] we assume the difference between these two values result from such additional spectral contributions. It therefore seems likely that the smaller value would be the upper limit. This is in agreement with the Raman difference data which is consistent with a 10–20% formation of aquometHb.

The ultraviolet difference spectrum has been proposed as an indicator of quaternary structural change in hemoglobin [1,8,10]. In hemoglobin derivatives in which such conformational change is known to occur, the difference spectrum is characterized by features in the 283–287 nm region and the 294–302 nm region. We obtain a 287 minus 283 nm $\Delta\epsilon_{mM} = 0.37$ for metHbA(H_2O)±IHP in good agreement with the value of 0.38 which we computed from the data in [8]. Azido-methemoglobin A and cyanomethemoglobin A, which do not undergo quaternary structure changes, have been reported to have similar IHP-induced ultraviolet difference spectra [8] from which we compute $\Delta\epsilon_{mM} = 0.1$; these difference spectra lack the features in the 294–302 nm region. Like azido-methemoglobin A and cyanomethemoglobin A, we find that the difference spectrum of metHbA-(Im^-)±IHP has a 287 minus 283 nm $\Delta\epsilon_{mM} = 0.11$ and appears to lack inflections in the 294–302 nm region. These data supply further evidence that in metHbA(Im^-) a complete conversion to the T structure cannot occur. To assess the consequences of partial replacement of imidazole by water as the sixth ligand on the ultraviolet difference spectrum, we examined the difference spectrum of metHbA-(H_2O) versus metHbA(Im^-) in the absence of IHP, but found no differences either in the 283–287 nm region or in the 294–302 region. On the other hand, the difference spectrum of metHbA(H_2O)+IHP vs metHbA(Im^-)–IHP shows a prominent feature in the 283–287 nm region, with a $\Delta\epsilon_{mM} = 0.33$, comparable to that of the metHbA(H_2O)±IHP difference spectrum in this region. The steep slope of the aquomethemoglobin versus imidazole–methemoglobin difference spectrum in the 294–302 nm region makes detection of any inflections in this region difficult. These experiments demonstrate that the ultraviolet differences are induced by the binding of IHP

and not simply by the differences in spin state of the two ligands.

Although the ultraviolet difference spectrum of metHbA(Im^-)±IHP is very similar to those of cyanomethemoglobin A and azidomethemoglobin A, it is difficult to assign it as resulting solely from the tertiary structural changes induced by the organic phosphate in the absence of a quaternary structural change, because, as discussed below, the conversion of a small amount of material to the *T* structure cannot be ruled out. Thus, we are uncertain whether the difference spectrum of metHbA(Im^-)±IHP arises solely from tertiary structural changes in the low spin liganded protein or whether there may be an additional small contribution from some *T* state tetramers formed by the IHP-induced displacement of imidazole. This additional contribution from *T* state tetramers is certainly $\leqslant$10–20% based on the sensitivity of our CD and ultraviolet difference data.

On the basis of these data we concluded that the quarternary structure of metHbA(Im^-) is unaffected by organic phosphates. The spectral data may be accounted for by displacement of the imidazole ligand by water induced by addition of IHP. Our data are not sufficiently extensive to establish whether those hemes in which imidazole is replaced by water are randomly distributed or whether the replacement is a cooperative process in which all 4 hemes of 10–20% of the tetramers are replaced by water. In the latter case the resulting aquometHbA would be in the *T* conformation and the relative affinity difference between water and imidazole would be induced by the change in quaternary structure. However we have no reason to expect that the cooperativity for IHP-induced ligand replacement is large enough to yield this equilibrium condition since ligand replacement of H_2O by CN^- and N_3^- has been found to be non-cooperative [8]. On the other hand if the replacement of imidazole by water is randomly distributed, then a substantial fraction of tetramers would have water as one of the heme ligands. In this case the IHP-induced replacement of imidazole by water would be due to tertiary structural changes in the protein. Tertiary structure-induced equilibrium changes have been reported for the *R* structure proteins metHb(CN^-) and metHbA(N_3^-) [8]. If this is the case for metHbA(Im^-), our data establishes conclusively that the tetramers with one water molecule as a ligand do not undergo a quaternary structure transition. Possible nonequivalence between α and β hemes in their affinity for the imidazole ligand also has not been determined. Confusion may arise from failure to distinguish between quaternary conformational changes and alterations in the thermodynamic properties of a single conformation state [1]. Because of the equilibrium between imidazole and water ligands which cannot be easily eliminated in this system, some of the criteria which are reliable monitors of conformational state in other systems become reporters of thermodynamic properties of the *R* state of metHbA(Im^-).

These results underscore the importance of ligand electronic properties in regulating quaternary conformation in hemoglobin. It appears that there are no low spin ligands which can be bound to methemoglobin A in the *T* structure. Only in fish hemoglobins with unusually stable *T* structures can such low spin methemoglobins be switched to the *T* structure by IHP. In mixed spin methemoglobins, the free energy difference between high and low spin states has been shown to be in the order of 1 kcal/mol heme smaller when the protein is in the *T* structure than when it assumes the *R* structure [1,3,11,12]. This shift in the spin state free energy difference accompanies the quarternary conformational transition in mixed spin methemoglobins. As long as the spin states are separated by an energy difference $>kT$ the narrowing of the energy difference will be reflected in the apparent spin state equilibrium. In all pure low spin human methemoglobins, evidently the electronic structure of the ligand–heme complexes specifically stabilizes the heme-protein interactions of the *R* structure to an extent that the quaternary structural change is blocked.

Acknowledgements

S.R.S. was supported by grants from the National Institutes of Health (HL-13527), the American Heart Association (76-688), and the New York State Health Research Council.

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