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Calorimetric studies of inositol hexaphosphate binding to aquomethemoglobin A[1]

B. George Barisas[a,*], Donna Barton[b]

[a] *Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523, USA*
[b] *Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104, USA*

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Abstract

Inositol hexaphosphate (IHP) binding to aquomethemoglobin A (metHb) has been studied by flow microcalorimetry and compared to IHP interactions with carbonmonoxyhemoglobin A (COHb) and deoxyhemoglobin A (deoxyHb). At 25°C and pH 6.8 in 0.05 M NaPhos containing 0.1 M NaCl and, after correction for heats of proton extraction from buffer, IHP binds to metHb, deoxyHb and COHb with enthalpies of −80.3, −104.2 and −105.9 kJ/mol IHP, respectively. None of the above processes exhibits large apparent heat capacity changes. The binding to metHb is strongly linked to proton uptake, 1.5 H^+ being absorbed per IHP bound at pH 6.8, with an enthalpic contribution of −21 kJ/mol H^+ absorbed. Inorganic anions, including chloride, phosphate and perchlorate, bind to metHb with dissociation constants in the 0.1 M range and are released upon IHP binding. At high salt concentrations 2–4 such ions are released with enthalpic contributions of +13 to +29 kJ/mol anion released. Thus observed heats of reaction represent a difference of large energetic terms, including not only intrinsic interaction of ligand with protein but also heats of proton extraction from buffer, proton uptake by protein and anion displacement by ligand, each of which can exceed 42 kJ/mol. These results are discussed in light of a possible allosteric transition accompanying IHP binding to metHbA. © 1998 Published by Elsevier Science B.V.

Keywords: Calorimetry; Hemoglobin; Inositol hexaphosphate; Methemoglobin; Thermodynamics

1. Introduction

Properties of water largely define the thermodynamics observed experimentally in biological systems. For example, the partition of free energies of ionic interactions into enthalpic and entropic components depends upon the temperature coefficient of the solvent dielectric constant. One system where ionic interactions between a biological macromolecule and solution species are particularly complex is the oxygen-carrying protein hemoglobin [1]. Besides the well-known binding of oxygen, carbon monoxide and nitric oxide to heme irons, adult human hemoglobin (hemoglobin A; Hb) interacts differentially with protons, small anions and organic phosphates according to its heme-ligation state [2]. Many of these interactions have been traced to particular Hb surface residues and differences in binding exhibited by deoxyHb and oxyHb have been broadly rationalized on the basis of the quaternary structural changes occurring upon heme ligation.

*Corresponding author. Tel.: 001 970 491 6641; fax: 001 970 491 1801; e-mail: barisas@lamar.colostate.edu

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Methemoglobin is of particular interest in relation to the allosteric linkages connecting the binding of gaseous ligands, inorganic ions, protons and organic phosphates. In aquomethemoglobin, the radius of high-spin Fe(III) is midway between high- and low-spin Fe(II). Thus in oxyHb the iron lies in the plane of the porphyrin ring, in deoxy Hb the iron is displaced by ~0.8 Å toward the proximal side and metHb represents an intermediate state [3]. Hence metHb exists, both in the crystal and solution, in thermal equilibrium between the R&T forms with the R form predominant [4]. However, methemoglobin also binds polyanions like inositol hexaphosphate strongly and such binding is accompanied with various spectral changes characteristic of a conformational transition to the T state which is known to have higher affinity for organic phosphates. Thus, it appears that IHP binding drives a methemoglobin conformational transition from R to T. This transition in oxy- and deoxyhemoglobin has been extensively studied since it occurs upon gaseous-ligand binding. If quaternary structures and structural transitions have real validity, then a single treatment of hemoglobin conformational transitions ought to accommodate, at least approximately, both transitions driven by oxygen binding or by allosteric effector. Thus, the detailed thermodynamics of IHP binding by metHb are of interest. (Fig. 1)

This binding is, however complicated by its linkages to protein binding both of the anions and protons. Since IHP binding is necessarily studied in buffers containing chloride or other anions and since IHP binding drives changes in protein protonation [5] with accompanying large thermal effects, quantitation of these linkages is necessary to interpret IHP binding thermodynamics in various solution conditions. Aquomethemoglobin is thought to exist primarily in the R-conformation [4] and, like oxyhemoglobin, to present several low-affinity sites for the binding of small anions like chloride [6]. At the same time, also like oxyhemoglobin, certain proton-binding sites, such as those responsible for the alkaline Bohr effect, would be variably deprotonated relative to deoxyhemoglobin [7,8]. On the other hand, IHP binding in the groove between the β-chains would displace the anions bound there and the R–T transition would encourage protonation of conformationally-sensitive sites. Thus, the net reaction would be accompanied by the *uptake* of some number n_H of protons and the *release* of n_A bound anions, as illustrated in Fig. 2. We present here a thermodynamic study of IHP binding by metHb in light of the above model and including the first detailed calorimetric investigations of this system.

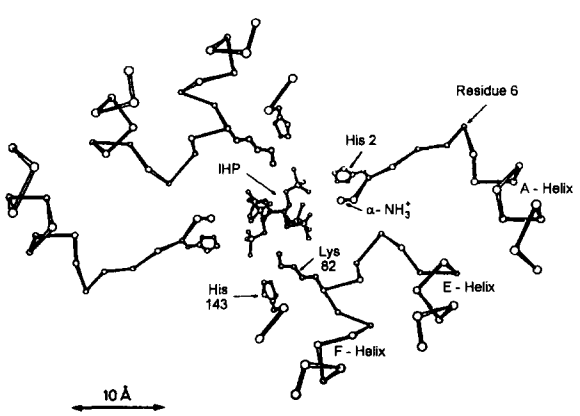

Fig. 1. Crystallographic structure of IHP bound between the β-chains of deoxyHb. Shown to suggest essential features of IHP binding to metHb. From Arnone and Perutz [8].

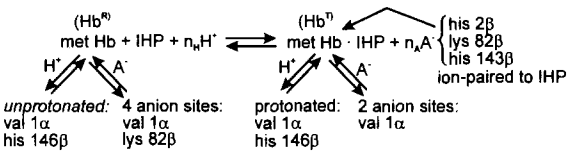

Fig. 2. Equation showing the linkage of metHb binding of the allosteric effector IHP to binding of protons and inorganic anions. The equation is written to indicate the uptake of protons and release of anions upon IHP binding. Specific residues are indicated only to suggest *possible* sites of particular types of interactions.

2. Experimental

2.1. Hemoglobins

Adult human hemoglobin was prepared from fresh blood collected by venipuncture from volunteer laboratory personnel and freed of intrinsically-bound DPG by gel filtration over Sephadex G50 [9]. CoHb and deoxyHb were prepared by dialysis of oxyhemoglobin stock solution into the desired buffer, adjusted

to a typical concentration of 8×10^4 M protein and treated in a tonometer with CO or high purity argon, respectively. Some COHb samples contained 0.1 mM triethylenetetramine to retard autoxidation from residual solution oxygen. MetHb was prepared by the treatment of oxyhemoglobin with 1.1 mol $K_3Fe(CN)_6$ per heme, removal of ferri- and ferrocyanide by filtration over Sephadex G25 [10], concentration adjustment and dialysis into buffer. Total concentrations of oxyHb or COHb, deoxyHb and metHb in each sample were determined spectrophotometrically in the tonometer. Typically at least 95% of hemes were in the desired state [11].

2.2. Calorimetry

An LKB Model 10700-1 heat conduction flow microcalorimeter [11a] mounted in a airtight, stainless steel submarine in a Tronac PTC-40 temperature-controlled water bath was used to measure heats of ligand binding to hemoglobin. The water bath was cooled with a Lauda K2-R refrigerator circulator, permitting calorimetric measurements at temperatures from 4 to 37°C. Samples were introduced through stainless steel tubes from a pair of computer-controlled LKB VarioPerpex peristaltic pumps mounted in an airtight plastic housing. Samples were aspirated from tonometers under argon blanket and both the submarine and pump housing were continuously purged with argon. Typical flow rates were 0.1 ml per minute per channel and flow calibration was performed by weighing water delivered in known time intervals. The thermopile output voltage, proportional to the rate of heat evolution, was amplified by a Keithley Model 150B microvolt ammeter and recorded on a strip chart recorder. The instrumental calibration constant in volts per watt was obtained both by the use of the calorimeter internal calibration heater and by measuring the heat of reaction of HCl and NaOH at known flow rates. IHP was purchased from Sigma Chemical and solutions standardized by phosphorous microassay [12]. Various IHP concentration in the buffer of interest were mixed with hemoglobin and the steady-state rates of heat production were established over at least 1 min interval. Corrections were applied for viscous heating and for heats of dilution of both protein and ligand [13] into the protein final dialysate buffer. Data were recorded as kilojoules of heat evolved per mole hemoglobin vs. the total analytical ligand concentration after mixing with hemoglobin. Representative thermal titration data are illustrated in Fig. 3.

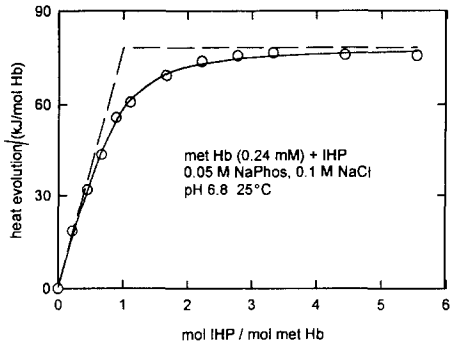

Fig. 3. Representative thermal titration curve for IHP binding to metHb. Temperature 25°C, pH 6.8, 0.05 M sodium phosphate containing 0.1 M sodium chloride. Circles are experimental points and the solid line is calculated from results of the non-linear least analysis of the data. The dashed line illustrates the hypothetical behavior of a sample exhibiting a binding enthalpy equal to that measured but an arbitrarily large binding constant.

2.3. Spectroscopic titrations

Methemoglobin samples of 3 ml at a concentration of 1×10^{-4} M were titrated with IHP in a Cary Model 118 spectrophotometer at 515 nm. The absorbance was recorded after each addition until at least a five-fold excess of titrant had been added. Data were corrected for dilution by titrant and recorded as sample absorbance vs. total analytical IHP concentration after mixing with hemoglobin.

2.4. Data analysis

Thermal titration curves were analyzed using the Marquardt non-linear least-squaring algorithm [14] to fit data to a single-site model containing the following parameters: total protein molar concentration, moles of ligand bound per mole protein at saturation, protein-ligand dissociation constant and heat of binding per mole ligand bound. Fitting yielded the fitted constants, the standard deviation of observed points about the fitted line and the linear estimates of standard errors of the fitted parameters. Spectral titrations were analyzed similarly, except that the model

involved a slightly different set of parameters: total protein molar concentration, moles of ligand bound per mole protein at saturation, protein-ligand dissociation constant, the maximum absorbance change and the baseline absorbance before ligand is added. The latter two parameters differ from the thermal titration model.

3. Results and discussion

3.1. Linkage of proton absorption to IHP binding

The linkage between proton uptake and IHP binding is reflected in both the pH dependence of the ligand dissociation constant and the differences in measured heats of binding in buffers with differing heats of protonation. We have explored both these phenomena. Fig. 4 shows the effects of varying pH on the apparent dissociation constant K_{IHP} for IHP binding to metHb. A plot of pK_{IHP} vs. pH near pH 6.8 has a slope of about −1.5, indicating the uptake of 1.5 protons upon IHP binding. A larger value of −2.4 is observed for this slope between pH 7.0 and 7.4 than at pH 6.8, a result similar to that seen with COHb [5]. The 43.1 kJ/mol IHP greater heat evolution at pH 7.4 relative to pH 6.2 can be equated to the heat of protonation to the 2.4 IHP-linked sites less the heat buffer protonation. This suggests

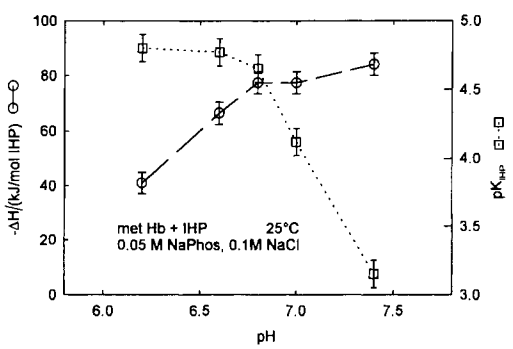

Fig. 4. pH effects on binding of IHP by metHb at 25°C in 0.05 M sodium phosphate containing 0.1 M sodium chloride. ΔH values are calorimetric heats of IHP binding to metHb. The estimated uncertainty in observed heats of reaction is estimated from the reproducibility of replicate measurements to be ±4 kJ/mol. Values of K_{IHP} are dissociation constants for IHP from metHb as obtained from thermal titrations. Estimated uncertainty is ±0.1 pK unit.

Table 1
IHP binding by metHb, COHb and deoxyHb in 0.05 M buffers containing 0.1 M NaCl, pH 6.8

		$-\Delta H^{BC}$/(kJ/mol IHP) [a]		
Protein	Buffer	5°C	25°C	37°C
metHb	Tris	nd	92.0 (20.9)	nd
	Bis–tris	82.0 (39.7)	83.3 (41.0)	70.3 (28.0)
	Phos	47.7 (41.4)	83.7 (77.4)	79.9 (73.6)
COHb	Bis–tris	103.8 (28.0)	104.2 (28.5)	115.5 (39.3)
	Phos	28.5 (17.2)	38.9 (31.0)	41.0 (29.7)
deoxyHb	Bis–tris	106.3 (53.1)	105.9 (48.5)	105.4 (48.1)
	Phos	30.1 (22.2)	60.2 (52.3)	77.8 (77.8)

[a] ΔH^{BC} values are calorimetric heats of IHP binding to the indicated protein, corrected for heats of buffer ionization using values of $1.5H^+$/IHP for proton absorption accompanying IHP binding to metHb, results of Brygier et al. [5] for proton absorption accompanying IHP binding to deoxyHb and COHb and values from Christensen et al. [16] for heats of buffer ionization. The estimated uncertainty of buffer-corrected heats is ±8 kJ/mol. Values in parentheses are uncorrected calorimetric heats of IHP binding. The estimated uncertainty in observed heats of reaction is estimated from the reproducibility of replicate measurements to be ±4 kJ/mol.

a protonation enthalpy of about −21 kJ/mol H^+, somewhat smaller than the −38 kJ/mol H^+ estimated for Bohr residue protonation near neutral pH [15].

Table 1 shows the heats of IHP binding by metHb as measured in three buffers with varying heats of proton ionization, namely, tris, bis–tris and phosphate, with heats of proton ionization of 47.3, 28.0 and 4.2 kJ/mol, respectively [16]. The observed heats of reaction are the sum of the intrinsic or 'buffer-corrected' heat of reaction, i.e. the heat of reaction in a buffer of zero heat of proton ionization, plus the molar heat of buffer ionization times the number of protons absorbed by the protein upon ligation. Thus, a plot of heat of reaction vs. molar heat of buffer ionization for the various buffers affords the number of protons absorbed as its slope. For metHb at 25°C this number is estimated as 1.2 H^+ absorbed/mole IHP bound. Any differential binding of 0.05 M phosphate relative to chloride would increase this figure for proton absorption, but the actual magnitude of such possible effects cannot be evaluated. Using the figure of 1.5 protons absorbed per IHP bound, we calculate the buffer-corrected heats of reaction at various temperatures. These buffer-corrected heats are discussed later in the paper.

Table 2
Chloride, phosphate and perchlorate effects on IHP binding by metHb at 25°C and pH 6.8

Salt	Conc (M)	pK_{IHP} [a]	$-\Delta H$(kJ/mol IHP) [b]
NaCl[c]	0	5.37 [d]	117.2
	0.05	5.33 [d]	83.7
	0.1	4.65 [d]	77.4
	0.2	4.27	65.3
	0.5	3.08	54.8
NaPhos [c]	0.01	6.06	65.7
	0.02	5.37	94.1
	0.05	4.65	77.4
	0.1	4.10	64.9
	0.2	3.59	19.2
$NaClO_4$ [c]	0	5.92	67.4
	0.05	5.21	49.4
	0.1	4.12	36.8
	0.2	nd	approx. 17

[a] K_{IHP} is the dissociation constant for IHP from metHb. Values from thermal titration except as indicated. Estimated uncertainty is ±0.1 pK unit.
[b] ΔH values are calorimetric heats of IHP binding to metHb. The estimated uncertainty in observed heats of reaction is estimated from the reproducibility of replicate measurements to be ±4 kJ/mol.
[c] All solutions contain 0.05 M NaPhos.
[d] Value from spectral titration.
[e] All solutions contain 0.1 M NaCl.

3.2. Linkage to chloride, phosphate and perchlorate binding

The linkage of inorganic anion binding to IHP binding is reflected in salt effects on both IHP binding equilibria and heats. Dissociation constants and heats for IHP binding to metHb in buffers containing various concentrations of added chloride, phosphate and perchlorate at pH 6.8 are summarized in Table 2. In all cases, both the heats and free energies of interaction decrease in magnitude with added salt, the heats falling from about −117 kJ/mol IHP at low salt concentrations to values as small as −17 kJ/mol IHP at high concentrations of added anions. The simplest model for salt effects on equilibrium is to assume that n_A sites, *each necessarily free* if IHP is to bind, react with anion with intrinsic dissociation constants K_A. The resulting equation for the anion dependence of the apparent IHP dissociation constant K_{IHP}^{app} is

$$K_{IHP}^{app} = K_{IHP}(1 + [A^-]/K_A)^n \quad (1)$$

Table 3
Linkage of inorganic anion and IHP binding by metHb at 25°C and pH 6.8

Anion	n_A [a]	$-\Delta H_A$ [b]/ (kJ/mol anion)	K_A (oxyHb) [c] (M)
Cl	2.2	29	0.15
Phosphate	1.8	25	0.04
ClO_4^-	~3.6	13	0.15 [d]

[a] Maximum number of anions released upon IHP binding calculated as limiting slope of plot of pK_{IHP} vs. log (anion concentration).
[b] ΔH is the estimated molar heat of differential anion binding to metHb. More negative heats might also be inferred from experimental data (see text).
[c] Dissociation constants for anions from deoxyHb assuming at total of 6 anion sites. From Nigen et al. [6].
[d] From comparison of data of Amiconi et al. [17] with analyses of Nigen et al. [6].

The maximum number of anions which must be displaced to permit IHP binding can thus be estimated from the slope of a plot of pK_{IHP} vs. log [salt] at high salt concentrations.

The results of this analysis are shown in Table 3 and suggest that 1.5–3 anions are displaced by IHP. The decreasing exothermicity of reaction with increasing salt concentration (Table 2) reflects the heat requirement for removing these bound inorganic anions from the effector site prior to IHP binding. If the fall in observed IHP binding heats with increasing salt concentrations is attributed solely to this requirement, then some limits can be set upon the actual heats of inorganic anion binding. The heat per anion bound must *at least* equal the heat of IHP binding at low salt minus the heat at high salt divided by the maximum number of anions which might be bound over this range. Table 4 shows that exothermic heats of −13 to −29 kJ/mol anion bound might be expected. In theory, the approximate affinities of anions for the metHb effector site can also be estimated from the shape of the pK_{IHP} vs. log (salt concentration) curve. Uncertainties in the data make it difficult to assign such affinities meaningfully, but clearly all three anions exert substantial effects on IHP binding in the region of 0.1–0.2 M. Given the structural similarities between unliganded metHb and oxyHb, one might appropriately compare these concentrations with those established spectroscopically by Nigen et al. [6] and Amiconi et al. [17] for binding of the same ions to oxyHb. These numbers are shown in Table 3.

Table 4
Free energies, buffer-corrected heats and differential phosphate binding heats for IHP binding by metHb, COHb and deoxyHb at 25°C in 0.05 M bis–tris buffers containing 0.1 M NaCl, pH 6.8

Protein	ΔG^{0} [a]/ (kJ/mol IHP)	ΔH^{BC} [b]/ (kJ/mol IHP)	ΔH_{diff} [c]/ (kJ/mol IHP)
metHb	−26.53	−83.3	0
COHb	−22.59	−104.2	−67
deoxyHb	−34.3 [a]	−105.9	−46

[a] Free energy of IHP binding to the indicated protein as determined from thermal titration.
[b] Buffer-corrected heat of IHP binding to hemoglobins. Buffer-correction was performed as described in the text and reported in Table 1. Estimated uncertainties are ±8 kJ/mol.
[c] Differential heat of phosphate binding at 0.05 M relative to buffers containing 0.12 M chloride, calculated as described in the text. Estimated uncertainties are ±17 kJ/mol.
[d] From spectral titration reported in [19]. Estimated uncertainty is −0.1 pK unit.

3.3. Comparison with COHb and deoxyHb

Binding thermodynamics of IHP to deoxyHb and COHb were measured in phosphate and bis–tris buffers for comparison to metHb. These results are summarized in Table 4. Values for proton uptake accompanying IHP binding were taken from Brygier [5] and used, with heats of buffer ionization, to calculate buffer-corrected heats of reaction. The buffer-corrected values for deoxyHb and COHb, obtained at pH 6.8 in 0.05 M bis–tris containing 0.12 M total chloride, −105.9 and −104.2 kJ/mol, respectively, agree well with values of −92 and −88 kJ/mol obtained calorimetrically by Gill et al. [18] in slightly higher chloride concentrations of ca. 0.2 M. The heat of reaction of metHb with IHP observed at pH 6.8 in 0.05 M bis–tris containing 0.12 M total chloride is −41 kJ/mol IHP. The only literature value for this reaction is that of Nelson et al. [19] who report a heat of −13.0 kJ/mol IHP at pH 7.4 in the same buffer. However, the difference in pH between our experiments and those of Nelson et al. [19] is highly significant. Our measurements show proton absorption increasing with pH from 1.5 H^+/IHP at pH 6.8 to at least 2.4 H^+/IHP at pH 7.2. Their experiments, relative to ours, require the additional extraction of about 1 H^+/IHP from their bis–tris buffer which would require an additional enthalpy *input* of about +25 kJ. Thus their result of −13.0 kJ/mol IHP would translate into about −38 kJ/mol IHP under our experimental conditions, a value well within experimental errors of both studies. While the free energies tabulated reflect the relative known affinities of hemoglobins for the allosteric effector, i.e. deoxyHb > metHb > oxyHbA, the buffer-corrected enthalpies do not determine these reaction specificities. On the other hand, the heats are all surprisingly large and provide the driving force for IHP binding to each protein.

One might expect the reaction of IHP with metHb, and perhaps that with COHb as well, to be characterized by a negative heat capacity change. The metHb reaction is thought to be accompanied by an R → T structural transition [4]. The T or deoxyHb structure is stabilized by 8 salt bridges relative to the R or oxyHb structure and the penultimate tyrosines are free in the R conformation but hydrophobically anchored in the T. Thus, an R→T transition might be expected to reduce the protein's vibrational degrees of freedom and so contribute to a negative ΔC_P Indeed a value of -20 kJ deg^{-1} (mol Hb tetramer)$^{-1}$ has been estimated for the transition as initiated by hemoglobin ligation with CO (S.J. Gill, personal communication). However, in bis–tris chloride, none of the proteins studied, neither metHb, COHb nor deoxyHb, exhibits a significant ΔC_P for IHP binding. In phosphate buffers, all reaction enthalpies do become increasingly negative with increased temperature. It seems likely that this reflects phosphate's stronger differential binding to the non-ligated hemoglobins [18]. This binding is exothermic and the phosphate concentrations used approximate that ion's dissociation constant from the unligated hemoglobin. Thus, temperature increases should weaken phosphate binding and reduce its endothermic contribution to the observed reaction enthalpy.

The buffer-corrected heats of IHP-binding to various hemoglobins at 25°C in chloride-only buffers minus the buffer-corrected heat in phosphate-containing chloride buffers is tabulated in Table 4 in analogy to [18] as the 'differential heat of phosphate binding.' This quantity certainly reflects phosphate's higher binding constant, relative to chloride, for interaction with hemoglobins and *may* also indicate a more negative ion binding enthalpy. For deoxyHb the value of −46 kJ/mol is somewhat less exothermic than the −67 kJ/mol obtained by Gill et al. [18] at the higher phosphate concentration of 0.2 M. The value of −67 kJ/mol we observed for COHb is, within the

uncertainties of the measurements, the same as that obtained both by ourselves and Gill et al. for deoxyHb. At 25°C metHb *apparently* exhibits a zero differential heat of phosphate binding. While this may appear substantively different from both deoxyHb and COHb, this may simply be an accident of large uncertainties in these measurements. Thus, one could use the data in Table 1 to calculate for metHb at 5°C a differential heat of phosphate binding of −34 kJ/mol, a value more nearly comparable to those exhibited by deoxyHb and COHb at that temperature.

In summary, calorimetric studies demonstrate that the binding of the allosteric effector inositol hexaphosphate to metHb, like its binding to deoxyHb and COHb, is driven by large negative enthalpies exceeding −83 kJ/mol IHP after correction for heat of buffer protonation (Table 4). The binding process can be rationalized by referring to Fig. 2. IHP binding is strongly linked to proton uptake, 1.5 H^+ being absorbed per IHP bound at pH 6.8, with an enthalpic contribution of −21 kJ/mol H^+ absorbed. The binding sites for these protons may be those residues ion-paired to bound IHP (his 2β, lys 82β, his 143β) and/or alkaline Bohr residues (val 1α, his 146β) protonated in the IHP binding T-conformation. Similarly, inorganic ions including chloride, phosphate and perchlorate bind to metHb, perhaps at lys 82β or val 1α, with dissociation constants in the 0.1 M range and a fraction of these are released upon IHP binding. 2–4 such ions are released at high salt concentrations with enthalpic contributions of +13 to +29 kJ/mol anion released. Thus observed heats of reaction represent a difference of large energetic terms, not only intrinsic interaction of ligand with protein but also heats of proton extraction from buffer, proton uptake by protein and anion displacement by ligand, each of which can exceed 42–63 kJ/mol. How these enthalpic terms arise from, and relate to, the more fundamental binding free energies is, as noted at the outset of this paper, a consequence of the dielectric properties of water.

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