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THE BINDING OF PHYSIOLOGICALLY SIGNIFICANT PROTONS TO 2,3-DIPHOSPHOGLYCERATE *

Mitchell K. HOBISH ** and Dennis A. POWERS ***

Department of Biology, The Johns Hopkins University, Baltimore, MD 21218, U.S.A.

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The intrinsic pK_a values of protons of 2,3-diphosphoglycerate (DPG) which titrate in the physiologically significant range (i.e., pH 6.8–7.8) have been determined by measuring the changes in chemical shifts of the two phosphate resonances of the molecule as a function of pH using ^{31}P-NMR spectroscopy. While conventional acid-base titration techniques resulted in apparent pK_a values of 6.39 and 7.39 for these protons, analysis of the ^{31}P-NMR data by statistical thermodynamic methods yielded intrinsic pK_a values of 6.99 ± 0.07 and 7.28 ± 0.04, for protons associated with the phosphates bound to carbon-3 (C-3) and carbon-2 (C-2), respectively, with an interaction energy of +0.77 kcal/mol. The free energies for the binding of protons to the C-2 and C-3 phosphates and the associated interaction energies determined by ^{31}P-NMR were used to generate a theoretical titration curve which was essentially identical to that determined by conventional acid-base titration. The physiological implications of this work are briefly discussed.

1. Introduction

A recent study from this laboratory on the binding of ATP to carp deoxyhemoglobin I [1] has examined a number of linked equilibria involving the various ionization states of ATP. The thermodynamic models generated in that study [1] demonstrate that the two ionized forms of ATP found in the physiological pH range (i.e., pH 6.8–7.8) bind to the protein with different affinities. Complete thermodynamic characterization required the inclusion of appropriate pK_a values of the organic phosphate groups.

The binding of 2,3-diphosphoglycerate (DPG) to hemoglobin is more complicated than the binding of ATP because of the presence of three ionic forms of DPG as opposed to two for ATP over the physiologically important pH range. Thermodynamic models generated to describe this system require the inclusion of these three pK_a values [2]. While some information on these values is available [3–5], they are inadequate for our purposes for a number of reasons, e.g., the published analyses neglect the electrostatic and steric interactions between phosphate groups, and experimental conditions were not identical to those of our experiments [2].

In an effort to extract the appropriate pK_a values for DPG, ^{31}P-NMR spectroscopy was used to determine the changes in chemical shift of the phosphate resonances as a function of pH (pH 3–9). Conventional potentiometric analysis was also performed under the same conditions. A sub-

* This is contribution No. 1219 from the Department of Biology and the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, MD 21218.

** Current Address: Laboratory of Chemical Evolution, University of Maryland, College Park, MD 20742, U.S.A.

*** To whom correspondence should be addressed.

set of ^{31}P-NMR data were analyzed in terms of statistical thermodynamic formulas [6] to obtain the intrinsic free energies of binding protons at the individual sites of the DPG molecule, as well as the free energy of interaction as both sites are protonated. The conditions of these experiments include the physiologically important pH range (i.e., pH 6.8–7.8) which is important for studies of ligand binding to human hemoglobin.

2. Experimental procedures

DPG was obtained from Sigma Chemical Co. as the pentacyclohexylammonium salt, and converted to the free acid form by ion-exchange chromatography on Dowex 50W (H^+ form). Samples were prepared to 10 mM in DPG in a solution containing 0.1 M KCl. For ^{31}P-NMR experiments, samples were made to 50 mM in either Bis-Tris (reagent grade, Sigma) for pH less than 7.50, or Tris (Trizma grade, Sigma) for pH greater than 7.50. All operations were carried out at 21.5°C. Conventional potentiometric titration of DPG was carried out using a Radiometer Model ABU-11 Auto-Burette. pH was determined with a Radiometer type GK2321C combination electrode and a Radiometer Model 26 pH meter. The system was calibrated using two standards (pH 4 and 10, VWR) at 21.5°C. pK_a values were determined from conventional acid-base titration data by finding the point corresponding to one-half the distance between integral numbers of equivalents added and extrapolating to the pH axis. In regions where there is no clear plateau (e.g., in the physiologically important pH range) such titration curves have been described by an average pK_a (see ref. 2). ^{31}P-NMR spectra were obtained on a JEOL FX-100 spectrometer using pulsed Fourier transform techniques at 40.25 MHz in proton decoupled mode. Up to 200 free induction decays were obtained for each sample. Chemical shifts were measured relative to a standard containing acidified orthophosphate in ^2H_2O. The ^{31}P-NMR data were analyzed by two methods: (a) the method of Moon and Richards [5], and (b) by converting the data to fractional saturation as a function of pH, and fitting the data by a nonlinear least-squares analysis to a Langmuir isotherm (see below). In order to assess the contribution of any energy of interaction between the neighboring phosphate groups to the total free energy of binding in the physiological pH range, a statistical thermodynamic approach was used. This formulation includes the free energy of cooperative interaction which is defined as the difference between the sum of free energies for binding the protons individually, and the free energy of binding them simultaneously. The possible binding sites for this system are represented in table 1, wherein are listed the contributions to the total free energy of binding by each of the available states. The subscript numbers 4 and 5 refer to the assigned pKa values of the DPG molecule, numbering from the most acidic (1) to the most basic (5), and correspond to groups which titrate in the physiological pH range. Specific assignment of pK_a is made later in the text. This nomenclature will be used throughout this manuscript. Each state of single proton binding has an intrinsic free energy of binding, which is related to an equilibrium constant through the relationship $\Delta G = -RT \ln K_i$, where: the microscopic binding constant, K_i, may be represented in terms of the pK_i for any given group. The total free energy of the system may therefore be represented by the sums of the free energies of binding for the individual protons considered singly, with the addition of a term representing the free energy of interaction of the protons. These relationships

Table 1

Microscopic configurations and free energy contributions for the binding of protons to 2,3-diphosphoglycerate in the physiological pH range

0, vacant site; 1, filled site $\Delta G_{4\,5}$ denctes the contributions of free energy of interaction.

Configuration		Total free energy
C-2	C-3	
0	0	0
1	0	ΔG_4
0	1	ΔG_5
1	1	$\Delta G_4 + \Delta G_5 + \Delta G_{4\,5}$

take the form:

$$f_4 = \frac{e^{-\Delta G_4/RT}[H] + e^{-(\Delta G_4+\Delta G_5+G_{45})/RT}[H]^2}{1+(e^{-\Delta G_4/RT}+e^{-\Delta G_5/RT})[H]+e^{-(\Delta G_4+\Delta G_5+\Delta G_{45})/RT}[H]^2}$$

$$f_5 = \frac{e^{-\Delta G_5/RT}[H] + e^{-(\Delta G_4+\Delta G_5+G_{45})/RT}[H]^2}{1+(e^{-\Delta G_4/RT}+e^{-\Delta G_5/RT})[H]+e^{-(\Delta G_4+\Delta G_5+\Delta G_{45})/RT}[H]^2}$$

where f_i represents the fraction of the available states populated by any given state, [H] the proton concentration, ΔG_4 and ΔG_5 the free energies of binding for each of the protons considered singly, and ΔG_{45} the contribution to the total free energy due to interactions between the neighboring phosphates (for a complete description of this approach, see ref. 6). The ^{31}P-NMR data were fitted to this function using non-linear least-squares regression analysis (see below).

2.1. Numerical analysis

Nonlinear least-squares regression analysis was used to analyze all data, using a program written in FORTRAN [7] and implemented on a Hewlett-Packard 1000 computer. The program provides a complete analysis of the fit, including cross-correlation coefficients betwen floated parameters, plots of residuals against both dependent and independent variables, tests of these relationships for randomness, and also provides the variance and confidence limits for the fit.

3. Results and discussion

The apparent pK_a values generated by conventional potentiometric methods are macroconstants, and, as such, do not provide explicit information concerning the effect that titration of one phosphate group has on the other. This information is necessary for a complete thermodynamic analysis of the DPG-hemoglobin system, for which studies are underway in our laboratory. At best, such standard techniques provide an average pK_a [3], largely due to the inability to identify specific groups and therefore to extract titration characteristics of the several groups individually. ^{31}P-NMR spectroscopy was employed in this study because it provides such information [5]. The rationale for

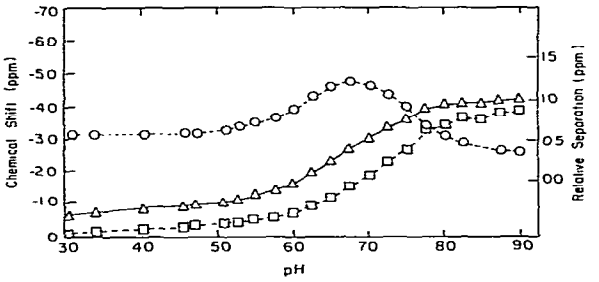

Fig. 1. pH-dependent chemical shift of phosphate resonances of DPG as determined by ^{31}P-NMR spectroscopy. Data were obtained as described in section 2. Data from the phosphate on C-2 (□); phosphate on C-3 (△); relative separation between peaks (○).

assignment of peaks has been described [5]. Our ^{31}P-NMR data were plotted as chemical shift as a function of pH (fig. 1). Also illustrated in fig. 1 is the relative separation between the two peaks as a function of pH. The position of the maximum in this plot differs from that found in a similar plot presented by Moon and Richards [5]. We ascribe this discrepancy to differences in experimental conditions. In order to determine the pK_a values of those groups which titrate in the physiologically important pH range, the data of fig. 1 over the range pH 5.5–9.0 were converted to a form suitable for analysis by standard binding techniques: levels of fractional saturation representing 0 and 1.0 were assigned to plateau values, and intermediate levels of saturation calculated accordingly. Application of the above equations to the fractional saturation form of the data of fig. 1 enabled us to determine the contributions of each phosphate group to the titration characteristics of the other. Results of this procedure and appropriate statistics of the fit are presented in table 2. The data were also fitted to eq. 1 while holding ΔG_{45} fixed at zero (i.e., assuming no energy of interaction). This resulted in ΔG values of -8.92 and -9.56 kcal/mol, corresponding to pK_a values of 6.62 and 7.10, respectively. The variance of the fit under these conditions was 1.8×10^{-3}, an order of magnitude greater than that reported for the fit taking interaction energy into account. Conversion of the data from fig. 2 of ref. 5 to a form ap-

Table 2

Fitted ΔG values and fitting parameters for statistical thermodynamic analysis of binding of protons to 2,3-diphosphoglycerate in the physiological pH range

Variance = 4.6×10^{-4}.

Phosphate on carbon No.	ΔG (confidence limits) (kcal/mol)	ΔG (confidence limits) (kcal/mol)
C-3	−9.42 (−9.51, −9.32)	
		+0.77 (0.69, 0.83)
C-2	−9.81 (−9.87, −9.74)	

propriate for analysis by our methods yielded values of $\Delta G_4 = -9.20$ and $\Delta G_5 = -9.59$ kcal/mol, respectively, corresponding to pK_a values of 6.83 and 7.12. The difference in these results may be ascribed to an apparent lack of salt in the experiments described in ref. 5, as well as some ambiguity as to the temperature of those experiments. Despite these differences, the energy of interaction derived from the data of ref. 5 (+0.76 kcal/mol) was virtually identical to the value reported in table 2. The free energies given in table 2 were used to generate a theoretical titration curve employing a two-step Adair equation. The resulting curve was essentially superimposable on a data set obtained by conventional acid-base titration (fig. 2). Curve b in fig. 2 is a computer-simulated titration curve based on macroconstants determined by potentiometric titration, which, of course, does not supply intrinsic constants. Use of these macroconstants in a two step Adair formulation demonstrates the inadequacy of this approach. Clearly, the resulting isotherm (curve b in fig. 2) differs significantly from the data upon which it is based (points in curve a, fig. 2).

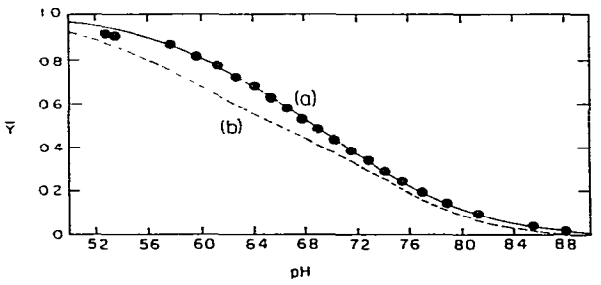

Fig. 2. Fraction saturation ($\bar{Y}$) as a function of pH. Solid circles (●) the potentiometric titration data converted to fractional saturation as described in the text. The solid line (curve a) was generated using constants derived from the ^{31}P-NMR data (table 2) and the Adair equation. The dotted line (curve b) was generated using constants obtained from potentiometric titration.

For a two-site system, the relationships between the micro and macroconstants may be designated as:

$$K_4' = K_4 + K_5$$

$$K_5' = (K_4 K_5 K_{4\,5})/(K_4 + K_5)$$

where K_4' and K_5' are the apparent macroscopic constants, K_4 and K_5 the microscopic constants, and $K_{4\,5}$ represents the constant derived from the interaction energy. If we use the fitted values for K_4, K_5 and $K_{4\,5}$ (see table 2), then the calculated $pK_5' = 7.46$ and $pK_4' = 6.42$, are in good agreement with the values obtained from standard titration analysis (7.39 and 6.39, respectively, data not shown). Our ability to use the derived micro constants to predict the gross behavior of the conventional acid-base titration curve as well as the measured pK_a values adds confidence to the calculated values reported in table 2.

The intrinsic pK_a values reported here for DPG take on significance in light of our studies on the binding of ATP to carp deoxyhemoglobin [1]. In that system it was shown that the two ionized forms of ATP which exist over the physiological pH range bind to the macromolecule with affinities that differ by as much as six orders of magnitude. This differential binding may play an important role in the modulation of several physiological phenomena. Since changes in pH affect the oxygen-binding equilibria of hemoglobin, and such effects are potentiated by the binding of organic phosphates, the situation for DPG is further complicated by the existence of three ionized states over the physiological pH range. The resolution of the pK_a values presented here is an important step toward a complete analysis of the DPG-hemoglobin interaction described previously [2]. Such models can provide accurate and testable equilibrium constants describing the several phenomena involved in the regulation of hemoglobin function.

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