Biophysical Chemistry 24 (1986) 295–309
Elsevier

BPC 01075

ANALYSIS OF ZEROS OF BINDING POLYNOMIALS FOR TETRAMERIC HEMOGLOBINS

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Received 6th January 1986
Accepted 8th May 1986

Key words: Binding polynomial; Patterns of zeros; Factorization; MWC model; Hemoglobin

A quantitative measure of the validity of the MWC description of cooperative binding equilibria has been obtained which uses only the Adair constants. This is accomplished through simple relationships using the zeros of the Adair binding polynomial and unique properties of the zeros of MWC polynomials as described in the accompanying paper (W.E. Briggs, Biophys. Chem. 24 (1986) 311). The method is applied to oxygen binding to a large number of hemoglobins under a wide variety of conditions. In most cases, exemplified by human hemoglobin under a wide range of conditions, the MWC model is allowed and the probability of its suitability is determined. The probability given by this method correlates directly with the deviation between the experimental binding curve and that derived from the theory. In several cases the pattern of the Adair polynomial zeros immediately excludes the MWC model, most notably for carp hemoglobins. A physical picture of cooperative binding site interactions is nevertheless obtained from the patterns of zeros as they relate to the factorization of the binding polynomial.

1. Introduction

The phenomenon of cooperativity in protein-ligand binding reactions has led to the formulation of several models that provide one with a picture of the molecular events that underlie observed binding behavior. In adopting or formulating a model to explain cooperativity, it is useful, where possible, to include structural and chemical information. One notable example is the information available on the distinct crystal structures for the oxygenated and deoxygenated forms of hemoglobin [1]. These data are incorporated into models which describe the cooperative binding of oxygen to hemoglobin. The two-state MWC model [2] was inspired from such information, and the Szabo and Karplus scheme [3,4] incorporated further structural and chemical detail into the model developed by Perutz [5].

Upon making equilibrium measurements of the binding of a single ligand to a nondissociating macromolecule, the usual procedure is to fit the data, represented as points of a binding curve (i.e., a plot of the degree of saturation vs. ligand activity), using the theoretical expression for a model binding curve. One then relies upon the 'goodness of fit' criteria to validate or disqualify the model [6].

Another approach, independent of data-fitting procedures, is the examination of mathematical features of the models, and the subsequent comparison with general properties of the data. The mathematical framework of ligand binding equilibria rests upon the binding polynomial introduced by Wyman [7] for nondissociating macromolecules. Expressions for most experimental observables can be derived from this function. The generality and utility of the binding polynomial make it a natural choice as a basis for comparison among commonly invoked models for phenomena such as cooperativity in ligand binding. The Adair formulation [8] of the binding polynomial is based solely on simple mass law considerations and accounts for the binding reactions in a most gen-

eral way. Within the dictates of the Adair formulation there are no assumptions about the mechanism of a binding process. Therefore, it provides a potentially exact representation of any ligand-binding system within the limits of the data. The Adair polynomial can thus be used as a standard against which other model polynomials can be compared in order to ascertain the validity or applicability of a model. Indeed, in a model-independent way, the factorization properties of the Adair polynomial can often provide insight into the grouping of interactions among binding sites.

In this paper we collect existing binding data for a variety of tetrameric hemoglobins under differing experimental conditions and analyze the zeros of the binding polynomials. The MWC model has been of widespread utility in providing a mechanistic means of explaining and generalizing cooperative binding behavior of hemoglobin. Here we outline and apply a test, developed rigorously in the previous paper [9], for determining the applicability of the two-state MWC model in a given case by comparison to the Adair polynomial. For making the comparison we show (1) how one may utilize the zeros of the Adair binding polynomial to assess whether or not the two-state MWC model is admissible (an idea introduced [10] and subsequently developed [9] by one of the authors previously); and, provided the admissibility requirement is satisfied, (2) how a probabilistic determination of the applicability of this model may be obtained. As will be shown, these criteria are satisfied by many binding studies on tetrameric hemoglobin from humans as well as that from some other animals. It is shown that insights into mechanistic aspects of the binding behavior of both the MWC-admissible and non-admissible cases can often be obtained from examination of patterns of zeros of the Adair binding polynomial.

2. Approach

Each term in an expanded binding polynomial represents the equilibrium concentration of each specifically ligated macromolecular species, relative to the unligated reference species. For a non-dissociating macromolecule with n ligand-binding sites, the Adair binding polynomial is a polynomial of degree n in x, where x is the activity of ligand X:

$$A(x) = 1 + \beta_1 x + \beta_2 x^2 + \ldots + \beta_n x^n \qquad (1)$$

where each β_i is the equilibrium constant for the reaction of the reference species with i ligand molecules X. Since the Adair formulation is a phenomenological one, it provides little physical insight into the mechanism of a process such as the cooperative binding of ligands.

Insight into the nature of cooperativity comes from more mechanistic thermodynamic models, for example, the Pauling [11] or KNF [12] models for subunit interactions in hemoglobin, or the allosteric models proposed by Wyman [13]. Briggs [14] has compared the nature of the zeros of binding polynomials for several of these models. The mathematical properties of the two-state MWC model make it particularly amenable to a critical examination of existing binding curve data. For a two-state MWC model describing an n-site macromolecule, the binding polynomial can be written

$$M(x) = \frac{1}{1+L}(1+\kappa_R x)^n + \frac{L}{1+L}(1+\kappa_T x)^n \qquad (2)$$

where κ_R and κ_T are the association constants for ligand X to each allosteric state of the macromolecule, and L the equilibrium constant between these forms when no ligand is present. We have included the factor $1/(1+L)$ so that the constant term becomes unity as in the Adair polynomial. Our concern is with the case $n = 4$, which is applicable to the large body of data available on the binding of oxygen to hemoglobin.

In comparing Adair and MWC binding polynomials we require that the median ligand activities [15] of the respective binding curves be coincident. This is equivalent to requiring the model to have the same overall binding free energy [15] as that of the experimental curve. Formally this is achieved by making use of normalized binding polynomials. In this way we examine only differences between the shapes of the two curves. The normalization procedure amounts to a simple

change of variable such that the last coefficient becomes unity. By setting $a^n = \beta_n x^n$, the normalized polynomial, $A_N(a)$, is given as

$$A_N(a) = 1 + B_1 a + B_2 a^2 + \ldots + a^n \tag{3}$$

where the coefficients B_i are given by $\beta_i(\beta_n)^{-i/n}$. The median activity, x_m, is given by $(1/\beta_n)^{1/n}$ showing that $a = x/x_m$. Expanding the MWC polynomial and performing a similar transformation gives the normalized MWC polynomial:

$$M_N(a) = \frac{1}{1+L}(1 + K_R a)^n + \frac{L}{1+L}(1 + K_T a)^n \tag{4}$$

where $K_R = \kappa_R x_m$, $K_T = \kappa_T x_m$, and $x_m = [1 + L)/(\kappa_R^n + L\kappa_T^n)]^{1/n}$. Expanding $M_N(a)$, the highest power term is a^n, and thus the median is seen to be the same as in the normalized Adair equation. In the following section we outline the formal mathematical development given by Briggs [9] in terms which are particularly appropriate for comparing general thermodynamic results of the experimental binding curves with the MWC model.

3. Theory

Given a set of n experimentally determined equilibrium constants for a macromolecular with n sites for a single ligand X, the zeros of the Adair binding polynomial, $A(x)$, can be readily computed. Dividing these zeros by the median ligand activity gives the zeros of the normalized polynomial, $A_N(a)$. We then seek to compare these zeros with the zeros of the normalized model polynomial, $M_N(a)$.

The zeros of $M_N(a)$ are found by evaluating the following expression for $z = \omega$, where ω is an n-th root of -1, and $t^n = L$:

$$\frac{(z - t)}{(-K_R z + tK_T)} = w \tag{5}$$

Eq. 5 is a bilinear transformation which maps the circle containing the n-th roots of -1 onto the circle containing the zeros of the MWC polynomial. The zeros of $M_N(a)$ are thus the images of the n-th roots of -1 under the transformation.

The zeros of a binding polynomial of degree four ($n = 4$) may reveal one of several patterns. Not all of these patterns are compatible with what we know to be true about the zeros of the MWC polynomial (see fig. 1). In particular, only cases D and E of fig. 1 arise in an MWC model. Thus, if experimental data give Adair coefficients which lead to the patterns of zeros A, B, or C, an MWC model is automatically excluded.

In considering the comparison of the zeros of MWC and Adair polynomials it was shown that in the preceding paper [9] three main questions need to be addressed: (1) Is there a normalized MWC polynomial having zeros that lie on a circle defined by the zeros of $A(a)$? (2) What is the probability that this can occur? and (3) Given admissibility, what is the probability that the zeros of the normalized Adair polynomial be located relative to the MWC zeros by chance alone?

The first of these questions is answered by whether or not the appropriate bilinear transformation can be determined with positive mapping parameters K_R, K_T, and L since they are equilibrium constants. These parameters for the four-site case ($n = 4$) can be determined from the real axis intersections (u and v) of the circle on which

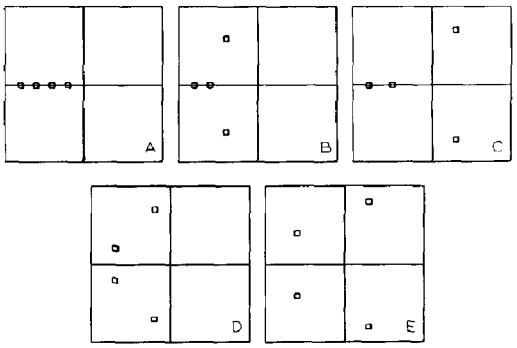

Fig. 1. Patterns of zeros of binding polynomials for four-site macromolecules. (A) Four linear factors representing four independent binding sites; (B) two linear factors with one positive quadratic factor representing two independent sites and a pair of interacting sites; (C) one linear and one positive cubic factor representing one independent and three interacting sites; (D) two positive quadratic factors representing two pairs of interacting sites; (E) an irreducible quadratic representing four interacting sites.

lie the zeros of $A_N(a)$ as follows:

$$K_R = -\frac{t(v-u)+(v+u)}{2vu} \tag{6}$$

$$K_T = -\frac{t(v+u)+(v-u)}{2vut} \tag{7}$$

$$t^4K_T^4 + K_R^4 = t+1 \tag{8}$$

Combining the above three equations, the value of t is found by a real solution of

$$at^4 + bt^3 + ct^2 + bt + a = 0 \tag{9}$$

where

$$a = 2(v^4 + 6v^2u^2 + u^4 - 8v^4u^4) \tag{10a}$$

$$b = 8(v^4 - u^4) \tag{10b}$$

$$c = 12(v^2 - u^2)^2 \tag{10c}$$

K_R and K_T are then obtained by substitution.

The answer to the second question, namely, the probability that a normalized Adair polynomial can be MWC-admissible, has been shown [9] to have a value of 0.41.

Suppose that we have an MWC-admissible polynomial, so that there is an MWC polynomial $M_N(a)$ whose zeros lie on the same circle as those of $A_N(a)$. Four of these (two from each polynomial) are in the upper half plane, and each determines an angle θ with respect to the center of the circle on the real axis, where θ ranges from 0 to π. These angles are designated θ_{A1}, θ_{A2}, θ_{M1} and θ_{M2}, respectively, for the Adair and MWC polynomials and are shown in fig. 2. Taking these angles as coordinates, the zeros of each polynomial can be represented as a point in the θ_1, θ_2 plane. The points representing zeros of positive normalized polynomials are restricted to lie on an arc in this space (fig. 3). This arc must lie within a region of the θ_1, θ_2 plane determined by conditions which insure that a polynomial with these zeros will have positive coefficients [9]. The arc thus represents normalized binding polynomials.

The third question is then answered by considering jointly the probability that the polynomial is MWC-admissible (0.41) together with the probability that the MWC point θ_{2M}, θ_{1M}, is within arc length ϵ of the Adair point θ_{A2}, θ_{A1}. The latter

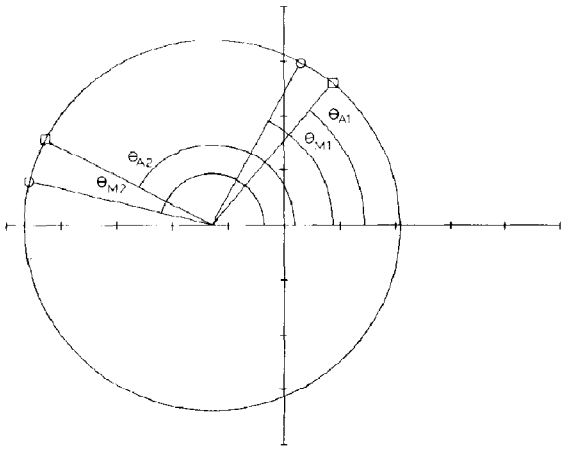

Fig. 2. The zeros of the Adair (□) and MWC (○) polynomials, and the angles which define their position relative to the center of the circle and the real axis.

probability is given by $2\epsilon/l$, where l is the length of the bold portion of the admissible arc in fig. 3. (When the MWC point is within a distance ϵ of a

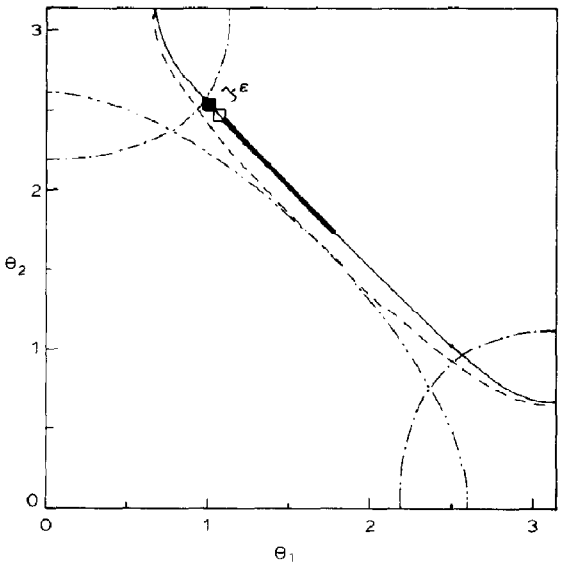

Fig. 3. Zero patterns of the Adair and MWC polynomials for a typical MWC-admissible case (horse Hb, sample no. 62) represented in θ_1, θ_2 space. The bold portion of the arc represents the sample space of zero of MWC admissible polynomials. The three hyperbolas, given by the various dashed lines, correspond to regions where each of the coefficients of the normalized Adair polynominal are positive.

boundary, the probability is given by $(\epsilon + \delta)/l$, where δ is the distance from the MWC point to the boundary.) The confidence in applicability of the two-state MWC model is thus taken as $1 - 0.41(2\epsilon/l)$ or $1 - 0.41(\epsilon + \delta)/l$, accordingly.

Regardless of the applicability of a particular model such as the MWC, there are characteristics of the zeros of the Adair binding polynomial as they relate to its factorization which can provide information concerning binding-site interactions in a model-independent way. Each positive factor of the binding polynomial contributes linearly to the binding curve. For example, if the binding polynomial for a four-site macromolecule has four positive factors, the binding curve is a sum of four 'sub'-binding curves, which we know corresponds to independent-site binding. Such a case of complete factorability gives a pattern of zeros like that shown in fig. 1A. Each of the other patterns of zeros shown in fig. 1 corresponds to other positive factorizations. In general the degree of each factor suggests the minimum number of sites which show positive interaction within the macromolecule [10]. Information of this sort about a binding process, while model-independent, often directs the choosing of a particular mechanistic model.

4. Results

The procedure described in the preceding sections was applied to gaseous ligand-binding data for tetrameric hemoglobins, since these are the only systems which have thus far been studied with sufficient precision to define Adair binding constants adequately. The hemoglobin data tested derive from a variety of sources under a wide range of solution conditions (see table 1). All examples involve data from experiments where oxygen served as the ligand, with the exception of trout hemoglobin I, in which CO was the ligand. Values of the coefficients of the Adair binding polynomials, both normalized and unnormalized, were calculated from literature values of the intrinsic association constants (κ_i) by $\beta_i = [n!/(i!(n - i)!]\Pi\kappa_j$ and appear in table 2.

The zeros of the normalized Adair binding polynomial for each sample case were calculated, and the equations of the circles that these zeros define were obtained. From the endpoints of the circle on the real axis, u and v, values of K_R, K_T and t were determined, when possible, from eqs. 6, 7 and 9, and the MWC zeros were found from eq. 5. Zeros of the Adair and MWC polynomials, in addition to the circle parameters u and v, are listed in table 3. Table 4 gives the values for κ_R, κ_T, and t ($= L^{1/4}$), which serve as parameters in the MWC binding polynomial. Also listed in table 4 are the confidence levels for the applicability of the two-state MWC model.

5. Discussion

The hemoglobin data analyzed reflect the wide variety of sources and conditions from which data were obtained (see table 1). The disparate values of the equilibrium parameters shown in table 2 and the varying patterns of the zeros given in table 3 demonstrate this variety.

As mentioned above the factors of the binding polynomial are of interest because they may indicate the existence of groups of interacting binding sites within the tetrameric structure. For example, sample no. 43, which is a hybrid of human and carp subunits, gives a binding polynomial that can be factored into a positive cubic term and a positive linear term. For such a four-site molecule, this suggests that three of the sites interact in a positively cooperative manner and the fourth site is independent in its binding of ligand. This type of information is often hidden from least-squares analysis of binding curves.

All the data on native human hemoglobin produce binding polynomials that are p-irreducible [10], i.e. they do not permit any positive factorizations. The appropriate pattern of zeros is shown in fig. 1E. Thus, under conditions of varying pH, buffer, $[Cl^-]$ and effector molecule concentration, this implies that all four binding sites interact in the oxygen-binding process. The same trend holds for the other mammalian hemoglobins (sheep and horse), and for clam and trout hemoglobins as well. The patterns of zeros for these cases are MWC-admissible, thus allowing the determination of the probability that the model is applicable.

Table 1

Hemoglobin types, and solution conditions for ligand-binding data

Experiments were carried out at 25°C, except where indicated in parentheses following the sample type.

Sample no.	Hemoglobin type	pH	Buffer	Chloride concentration	Other effector	Ref.
1	Hb A	9.1	0.05 M Tris	2.6 mM	–	21
2	Hb A	9.1	0.05 M Tris	0.1 M	–	21
3	Hb A	7.4	0.05 M Bis-Tris	7 mM	–	21
4	Hb A	7.4	0.05 M Bis-Tris	0.1 M	–	21
5	Hb A	7.4	0.05 M Bis-Tris	0.005 M	–	22
6	Hb A	7.4	0.05 M Bis-Tris	0.005 M	0.66 mM CO_2	22
7	Hb A	7.4	0.05 M Bis-Tris	0.005 M	1.6 mM CO_2	22
8	Hb A	7.4	0.05 M Bis-Tris	0.005 M	4.0 mM CO_2	22
9	Hb A	7.4	0.05 M Bis-Tris	0.005 M	8.2 mM CO_2	22
10	Hb A	7.4	0.05 M Bis-Tris	0.1 M	–	22
11	Hb A	7.4	0.05 M Bis-Tris	0.1 M	0.66 mM CO_2	22
12	Hb A	7.4	0.05 M Bis-Tris	0.1 M	1.6 mM CO_2	22
13	Hb A	7.4	0.05 M Bis-Tris	0.1 M	4.0 mM CO_2	22
14	Hb A	7.4	0.05 M Bis-Tris	0.1 M	8.2 mM CO_2	22
15	Hb A	7.4	0.1 M phosphate	–	–	21
16	HbA	7.4	0.05 M Bis-Tris	0.1 M	2 mM DPG	21
17	Hb A	7.4	0.05 M Bis-Tris	0.1 M	2 mM IHP	21
18	Hb A	6.5	0.05 M Bis-Tris	0.1 M	–	21
19	Hb A	6.5	0.05 M Bis-Tris	0.1 M	2 mM IHP	21
20	Hb A	7.4	0.05 M Bis-Tris	–	–	17
21	Hb A	7.4	0.05 M Bis-Tris	–	2 mM DPG	17
22	Hb(AcAm)	7.4	0.05 M Bis-Tris	–	–	17
23	Hb(AcAm)	7.4	0.05 M Bis-Tris	–	2 mM DPG	17
24	Hb(MalN)	7.4	0.05 M Bis-Tris	–	–	17
25	Hb(MalN)	7.4	0.05 M Bis-Tris	–	2 mM DPG	17
26	Hb(CPase)	7.4	0.05 M Bis-Tris	–	–	17
27	Hb(CPase)	7.4	0.05 M Bis-Tris	–	2 mM DPG	17
28	carp Hb	6.43	0.05 M Bis-Tris	–	–	19
29	carp Hb	6.61	phosphate/citrate	–	–	19
30	carp Hb	6.92	0.1 M phosphate	–	–	19
31	carp Hb	7.20	0.1 M phosphate	–	–	19
32	carp Hb	7.65	phosphate/borate	–	–	19
33	carp Hb	7.83	phosphate/borate	–	–	19
34	carp Hb	9.11	0.1 M borate	–	–	19
35	carp Hb	6.93	0.1 M phosphate	–	1.4 mM IHP	19
36	carp Hb	7.35	0.1 M phosphate	–	1.4 mM IHP	19
37	carp Hb	7.65	phosphate/borate	–	1.4 mM IHP	19
38	carp Hb	7.98	phosphate/borate	–	1.4 mM IHP	19
39	carp Hb	8.33	phosphate/borate	–	1.4 mM IHP	19
40	carp Hb	8.58	0.1 M borate	–	1.4 mM IHP	19
41	carp Hb	8.95	0.1 M borate	–	1.4 mM IHP	19
42	carp Hb	6.85	– phosphate	–	–	20
43	hybrid I	7.03	– phosphate	–	–	20
44	hybrid II	6.88	– phosphate	–	–	20
45	carp Hb	6.85	0.2 M phosphate	–	–	23
46	desHis-Carp	6.71	0.2 M phosphate	–	–	23
47	carp Hb (10°C)	7.3	0.1 M phosphate	–	–	24
48	carp Hb (15°C)	7.3	0.1 M phosphate	–	–	24
49	carp Hb (20°C)	7.3	0.1 M phosphate	–	–	24
50	carp Hb (25°C)	7.3	0.1 M phosphate	–	–	24

Table 1 (continued)

Sample no.	Hemoglobin type	pH	Buffer	Chloride concentration	Other effector	Ref.
51	carp Hb (10°C)	8.3	0.1 M phosphate	–	–	24
52	carp Hb (15°C)	8.3	0.1 M phosphate	–	–	24
53	carp Hb (20°C)	8.3	0.1 M phosphate	–	–	24
54	carp Hb (25°C)	8.3	0.1 M phosphate	–	–	24
55	carp Hb (10°C)	9.1	0.1 M phosphate		–	24
56	carp Hb (15°C)	9.1	0.1 M phosphate	–	–	24
57	carp Hb (20°C)	9.1	0.1 M phosphate	–	–	24
58	carp Hb (25°C)	9.1	0.1 M phosphate	–	–	24
59	trout I (25°C)	6.8	0.1 M phosphate	–	–	25
60	trout I (20°C)	6.8	0.1 M phosphate	–	–	25
61	horse Hb	9.1	0.2 M borate	–	–	21
62	horse Hb	7.0	0.6 M phosphate	–	–	21
63	sheep Hb	9.1	0.2 M borate	–	–	21
64	sheep Hb	7.1	0.05 M phosphate	–	–	21
65	Hb A	7.4	0.1 M Tris	0.18 M	–	26
66	clam Hb (10°C)	7.8	0.1 M phosphate	–	–	27
67	clam Hb (15°C)	7.8	0.1 M phosphate	–	–	27
68	clam Hb (20°C)	7.8	0.1 M phosphate	–	–	27
69	clam Hb (25°C)	7.8	0.1 M phosphate	–	–	27
70	clam Hb (30°C)	7.8	0.1 M phosphate	–	–	27

The results of these calculations, given as % confidence in the MWC model, are shown in table 4.

The suitability of the MWC description for these hemoglobins is strongly evidenced. For these cases the confidence levels in the MWC representability are clustered toward high probabilities (table 4). It should be pointed out that the confidence level can never drop below 59%, since there is only a 41% probability that any normalized, positive polynomial will be MWC-admissible at all, as stated above. However, the high probabilities obtained, in excess of 90%, provide strong evidence of the suitability of the MWC model for describing ligand binding to these hemoglobins. Of course in some cases, alternative experiments may allow one to test further the MWC model. The utility of the present method lies in providing a direct test of the MWC model based on single binding curves, keying on more specific knowledge of the model than is emphasized by simple data-fitting techniques.

A more intuitive measure of the appropriateness of an MWC model for binding data is given by comparison between the MWC-predicted binding curve and the Adair or actual experimental one. The binding curves of an example (case no. 62, horse hemoglobin) which fits the MWC model moderately well are depicted in fig. 4. For this example the difference between the curves is shown in fig. 5 as the dotted line. The solid line represents the difference between binding curves for the MWC case and the Adair case with zeros now located as far away as possible. This corresponds to using the Adair polynomial defined by the zeros at the extremes of the allowed arc of fig. 3. A more general average measure of the deviation between the model prediction and the best Adair curve is given by the area between the two curves. This area, when compared to the area that results from the extreme possible Adair case, gives the degree of departure of the MWC curve from the best Adair curve. This heuristic measure of the goodness of fit can then be compared with the probability values already evaluated for the various admissible cases. Fig. 6 shows the direct correlation between these two measures of the applicability of the MWC model.

In contrast to the cases discussed so far, the

Table 2

Unnormalized coefficients, β_i, and normalized coefficients, B_i, of binding polynomials with median ligand activities, x_m

Unnormalized coefficients, β_i, are expressed in $torr^{-i}$ and x_m in torr, except for samples 43–46, where β_i are expressed in μM^{-i} and x_m in μM, and samples 59–60 where β_i are expressed in M^{-i} and x_m in M. All parameters are reported with two or three significant figures (depending on the precision of the original data) and treated as exact values.

Sample no.	β_1	β_2	β_3	β_4	x_m	B_1	B_2	B_3
1	0.680	0.367	0.832	0.768	1.07	0.727	0.419	1.01
2	0.238	0.571	0.0571	0.0476	2.14	0.510	0.262	0.561
3	0.259	0.186	0.0559	0.0593	2.03	0.524	0.765	0.465
4	0.0872	0.00811	0.00160	0.00140	5.17	0.451	0.217	0.224
5	0.360	0.0977	0.0854	0.102	1.77	0.636	0.305	0.471
6	0.232	0.00766	0.0141	0.0197	2.70	0.619	0.0546	0.269
7	0.116	0.00592	0.00217	0.00310	4.24	0.492	0.106	0.165
8	0.096	0.00446	0.000446	0.000543	6.55	0.629	0.191	0.125
9	0.064	0.0024	8.00×10^{-5}	0.000114	9.67	0.619	0.225	0.0724
10	0.092	0.00566	0.000830	0.00126	5.31	0.488	0.159	0.124
11	0.080	0.00576	0.000576	0.000943	5.71	0.456	0.188	0.107
12	0.076	0.00319	0.000340	0.000393	7.10	0.540	0.161	0.122
13	0.068	0.00235	0.000109	0.000133	9.31	0.633	0.203	0.0883
14	0.064	0.00134	7.17×10^{-5}	6.59×10^{-5}	11.1	0.710	0.166	0.098
15	0.0544	0.00245	0.000245	0.000210	8.31	0.452	0.169	0.140
16	0.0326	0.00161	3.22×10^{-5}	3.08×10^{-5}	13.4	0.437	0.291	0.078
17	0.0201	0.000392	1.10×10^{-6}	2.57×10^{-7}	44.7	0.897	0.782	0.0977
18	0.0476	0.000785	3.61×10^{-5}	1.12×10^{-5}	17.3	0.823	0.235	0.187
19	0.0161	0.000106	5.18×10^{-7}	4.39×10^{-9}	123	1.98	1.61	0.961
20	0.456	0.113	0.0880	0.0889	1.83	0.835	0.379	0.541
21	0.0540	0.000724	2.08×10^{-5}	2.2×10^{-5}	14.6	0.789	0.154	0.0648
22	1.19	1.70	0.443	0.494	1.19	1.42	2.42	0.752
23	0.0828	0.00929	0.000206	0.000268	7.82	0.647	0.568	0.0986
24	1.62	1.52	1.08	0.430	1.24	2.00	2.31	2.04
25	0.118	0.0435	0.00581	0.00229	4.57	0.539	0.909	0.554
26	8.36	27.6	38.8	33.9	0.41	3.47	4.74	2.76
27	8.04	32.7	33.6	37.2	0.41	3.26	5.36	2.23
28	0.092	0.011	0.0014	0.00027	7.8	0.72	0.69	0.65
29	0.10	0.0022	7.4×10^{-5}	3.9×10^{-6}	23	2.3	1.1	0.85
30	0.084	0.0040	0.0002	3.5×10^{-5}	13	1.1	0.68	0.43
31	0.25	0.030	0.0064	0.0008	6.0	1.5	1.1	1.4
32	1.3	0.84	0.30	0.10	1.8	2.3	2.6	1.7
33	2.1	2.3	1.0	0.45	1.2	2.6	3.3	1.9
34	10.0	31.0	32.3	17.5	0.49	4.91	7.42	3.77
35	0.0392	0.00138	2.04×10^{-5}	1.81×10^{-7}	48.4	1.90	3.24	2.33
36	0.157	0.0125	0.000340	1.73×10^{-5}	15.5	2.44	3.00	1.27
37	0.218	0.0337	0.00241	0.000280	7.73	1.69	2.02	1.11
38	0.154	0.0679	0.00647	0.00100	5.62	0.868	2.14	1.15
39	1.38	0.660	0.195	0.0249	2.52	3.48	4.18	3.12
40	2.11	2.53	1.50	0.238	1.43	3.02	5.19	4.40
41	5.32	7.68	7.22	1.30	0.936	4.98	6.73	5.92
42	0.056	0.0016	6.4×10^{-6}	5.1×10^{-7}	37	2.1	2.2	0.34
43	0.22	0.0051	9.1×10^{-5}	2.3×10^{-6}	26	5.7	3.4	1.6
44	0.31	0.040	0.00017	4.8×10^{-6}	21	6.6	18	1.6
45	0.056	0.0016	6.4×10^{-6}	4.9×10^{-7}	38	2.1	2.2	0.35
46	0.071	0.00028	4.3×10^{-6}	2.4×10^{-7}	45	3.2	0.58	0.39
47	0.788	0.0567	0.0775	0.0438	2.19	1.72	0.271	0.810
48	0.524	0.196	0.0324	0.00906	3.24	1.70	2.06	1.10
49	0.636	0.0782	0.0335	0.00415	3.94	2.51	1.21	2.05

Table 2 (continued)

Sample no.	β_1	β_2	β_3	β_4	x_m	B_1	B_2	B_3
50	0.492	0.0812	0.0139	0.00130	5.27	2.59	2.26	2.03
51	6.52	21.0	27.9	30.5	0.425	2.77	3.80	2.15
52	4.24	4.35	3.51	1.61	0.887	3.76	3.42	2.45
53	3.62	3.63	2.88	0.562	1.16	4.18	4.84	4.43
54	3.30	2.39	1.12	0.142	1.63	5.39	6.36	4.83
55	11.4	44.6	63.4	50.5	0.375	4.29	6.28	3.34
56	10.0	31.0	32.3	17.5	0.489	4.91	7.41	3.77
57	8.20	22.4	19.6	8.70	0.582	4.77	7.59	3.86
58	6.64	10.9	9.77	2.64	0.785	5.21	6.68	4.72
59	1.49×10^{6}	1.83×10^{12}	7.00×10^{18}	2.00×10^{25}	4.73×10^{7}	0.801	0.528	1.09
60	1.29×10^{6}	1.57×10^{12}	7.07×10^{18}	2.80×10^{25}	4.35×10^{7}	0.561	0.297	0.581
61	0.213	0.704	0.352	0.916	1.02	0.218	0.735	0.376
62	0.0572	0.00434	0.000243	0.0000140	16.3	0.525	0.366	0.188
63	0.109	0.0238	0.00440	0.00880	3.26	0.357	0.253	0.153
64	0.0744	0.00187	4.69×10^{5}	7.50×10^{5}	10.7	0.799	0.216	0.0582
65	0.0644	0.00187	0.000266	0.000580	6.44	0.415	0.0776	0.0712
66	0.23	0.029	0.0029	0.00078	6.0	1.4	1.0	0.62
67	0.18	0.016	0.0012	0.00028	7.7	1.4	0.96	0.60
68	0.12	0.0087	0.00064	0.000088	10.3	1.2	0.93	0.70
69	0.11	0.0062	0.00033	0.000043	12.3	1.4	0.95	0.62
70	0.072	0.0039	0.00015	0.00016	15.8	1.1	0.98	0.59

remainder of the binding data we examined yield binding polynomials which are not p-irreducible, i.e., they are factorable. Except in a few cases, these polynomials are not MWC-admissible. Nevertheless, the pattern of zeros is often in itself useful for detecting information about site linkages. Among these factorable cases are some of the chemically modified derivatives of human hemoglobin. In particular, the two binding polynomials for Hb(CPase) (a hemoglobin partially digested with carboxypeptidase), one stripped of phosphate and the other under conditions of 2 mM 2,3-diphosphoglycerate (DPG), are both factorable into two quadratic terms. The pattern of zeros corresponds to that shown in fig. 1D. This pattern suggests the presence of two pairs of interacting binding sites. It is noteworthy that the maximum Hill slope can never be greater than the degree of the highest degree factor of the binding polynomial [16]. For Hb(CPase), the factor of highest degree is a quadratic – degree 2. The reported maximum Hill slopes are 1.15 and 1.23 for Hb(CPase) with and without DPG, respectively [17], in agreement with the prediction that this value does not exceed 2.

Another modified hemoglobin, Hb(MalN) [17], has the pattern of zeros which depends upon the presence of DPG. For stripped Hb(MalN) the binding polynomial can be factored into two quadratics, again implying two pairs of interacting sites. The maximum Hill slope is reported to be 1.44 – less than 2, as expected for independent quadratics. However, under conditions of 2 mM DPG, the binding polynomial produces no positive factorizations (p-irreducible), indicating the presence of four interacting sites. As expected, the Hill slope in this case exceeds 2. The addition of DPG has the effect of coordinating the two pairs of interacting sites into a set of four interacting sites. This fact is intriguing in that the modification of this hemoglobin affects a salt bridge in the deoxy form between the β-chains [5], thus disturbing the interface between the two $\alpha\beta$ dimers. The binding of DPG, which is known to span the termini of the β-chains in the deoxy form [18], effectively restores the linkage between all four sites in the molecule.

The binding polynomials for Hb(AcAm) [17] are not factorable, corresponding to the pattern of zeros labelled E in fig. 1. Under both phosphate-

Table 3

Zeros of the normalized Adair polynomial, $A_N(a)$, and normalized MWC polynomial, $M_N(a)$, and the endpoints of the circle which their zeros define

Missing entries indicate MWC-nonadmissible cases.

Sample no.	Adair zeros		v	u	MWC zeros	
1	$0.426 \pm 0.846i$;	$-0.934 \pm 0.492i$	0.906	−1.07	$0.405 \pm 0.858i$;	$-0.908 \pm 0.534i$
2	$0.534 \pm 0.834i$;	$-0.814 \pm 0.597i$	0.984	−1.01	$0.544 \pm 0.828i$;	$-0.824 \pm 0.583i$
3	$0.451 \pm 0.907i$;	$-0.684 \pm 0.712i$	1.03	−0.980	$0.572 \pm 0.839i$;	$-0.797 \pm 0.578i$
4	$0.615 \pm 0.842i$;	$-0.727 \pm 0.625i$	1.07	−0.945	$0.639 \pm 0.826i$;	$-0.748 \pm 0.598i$
5	$0.544 \pm 0.876i$;	$-0.780 + 0.576i$	1.05	−0.961	$0.559 \pm 0.868i$;	$-0.794 \pm 0.555i$
6	$0.636 \pm 0.853i$;	$-0.771 \pm 0.539i$	1.10	−0.925	$0.614 \pm 0.886i$;	$-0.753 \pm 0.566i$
7	$0.651 \pm 0.837i$;	$-0.733 \pm 0.593i$	1.10	−0.926	$0.652 \pm 0.837i$;	$-0.734 \pm 0.592i$
8	$0.648 \pm 0.884i$;	$-0.711 \pm 0.572i$	1.16	−0.886	$0.656 \pm 0.879i$;	$-0.716 \pm 0.564i$
9	$0.656 \pm 0.890i$;	$-0.692 \pm 0.582i$	1.17	−0.874	$0.673 \pm 0.880i$;	$-0.703 \pm 0.566i$
10	$0.651 \pm 0.847i$;	$-0.713 \pm 0.606i$	1.11	−0.915	$0.664 \pm 0.838i$;	$-0.723 \pm 0.592i$
11	$0.650 \pm 0.845i$;	$-0.704 \pm 0.620i$	1.11	−0.916	$0.671 \pm 0.831i$;	$-0.720 \pm 0.598i$
12	$0.653 \pm 0.859i$;	$-0.714 \pm 0.592i$	1.13	−0.904	$0.662 \pm 0.853i$;	$-0.721 \pm 0.582i$
13	$0.656 \pm 0.889i$;	$-0.700 \pm 0.574i$	1.17	−0.876	$0.667 \pm 0.882i$;	$-0.707 \pm 0.564i$
14	$0.662 \pm 0.899i$;	$-0.711 \pm 0.545i$	1.19	−0.867	$0.657 \pm 0.902i$;	$-0.708 \pm 0.550i$
15	$0.645 \pm 0.840i$;	$-0.715 \pm 0.617i$	1.09	−0.926	$0.662 \pm 0.828i$;	$-0.728 \pm 0.599i$
16	$0.638 \pm 0.859i$;	$-0.677 \pm 0.644i$	1.12	−0.908	$0.682 \pm 0.830i$;	$-0.711 \pm 0.600i$
17	$0.553 \pm 1.05i$;	$-0.602 \pm 0.593i$	1.37	−0.780	$0.583 \pm 1.04i$;	$-0.676 \pm 0.462i$
18	$0.629 \pm 0.930i$;	$-0.723 \pm 0.521i$	1.21	−0.863	$0.624 \pm 0.932i$;	$-0.719 \pm 0.527i$
19	$0.222 \pm 1.25i$;	$-0.702 \pm 0.360i$	1.82	−0.753	$0.235 \pm 1.25i$;	$-0.707 \pm 0.342i$
20	$0.518 \pm 0.922i$;	$-0.788 \pm 0.523i$	1.10	−0.933	$0.523 \pm 0.919i$;	$-0.793 \pm 0.515i$
21	$0.676 \pm 0.915i$;	$-0.708 \pm 0.521i$	1.23	−0.848	$0.659 \pm 0.924i$;	$-0.698 \pm 0.538i$
22	$-8 \times 10^4 \pm 1.37i$;	$-0.375 \pm 0.624i$	4.09	−0.462	–	–
23	$0.585 \pm 0.953i$;	$-0.634 \pm 0.632i$	1.22	−0.849	$0.676 \pm 0.909i$;	$-0.691 \pm 0.550i$
24	$-0.0834 \pm 0.986i$;	$-0.935 \pm 0.384i$	0.962	−1.01	$-0.0603 \pm 0.987i$;	$-0.960 \pm 0.318i$
25	$0.402 \pm 0.912i$;	$-0.679 \pm 0.739i$	0.993	−1.01	$0.533 \pm 0.841i$;	$-0.826 \pm 0.571i$
26	$-0.563 \pm 0.290i$;	$-0.820 \pm 1.35i$	−0.551	−7.61	–	–
27	$-0.357 \pm 0.347i$;	$-0.757 \pm 1.86i$	−0.344	−9.11	–	–
28	$0.433 \pm 0.981i$;	$-0.757 \pm 0.631i$	1.03	−0.980	$0.505 \pm 0.882i$;	$-0.825 \pm 0.535i$
29	$0.377 \pm 1.26i$;	-0.548; -1.05	–	–	–	–
30	$0.491 \pm 1.03i$;	$-0.708 \pm 0.516i$	1.29	−0.842	$0.534 \pm 1.02i$;	$-0.733 \pm 0.468i$
31	$0.246 \pm 0.998i$;	$-0.939 \pm 0.258i$	1.06	0.972	$0.197 \pm 1.01i$;	$-0.894 \pm 0.390i$
32	$-0.175 \pm 1.22i$;	$-0.669 \pm 0.458i$	2.49	−0.735	$-0.0657 \pm 1.31i$;	$-0.710 \pm 0.281i$
33	$-0.388 \pm 1.36i$;	$-0.550 \pm 0.446i$	9.81	−0.569	–	–
34	$-1.41 \pm 1.59i$;	-0.416; -0.533	–	–	–	–
35	$-0.244 \pm 0.707i$;	$-0.919 \pm 0.972i$	0.071	−1.87	–	–
36	$-0.127 \pm 1.51i$;	$-0.506 \pm 0.423i$	5.45	−0.536	–	–
37	$0.0512 \pm 1.20i$;	$-0.607 \pm 0.567i$	1.65	−0.749	$0.414 \pm 1.20i$;	$-0.685 \pm 0.389i$
38	$-0.021 \pm 0.855i$;	$-0.554 \pm 1.03i$	0.434	−1.63	–	–
39	$-0.503 \pm 1.01i$;	-1.63; -0.480	–	–	–	–
40	$-0.301 \pm 0.556i$;	-2.95; -0.850	–	–	–	–
41	$-0.465 \pm 0.730i$;	-4.70; -0.284	–	–	–	–
42	$-0.490 \pm 0.418i$;	$-0.321 \pm 1.52i$	3.01	−0.540	–	–
43	$0.0807 \pm 1.82i$;	-0.198; -1.52	–	–	–	–
44	$-0.623 \pm 4.18i$;	$-0.183 \pm 0.149i$	−0.183	−40.4	–	–
45	$0.314 \pm 1.52i$;	$-0.487 \pm 0.417i$	3.06	−0.536	–	–
46	$0.638 \pm 1.36i$;	-0.332; -1.33	–	–	–	–
47	$0.502 \pm 1.05i$;	-1.20; -0.614	–	–	–	–
48	$0.042 \pm 1.22i$;	$-0.593 \pm 0.569i$	2.00	−0.718	–	–
49	$0.170 \pm 1.07i$;	-1.95; -0.438	–	–	–	–
50	$-0.0478 \pm 1.41i$;	-1.38; 0.558	–	–	–	–

Table 3 (continued)

Sample no.	Adair zeros		v	u	MWC zeros	
51	$-0.558 \pm 1.37i$;	$-0.515 \pm 0.435i$	-0.510	-39.8	–	–
52	$-0.277 \pm 1.32i$;	-1.54; -0.356	–	–	–	–
53	$-0.387 \pm 0.872i$;	-3.33; -0.330	–	–	–	–
54	$-0.593 \pm 0.925i$;	-3.40; -0.243	–	–	–	–
55	$-0.498 \pm 0.158i$;	$-1.17 \pm 1.51i$	-0.491	-4.53	–	–
56	$-1.41 \pm 1.59i$;	-0.533; -0.416	–	–	–	–
57	$-0.434 \pm 0.145i$;	$-1.50 \pm 1.60i$	–	–	–	–
58	$-0.699 \pm 0.858i$;	-3.06; -0.267	–	–	–	–
59	$0.472 \pm 0.874i$;	$-0.843 \pm 0.551i$	0.988	-1.01	$0.483 \pm 0.868i$;	$-0.849 \pm 0.542i$
60	$0.523 \pm 0.848i$;	$-0.814 \pm 0.588i$	0.994	-1.00	$0.527 \pm 0.845i$;	$-0.829 \pm 0.567i$
61	$0.477 \pm 0.840i$;	$-0.665 \pm 0.793i$	0.936	-1.06	$0.598 \pm 0.748i$;	$-0.807 \pm 0.662i$
62	$0.579 \pm 0.885i$;	$-0.691 \pm 0.633i$	1.12	-0.913	$0.649 \pm 0.853i$;	$-0.734 \pm 0.619i$
63	$0.625 \pm 0.830i$;	$-0.701 \pm 0.659i$	1.06	-0.948	$0.664 \pm 0.802i$;	$-0.734 \pm 0.619i$
64	$0.667 \pm 0.928i$;	$-0.696 \pm 0.530i$	1.24	-0.841	$0.622 \pm 0.931i$;	$-0.693 \pm 0.535i$
65	$0.678 \pm 0.819i$;	$-0.714 \pm 0.612i$	1.10	-0.921	$0.681 \pm 0.817i$;	$-0.716 \pm 0.610i$
66	$0.385 \pm 1.11i$;	$-0.695 \pm 0.486i$	1.43	-0.806	$0.445 \pm 1.10i$;	$-0.695 \pm 0.486i$
67	$0.408 \pm 1.11i$;	$-0.708 \pm 0.464i$	1.42	-0.810	$0.446 \pm 1.10i$;	$-0.728 \pm 0.419i$
68	$0.383 \pm 1.05i$;	$-0.735 \pm 0.505i$	1.28	-0.862	$0.437 \pm 1.04i$;	$-0.769 \pm 0.437i$
69	$0.405 \pm 1.10i$;	$-0.715 \pm 0.464i$	1.40	-0.817	$0.442 \pm 1.10i$;	$-0.734 \pm 0.420i$
70	$0.393 \pm 1.06i$;	$-0.689 \pm 0.557i$	1.30	-0.846	$0.488 \pm 1.04i$;	$-0.746 \pm 0.450i$

stripped conditions and conditions of 2 mM DPG, the zeros appear to be intermediate between those of native hemoglobin (pattern E, fig. 1) and Hb(MalN) (pattern D, fig. 1). One of the conjugate pairs of zeros for Hb(AcAm), when stripped of phosphate, lies very nearly on the imaginary axis, having a real part which is only slightly positive. The modification in this case is a simple

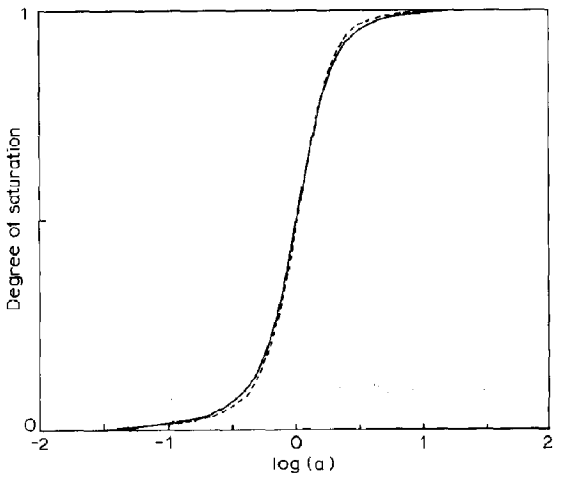

Fig. 4. Binding curves derived from the normalized Adair (———) and normalized MWC (- - - - - -) polynomials. (Same example as that used in fig. 3.)

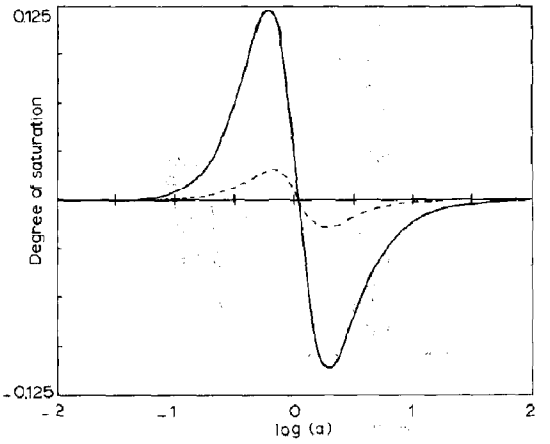

Fig. 5. Difference between binding curves derived from Adair and MWC polynomials (· · · · · ·), for a typical MWC-admissible case. Maximum difference between the binding curves (———), defined as the difference between the polynomials represented by the endpoints of the bold arc shown in fig. 3. (Same example as that used in fig. 3).

Table 4

Binding parameters for the unnormalized MWC binding polynominal, $M(x)$, and confidence levels in MWC applicability

κ_R and κ_T (units given by corresponding β_1) were calculated from eqs. 6 and 7, respectively, and t was obtained from positive solutions to eq. 9.

Sample no.	κ_R	κ_T	t	L	% confidence
1	3.79	0.159	4.05	2.56×10^{2}	97
2	3.38	0.0581	7.23	2.74×10^{3}	99
3	4.48	0.0652	9.10	6.85×10^{3}	92
4	3.67	0.0222	19.0	1.3×10^{5}	98
5	4.98	0.0900	8.81	6.02×10^{3}	99
6	5.62	0.0563	15.2	5.29×10^{4}	99
7	5.98	0.0291	25.4	4.13×10^{5}	100
8	5.84	0.0242	38.2	2.14×10^{6}	100
9	9.15	0.0163	88.5	6.15×10^{7}	99
10	6.85	0.0233	36.4	1.75×10^{6}	99
11	7.63	0.0205	43.5	3.60×10^{6}	99
12	5.23	0.0192	37.2	1.91×10^{6}	99
13	6.70	0.0172	62.4	1.52×10^{7}	99
14	4.49	0.0159	49.8	6.17×10^{6}	100
15	3.79	0.0138	31.5	9.85×10^{5}	98
16	5.76	0.00859	77.2	3.55×10^{7}	97
17	1.05	0.00669	46.3	4.60×10^{6}	98
18	1.48	0.0118	25.5	4.23×10^{5}	99
19	0.0682	0.00406	8.52	5.27×10^{3}	99
20	4.34	0.113	7.94	3.97×10^{3}	99
21	5.68	0.0132	82.9	4.72×10^{7}	98
23	75.4	0.0230	589	1.20×10^{11}	94
24	1.80	0.348	2.22	2.43×10	96
25	1.52	0.0302	6.93	2.31×10^{3}	92
28	0.830	0.0229	6.48	1.76×10^{3}	95
30	0.991	0.0217	12.9	2.77×10^{4}	96
31	0.536	0.0583	3.22	1.08×10^{2}	95
32	3.30	0.353	6.09	1.38×10^{3}	97
37	2.12	0.0548	16.5	9.41×10^{4}	95
59	1.18×10^{7}	3.56×10^{5}	5.60	9.82×10^{2}	98
60	1.56×10^{7}	3.27×10^{5}	6.77	2.10×10^{2}	99
61	9.40	0.043	9.59	8.46×10^{3}	91
62	2.83	0.0150	26.0	4.57×10^{5}	96
63	8.89	0.0281	29.0	7.07×10^{5}	97
64	12.3	0.0186	131	2.94×10^{8}	100
65	9.39	0.0161	60.5	1.34×10^{7}	100
66	1.76	0.0605	10.6	1.26×10^{4}	95
67	1.35	0.0465	10.4	1.17×10^{4}	97
68	0.720	0.0309	7.44	3.06×10^{3}	95
69	0.785	0.0288	9.69	8.82×10^{3}	97
70	0.623	0.0193	9.87	9.48×10^{3}	91

alkylation of the same cysteine that is modified with *N*-ethylsuccinamide in the Hb(MalN).

A summary of the patterns of zeros for the chemically modified hemoglobins is provided by fig. 7. The progression of cooperativity (as measured by the maximum Hill slope) for the sequence, native Hb: Hb(AcAm): Hb(MalN): Hb(CPase), runs from a high of 3.09 to a low of 1.15 [17]. In fig. 7 the patterns for these hemoglobins under stripped conditions are shown on

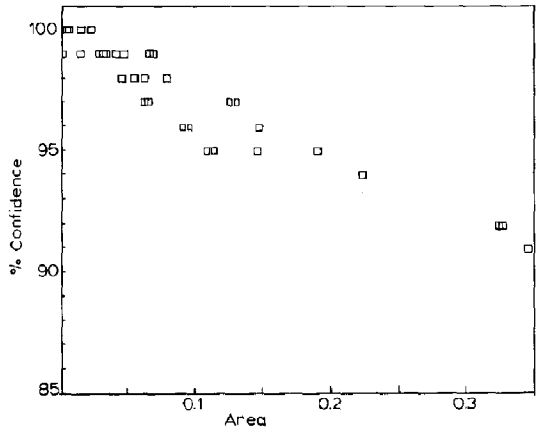

Fig. 6. A plot of the % confidence in the applicability of the MWC model vs. the area between normalized binding curves representing the data (Adair) and the MWC description for all MWC-admissible cases. The areas have been normalized to that area bounded by the binding curves derived from the extreme possible admissible cases, as described in the text.

the left and those in the presence of the effector molecule DPG on the right. The patterns of zeros can be seen to reveal the progressive drop in cooperatively as they change from a pattern like that of fig. 1E to that of fig. 1D. From top to bottom in fig. 7, the hemoglobins are placed to reflect the increasing severity of chemical alterations to the interface region between the two $\alpha\beta$ dimers. In each case except the last (Hb(CPase)), the addition of DPG results in the reestablishment of functional linkage between pairs of interacting sites. The last of these cases is the hemoglobin partially digested by carboxypeptidase A. This modification is known to destroy some of the residues normally involved in DPG binding. Taken together, the patterns of zeros for these cases strongly suggest identification of the $\alpha\beta$ dimers as the interacting pairs of sites.

Another subset of the binding data collected here is the data from the carp hemoglobins, and hybrids constructed from subunits of carp and human hemoglobins, the so-called 'mermaid' hemoglobins. In the majority of these cases the MWC model is not admissible, but again, in the nonadmissible cases information regarding site linkage can be obtained from an examination of the zeros alone.

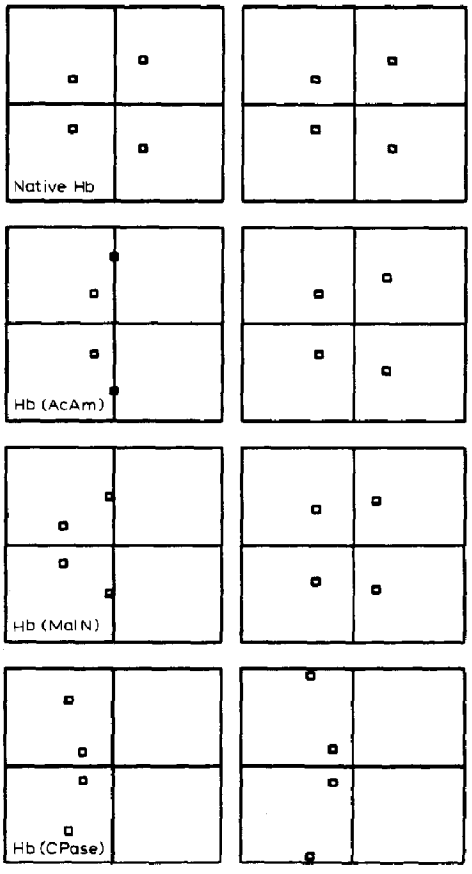

Fig. 7. Patterns of zeros of the normalized Adair binding polynomial for native human hemoglobin, pH 7.4, 25°C (sample nos. 20 and 21), and for human hemoglobins chemically modified in the β-chains (sample no. 22–27). Patterns in the left column correspond to phosphate-stripped conditions; those in the right column correspond to conditions of 2 mM DPG.

As regards the MWC model, only five of the 28 carp samples analyzed here yield Adair binding polynomials that are MWC-admissible. Thus, an analysis of the zeros of the binding polynomials indicates that the MWC model is inappropriate for these cases. Chien and Mayo [19] reported that nonlinear least squares fits of the two-state MWC model to oxygen-binding data for carp Hb at certain pH values failed to converge. Similarly, for the hybrid hemoglobins, a non-linear least-squares analysis did not provide well-determined model parameters for the two-state MWC model [20].

The zeros for hybrid I (set no. 43) include two reals (pattern C in fig. 1), a nonadmissible pattern. In attempting to explain the data, the investigators extended the MWC model to include a third allosteric form. However, it can be shown that the zeros of the MWC model, regardless of the number of allosteric forms, cannot have real zeros in the four-site case. Thus, the three-state model is inappropriate as well.

The analysis of zeros of binding polynomials describing binding curves performed on carp hemoglobin at a series of pH values provides insight into the so-called 'Root effect', analogous to but greater in extent than the Bohr effect in human hemoglobin. In phosphate buffer with the effector molecule IHP present, the binding polynomial factors into two quadratics at low pH (6.9, 7.4), suggesting two pairs of interacting sites (see fig. 8). However, at pH 7.7, in the same buffer and under the same effector conditions the binding polynomial produces no positive factorization, indicating four interacting sites. At pH 8.0, two pairs of interacting sites are again suggested; and at still higher pH values (8.3, 8.6) the binding polynomial factors into two linear terms and a quadratic term. There are two ways to interpret linear factors (associated with distinct real zeros): either the two identical sites are linked anticooperatively or the two sites are heterogeneous in their binding affinities. Thus, under the latter pH conditions, the carp hemoglobin has either two heterogeneous, independent sites and a pair of cooperative sites or it has a pair of negatively cooperative sites and a pair of cooperative sites. The Hill slope for these data is reported to be 0.98. This suggests that the effect of the positively cooperative pair of sites negates the influence of the other pair to give rise to noncooperative binding overall, in the presence of the effector molecule IHP.

The binding polynomials for carp Hb in phosphate buffer in the absence of IHP at pH 6.6 can be factored into a cubic and a linear term suggestive of an independent site and a group of three interacting sites (see fig. 9). As the pH is increased to around 7 all four sites become linked. At higher pH values (7.7, 7.8) there are seen to be two pairs of interacting sites. As the pH is raised still further to 9.1, a pair of interacting sites and a pair of sites which are heterogeneous and independent or are anticooperative are revealed. Similar behavior is exhibited at different temperatures, as can be seen from fig. 10.

The overall picture of the pH dependence of oxygen binding to carp hemoglobin is that at intermediate pH the factorability of the binding polynomial indicates more extensive linkage between sites, while the extent of site interaction is reduced to pairs of sites or independent sites at low and high pH. Since the degree of factorization of the binding polynomial imposes limits on the observed cooperativity, we expect to see this reflected in the values of the Hill slopes as a func-

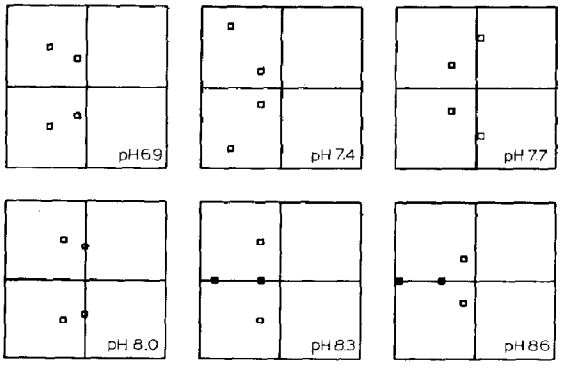

Fig. 8. Patterns of zeros of carp hemoglobin at various pH values with IHP present (sample nos. 35–40).

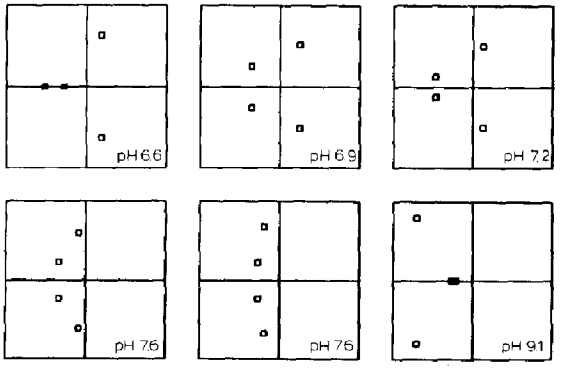

Fig. 9. Patterns of zeros for carp hemoglobins at various pH values in the absence of IHP (sample nos. 29–34), 25°C.

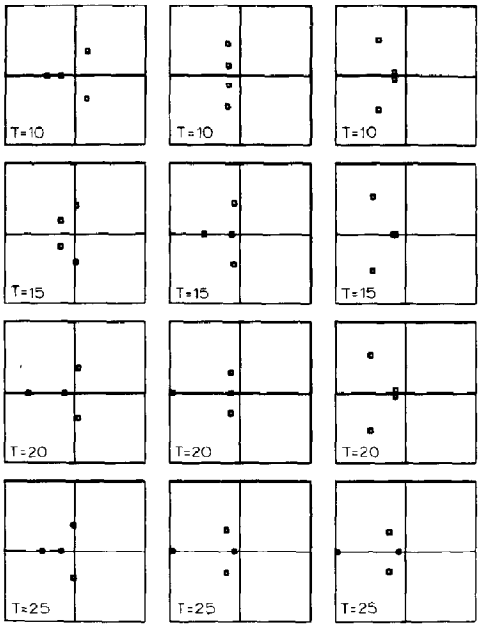

Fig. 10. Patterns of zeros for carp hemoglobin at various temperatures (sample nos. 47–58). Patterns in the left column correspond to pH 7.3; those in the center column to pH 8.3; and those in the right column to pH 9.1.

tion of pH, as is in fact observed. The distinct real zeros arising from the carp data immediately rule out the application of the simple MWC model.

We feel that the examples presented here demonstrate that the analysis of simple mathematical properties of binding polynomials can provide insight into macromolecular ligand-binding processes. The MWC model is particularly amenable to further analysis, enabling one to obtain a quantitative, probabilistic measure of its applicability. However, in a model-independent manner, the factorization of the Adair binding polynominal enables one to determine the grouping of site-site interactions in the macromolecule. This information can itself provide a guide for the selection of an appropriate mechanistic model.

Acknowledgements

This work was supported by National Institute of Health Grants HL 22325 (S.J.G.).

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