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Nonlinear optical effects in oxygen-binding reactions of hemoglobin A_0

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Optical spectra have been taken in the Soret band (440–400 nm) under different oxygen partial pressures for hemoglobin (Hb) A_0 at pH 7.0, 15°C, 2–3 mM heme, 30 mM inositol hexaphosphate, 0.1 Hepes and 0.1 M NaCl. Application of the matrix method of singular value decomposition (SVD) to the difference spectra for different oxygen pressures shows the presence of at least two distinct optical transitions. From this result one concludes that the optical response to oxygen binding is nonlinear in the Soret band. The degree of nonlinearity has been determined by fitting the data at different wavelengths to the four-step reaction Adair equation with the inclusion of optical parameters that describe the intermediate oxygenated species. It is found that the data are well-represented by two optical parameters at each wavelength, one which represents the optical change for the addition of the first and second oxygen molecules and the other which corresponds to the change for the addition of the third and fourth oxygen molecules. The ratio of these optical parameters depends only moderately upon wavelength with an average value of 0.8 over the Soret band. Thus, there is an approx. 20% smaller optical response for the first two ligated species than that for the last two ligated species. The overall Adair equilibrium constants are evaluated as follows: $\beta_1 = 0.081 \pm 0.003$ Torr^{-1}, $\beta_2 = 2.53 \times 10^{-3} \pm 2.4 \times 10^{-4}$ Torr^{-2}, $\beta_3 = 1.25 \times 10^{-5} \pm 1.0 \times 10^{-6}$ Torr^{-3}, $\beta_4 = 1.77 \times 10^{-6} \pm 1.5 \times 10^{-7}$ Torr^{-4}.

1. Introduction

The determination of high-precision ligand binding curves to hemoglobin has been accomplished in recent years by the use of spectrophotometric measurements as a function of ligand concentration [1–3]. Results from our laboratory using a thin-layer equilibrium optical method [4] with concentrated hemoglobin solutions have led to an unexpected observation that triply ligated species are only slightly populated at all stages of the ligand binding curve. These findings do not agree with results obtained in general from other laboratories and consequently have instigated further investigation into possible sources of the discrepancy, for example, analysis of residuals in data fitting (Di Cera, personal communication).

A key assumption in all of these studies is that the optical absorbance of hemoglobin depends linearly upon the extent of ligand binding. Deviation from linearity has been suggested some years ago in a brief report by Rifkind and Lumry [5], and has been indicated from careful examination of 'isosbestic' regions at different degrees of binding [6,7]. However, in these most recent studies, the extent of the deviation was of a nature to suggest that linearity would closely be followed in regions far from the isosbestic points. Indeed, binding studies, carried out at different wavelengths where large optical changes occur upon ligation, have been found within experimental error to be independent of the wavelength used [8]. These results are consistent with the near isosbes-

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tic behavior that is observed in intermediate stages of ligation. The implication is that, to a very good approximation, only two optical states are needed to describe the system. On the other hand, recent optical measurements at two wavelengths as a function of oxygen activity measured from spectral changes of added myoglobin have led Parkhurst [9] to conclude that nonlinear effects are significant.

However, though it is not generally considered, it is also possible to obtain isosbestic behavior for systems of more than two optical states where the states (absorbance vs wavelength) scale with different proportionality factors. The binding curves obtained for such systems would be independent of wavelength. With three or more optically distinct species there would be a nonlinear optical response to ligand binding, and thus the determination of the extent of reaction based upon a proportional absorbance change would be in error.

Shrager [10–12] has applied the matrix method of singular value decomposition (SVD) to the problem of mixtures of several reacting species and has shown that the minimum number of transitions, necessary to decribe the system, can be determined even though the transitions or the associated spectral features may be quite similar. The method requires that under each set of experimental conditions (pH, e.m.f., p_{O_2}, time, etc.) a spectrum be collected over a wavelength range sufficient to include the spectral change associated with the reaction of each point. In this paper, we have applied SVD analysis to oxygen binding in HbA_0 to determine whether more than two optical states are required to describe the optical behavior of hemoglobin as a function of the degree of oxygenation.

Guided by the SVD results we have performed a nonlinear least-squares analysis of the differential optical binding curve obtained at various wavelengths as a function of changes in oxygen partial pressure, recognizing the optical characteristics of each stoichiometrically ligated species. The Adair constants are found to be well resolved along with optical parameters that show clear differences between the optical features of the first two and last two ligated states.

2. Theory

2.1. Singular value decomposition

The theory of the singular value decomposition of a matrix [13,14], and its application to the analysis of a multiple-component system [10–13] have been discussed in depth elsewhere; only a brief summary of the method will be given here.

The data consists of the spectra of the sample taken at different oxygen partial pressures. It is expressed as a matrix $\boldsymbol{A}$, of m rows, each of a different wavelength, and n columns, each of a different degree of reaction. We have constructed the columns of this matrix by taking the difference of the spectra between two degrees of saturation. Each column is then a stepwise difference spectrum, and $\boldsymbol{A}$ will approximate the derivative of the optical binding curve. Typically, in our experiments, $\boldsymbol{A}$ consists of 81 wavelengths (rows) per spectrum taken at 16 different p_{O_2} (columns). The SVD of $\boldsymbol{A}$ is written as the product of three matrices

$$\boldsymbol{A} = \boldsymbol{U}\boldsymbol{S}\boldsymbol{V}^{\mathrm{T}} \tag{1}$$

where $\boldsymbol{U}$ is an $m \times n$ orthogonal matrix, $\boldsymbol{V}$ is an $n \times n$ orthogonal, and $\boldsymbol{S}$ is an $n \times n$ diagonal matrix containing the singular values of $\boldsymbol{A}$, with elements $s_{1,1} > s_{2,2} > \ldots \gg s_{n,n}$. In the ideal case of no noise in $\boldsymbol{A}$, the number of nonzero diagonal elements in $\boldsymbol{S}$ indicates the rank, r, or number of linearly independent transitions or components necessary to generate $\boldsymbol{A}$. In the case of real data, all the diagonal elements of $\boldsymbol{S}$ will be positive, although those solely attributable to noise will be very small. If the contribution to $\boldsymbol{A}$ by a component is small, it may be difficult to determine the rank of $\boldsymbol{A}$ from examination of the elements of $\boldsymbol{S}$. Shrager [12] has shown that the number of significant $s_{i,i}$ can be estimated by choosing r such that

$$\sum_{i=r+1}^{n} s_{i,i}^2 \leq mn\sigma^2 < \sum_{i=r}^{n} s_{i,i}^2 \tag{2}$$

where m is the number of rows of $\boldsymbol{A}$, n the number of columns of $\boldsymbol{A}$, and σ^2 the average variance in $\boldsymbol{A}$.

The eigenvectors U and V are obtained from AA^T and A^TA, respectively [15]. This means that the columns of U are linear combinations of the independent difference spectra of each component in A, and the columns of V are linear combinations of the transitions of each component. Those columns of U and V which have very small corresponding singular values contribute primarily noise to A, while the first r columns contain the spectra and transitions of interest. In other words, a matrix A' constructed from the first r columns of U and V and the first r diagonal elements of S will differ from the original A only by noise. Often, however, the rank of A is not determined unambiguously from eq. 2 (e.g., when the variance in the data is not constant within a row or column). The autocorrelation coefficients (A.C.) of the columns of U and V, given as follows:

$$\text{A.C.}_{U\,\text{col}\,j} = \sum_{i=1}^{m-1} u_{i,j}u_{i+1,j};\ \text{A.C.}_{V\,\text{col}\,j} = \sum_{i=1}^{n-1} v_{i,j}v_{i+1,j} \tag{3}$$

are an additional tool for determining r.

The autocorrelation coefficient is essentially a measure of the high-frequency noise vs low-frequency signal for each column. The smoother the column, the larger the A.C.; generally, a column can be regarded as primarily noise-containing if A.C. < 0.5. Eqs 2 and 3 combined yield the effective rank of A.

Recovery of the difference spectra or transition curve of each component from U and V requires fitting the elements of the first r columns of each matrix with some reasonable model for each transition or spectrum with parameters that determine the amount of each component present in the column. In general, the fitting function will be of the form

$$V\,\text{col}\ j = H_{0,j} + H_{1,j}f_1(j) + \ldots + H_{r,j}f_r(j) \tag{4}$$

The Hs are parameters which determine the linear combination of the model defined transition curves that best describe V. The $f(j)$s are the functional values which account for the physical behavior of the system. The columns are best fitted simultaneously [15], minimizing the number of parameters to be fitted as well as computation time.

2.2. Adair analysis of optical response

The overall binding reactions, known as the Adair equations, can be written as

$$\text{Hb} + i\text{O}_2 \rightarrow \text{Hb}(\text{O}_2)_i \tag{5}$$

Each of these equations has an equilibrium constant denoted by β_i, and each has an average change in molar optical absorbance per mole of oxygen bound, denoted by $\Delta\bar{A}_i$, which in general depends upon the wavelength. The total absorbance at a given partial pressure, x, with reference to the value at a zero partial pressure is then given by

$$A(x) = A(0) + \left[\Delta\bar{A}_1\beta_1 x + 2\Delta\bar{A}_2\beta_2 x^2 + 3\Delta\bar{A}_3\beta_3 x^3 + 4\Delta\bar{A}_4\beta_4 x^4\right] / \left[1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4\right] \tag{6}$$

It is convenient to scale the intermediate absorbance changes to the overall $\Delta\bar{A}_4$ value as follows: $\Delta\bar{A}_i = (1 + \delta_i)\Delta\bar{A}_4$ with $\delta_4 = 0$. The result is given as follows:

$$\Delta A = A(0) + \left\{\left[(1+\delta_1)\beta_1 x + 2(1+\delta_2)\beta_2 x^2 + 3(1+\delta_3)\beta_3 x^3 + 4\beta_4 x^4\right] / \left[1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4\right]\right\}\Delta\bar{A}_4 \tag{7}$$

The difference in optical absorbance between two spectra taken at different partial pressures, x_j and x_{j-1}, is then obtained from eq. 7 as $\Delta A_j = A(x_j) - A(x_{j-1})$. In the case of interest using the thin-layer technique, $x_j = x_{j-1}D$, where D is a constant dilution factor of the cell, and thus $\Delta \ln x = -\ln D$; the change ΔA_j will be a close equivalent to the derivative of the optical binding curve given by eq. 7; $A(\xi)$ vs $\ln \xi$, where ξ is given by the geometric mean of the end partial pressures of the step. The absorbance difference is then given to a reasonable approximation [16] by the first term in the Taylor expansion evaluated at ξ_j. In

practice, the data was actually fitted to the difference equation given by the full expression of eq. 7.

The degree of reaction θ is given by

$$\theta = \frac{1}{4}\frac{\beta_1 x + 2\beta_2 x^2 + 3\beta_3 x^3 + 4\beta_4 x^4}{1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4} \quad (8)$$

A linear optical response to θ is obtained when the δ_is are zero. Isosbestic behavior will be observed when the δ_is are independent of wavelength, but clearly the optical response will then be nonlinear with θ, even though an SVD analysis would reveal only one independent transition.

3. Materials and methods

HbA_0 was prepared according to the method of Williams and Tsay [17], concentrated by pressure ultrafiltration, and stored in deionized water in liquid N_2. Final solutions were prepared by mixing the deionized water-Hb solution with concentrated buffer, and were reduced using the enzyme system of Hayashi et al. [18] at 6°C for 12 h, the components of which were obtained from Sigma. The solution conditions were: 0.1 M Hepes, 30 mM inositol hexaphosphate, 1 mM EDTA, 0.1 M chloride, pH 7.0; and 2–3 mM heme. These conditions have been found to maximize sample stability against met-Hb formation [2], and also to maximize the populations of intermediate ligation states. A temperature of 15°C was chosen to give an optimum balance between relatively short equilibration times between dilution steps and minimal met-Hb formation during the course of the experiment. Very high solution stability is mandatory in order to obtain data of sufficient quality for the detailed analysis of the binding curve in terms of optical and binding constant parameters.

Optical spectra were measured with an Aviv 14DS Spectrophotometer and a thermostated thin layer cell described previously [4]. Sample layer thickness was approx. 0.005 cm. A precision gas dilution valve ($D = 0.602$ or 0.595) was used to vary the oxygen partial pressure from an initial value determined by atmospheric pressure minus vapor pressure of water.

In a typical experiment, the HbA_0 sample in the thin-layer cell was equilibrated with an O_2 atmosphere and a spectrum was collected from 440 to 400 nm, every 0.5 nm. This wavelength range includes the near isosbestic point between the oxy-deoxy peaks in the Soret region, and was chosen (1) to allow use of very thin sample layers with high optical absorbance, (2) to take advantage of large positive and negative absorbance changes upon oxygenation and (3) to coordinate these studies with previous determinations of the 421 nm 'isosbestic' point [7]. Furthermore, the known met-Hb peak at 405 nm provides a sensitive means to assess whether significant met formation occurred during the course of the experiments. A stepwise dilution of the cell atmosphere with N_2 is then made. The approach to equilibrium under the new p_{O_2} is monitored at 415 nm; at the new equilbrium another spectrum is collected, and the next dilution step made. When no further change in absorbance is observed, the sample is reoxygenated and a final spectrum is collected. Typical recovery of the original absorbance spectra was better than 98%.

Singular value decompositions and nonlinear least-squares calculations were performed in the Gauss 2.0 operating system (Aptech Systems, Kent, WA) for the AT&T PC 6300, which also controls the Aviv 14DS. Parameters for the functions fitted to the columns of V were estimated using the Gauss-Newton method for least-squares minimization as modified by Marquardt [19], and with the method of Lawton and Sylvestre [20] for eliminating linear parameters from a nonlinear fitting function, which analytically calculates the best linear parameters at each successive estimation of the nonlinear parameters. This was found to be especially useful as an aid to convergence when simultaneously fitting all the wavelengths in A, where there is a small number of nonlinear parameters common to all wavelengths (the equilibrium binding constants) and a large number of wavelength-specific linear parameters (change in absorbance and the δs).

The different wavelengths in A were fitted to two special cases of eq. 7: (1) the δs were set to zero so that $\Delta\bar{A}_4 = \Delta\bar{A}_3 = \Delta\bar{A}_2 = \Delta\bar{A}_1$ and thus linearity of optical response is assumed; (2) $\delta_1 = \delta_2$

and $\delta_3 = 0$. In the second case, the first and second ligation states are described by $\Delta\bar{A}_4(1+\delta)$ and binding of the third and fourth ligands is described by $\Delta\bar{A}_4$. The wavelength dependence of δ was examined by comparing the fit obtained when δ was allowed to vary with wavelength and when it was constrained to a single proportionality constant for all wavelengths. Error estimation of parameters at the 67% confidence level was made by use of the linear approximation of the curvature matrix [21].

An attempt was made to fit the general case in which all three δs were not constrained. This situation showed unacceptably high correlation between the δs and the βs, making resolution of these parameters difficult. Also, the inclusion of all δs was not found to improve the fit significantly.

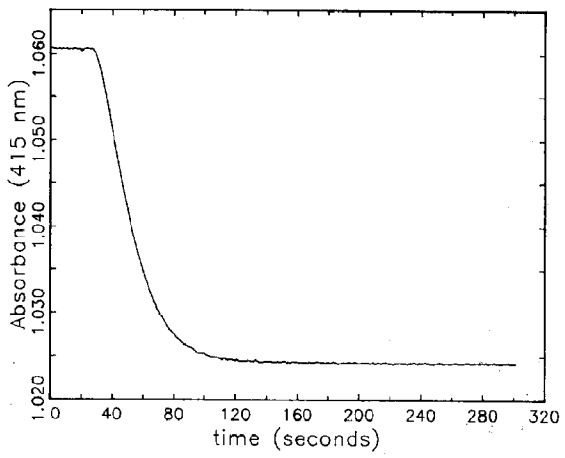


Fig. 1. A representative dilution step in the equilibrium binding curve, monitored at 415 nm. Solution conditions: 0.1 M Hepes, 30 mM IHP, 1 mM EDTA, 0.1 Cl^-, pH 7.0; and 2–3 mM heme.

4. Results

The attainment of equilibrium and the optical stability of each stepwise change in partial pressure is indicated by the time course of the absorbance at a typical wavelength (415 nm) and is illustrated for the solution conditions selected for the present studies in fig. 1. The constant absorbance at the end of each step verifies the attainment of equilibrium and also indicates the high stability of the sample to the imposed oxygen partial pressures.

A complete data set collected in a typical experiment is shown in fig. 2a as the spectrum measured at each oxygen partial pressure over the

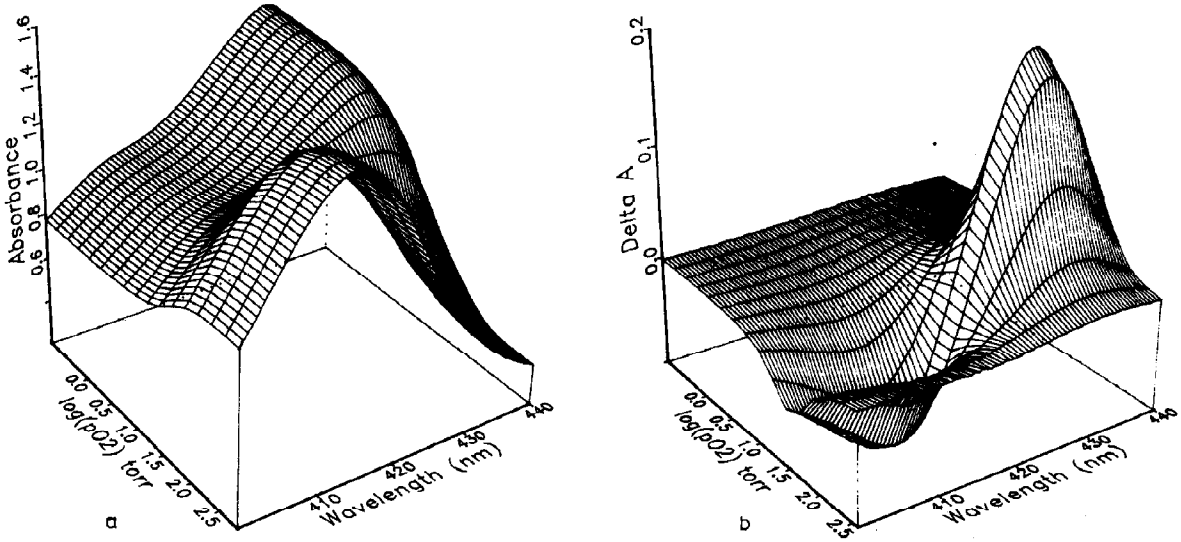


Fig. 2. (a) A representative data set consisting of the spectra collected from 440 to 400 nm, every 0.5 nm, at each O_2 partial pressure over the binding curve. (b) The data in panel a in the stepwise difference form of A.

wavelength range 440–400 nm. The data was then put into the form of the stepwise difference matrix A as shown in fig. 2b for further analysis. As a check upon sample degradation, at the end of the experiment the sample was reoxygenated and a final spectrum compared with the initial spectrum. Met-Hb formation, as estimated from the change in absorbance at 405 nm, was found to be less than 2% in all cases.

A critical triplicate set of experiments was obtained upon identical starting material prepared to the same solution conditions in order to ensure reproducibility. The SVD of a representative data set is presented in fig. 3 and the results for all three experiments are summarized in an abbreviated form in table 1. The analyses of the results for all three experiments correspond closely to one another. The autocorrelation coefficients for the columns of U and V indicate the presence of three significant components in A; the values of the $\Sigma_{i=r}^{n} s_{i,i}^2$ indicate the rank of A to be at least two but no more than three by the criteria of eq. 2. Examination of the first columns of U and V in fig. 3 clearly indicates the presence of signal in the first three columns, while the fourth and higher columns are predominantly noise. As a further test of the value of r, the reduced matrix A' was constructed with $r = 1$–3 and compared with the original A. The difference between A' and A for $r = 1$–3 is shown in fig. 4 as the deviation for each of the 16 spectra. It is apparent that $r = 1$ is not an adequate description of the system, and that the differences are systematic and not merely a larger random error. It is important to note here that if the assumption of linear optical response is valid then only one significant component would be sufficient.

Table 1

First five autocorrelation coefficients for the columns of U, V and the $\Sigma_{i=r}^{n} s_{i,i}^2$ for the SVD of data in fig. 2b

	Column (i)	A.C. of U col i	A.C. of V col i	$\Sigma_{i=r}^{n} s_{i,i}^2$
Data set 1	1	0.9944	0.9001	2.877
	2	0.9907	0.4606	0.00143
	3	0.9867	0.8045	0.00058
	4	0.7909	−0.0094	0.00013
	5	0.3752	−0.6598	8.63×10^{-5}
Data set 2	1	0.9966	0.9020	2.718
	2	0.9863	0.6595	0.00127
	3	0.9790	0.6423	0.00043
	4	0.8628	0.0805	0.00025
	5	0.4737	−0.6600	0.00018
Data set 3	1	0.9979	0.9040	2.579
	2	0.9923	0.7909	0.00122
	3	0.9821	0.5907	0.00045
	4	0.5306	−0.5662	0.00014
	5	0.5657	−0.1444	0.00011

These results show in a general, model-independent manner the presence of nonlinear optical

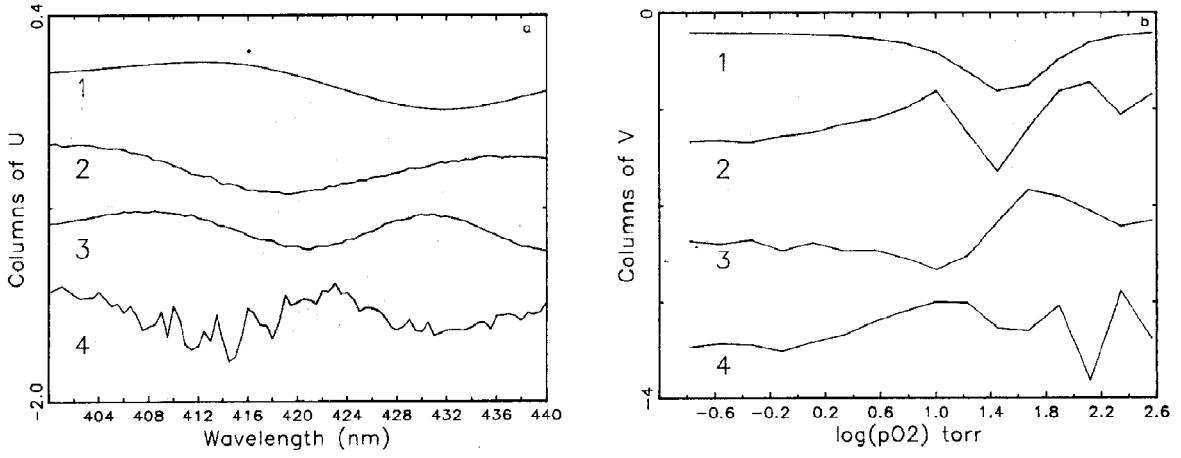
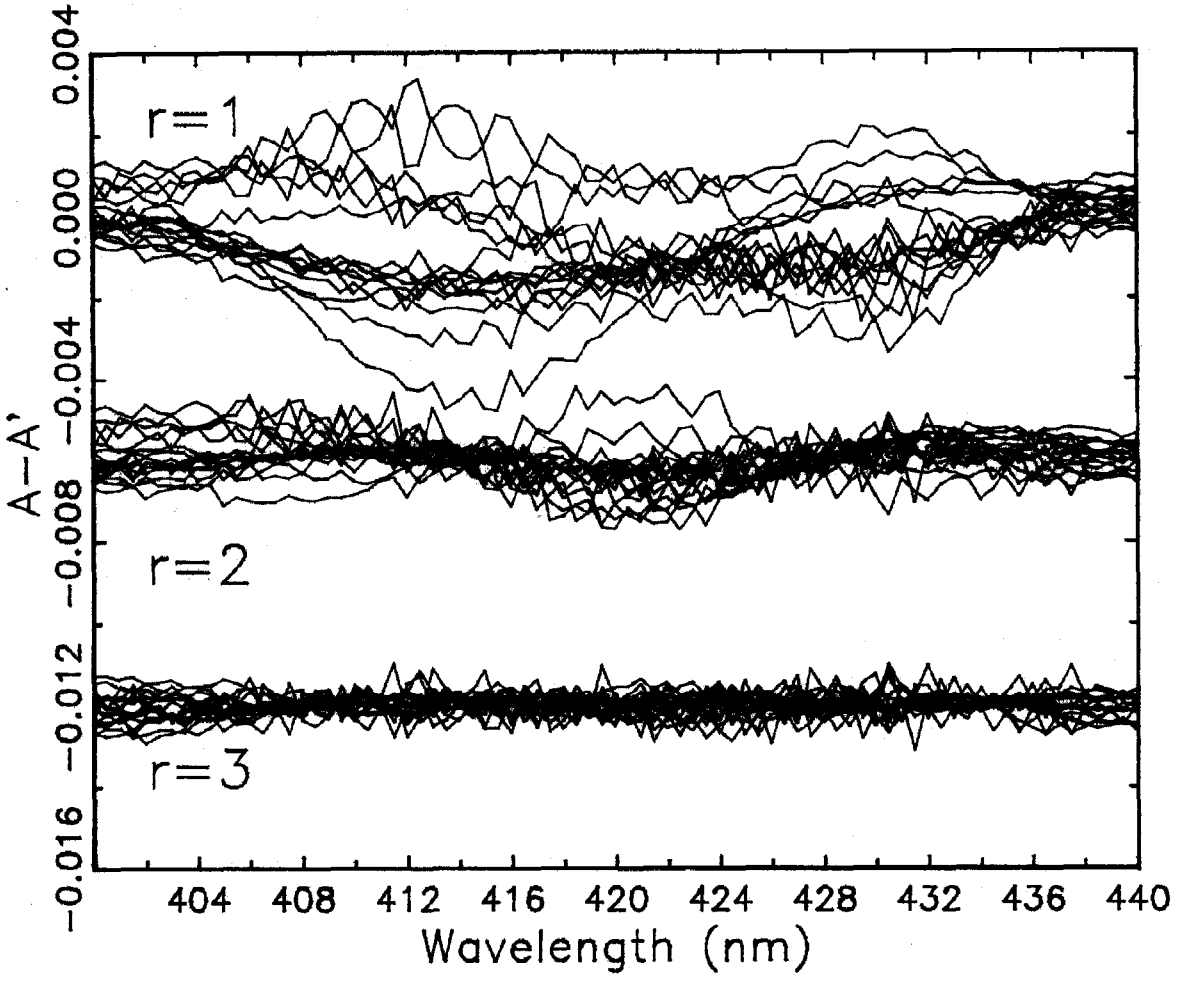


Fig. 3. (a) The first four columns of U from the SVD analysis of data set 1. (b) The first four columns of V from the SVD analysis of data set 1. Columns offset for clarity.



response to the degree of oxygen binding. In order to ascertain the possible molecular basis of the SVD analysis it is necessary to postulate molecular species with three different optical characteristics. The principal component is presumed to be the main change of absorbance upon oxygenation of any heme group, irrespective of whether it is located in α- or β-subunits or in different quaternary states of the hemoglobin molecule. The second optical component is assumed to arise due to small optical differences among the subunits or quaternary states of the molecule. Such a possibility is perhaps indicated by structural observations that suggest the first two stoichiometric oxygenated species are characterized by oxygen bound to the α chains in the T quaternary form, while the last two oxygenated species would be predominantly in the R quaternary form with oxygen added to the β chains [22,23]. The third and smallest component is more difficult to rationalize, but is possibly attributable to the small amount of methemoglobin which is inevitably formed during the experiment.

The equations that describe the optical response of the three components to changes in oxygen partial pressure indicated by $\Delta\{\}$ are given

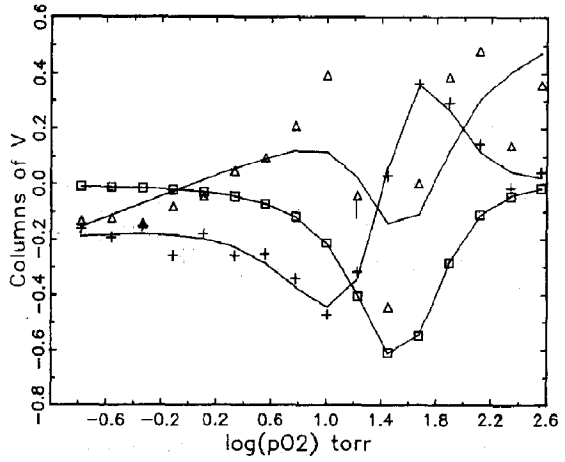


Fig. 5. Least-squares fit to the first three columns of V using eqs 9–11 in eq. 4. (□) Column 1, (△) column 2, (+) column 3.

from application of eq. 6 as follows:

$$f_1 = \Delta\left\{\frac{\beta_1 x + 2\beta_2 x^2 + 3\beta_3 x^3 + 4\beta_4 x^4}{1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4}\right\}\Delta\bar{A}_4 \quad (9)$$

$$f_2 = \Delta\left\{\frac{\beta_1 x + 2\beta_2 x^2}{1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4}\right\}\delta\,\Delta\bar{A}_4 \quad (10)$$

$$f_3 = a + b\frac{\log(x_i/x_0)}{\log D} \quad (11)$$

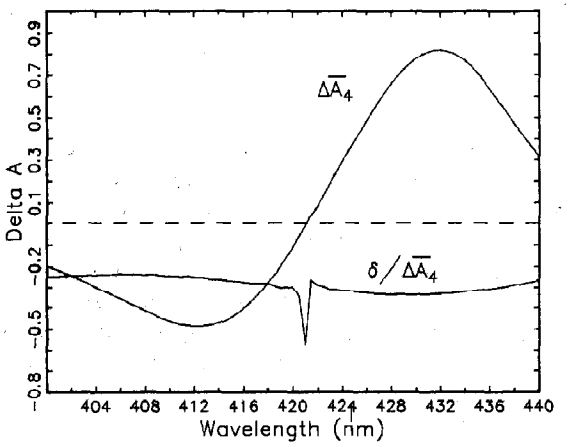
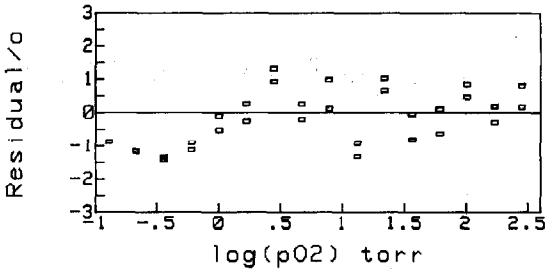


Fig. 6. $\Delta\bar{A}_4$ and $\delta/\Delta\bar{A}_4$ obtained at each of the 81 wavelengths in the global fit of data set 1 to eq. 7 with $\delta = \delta_1 = \delta_2$ and $\delta_3 = 0$.

where the last expression formulates the small growth of met formation with the i-th dilution step, proportional to the time from the initiation of the experiment. The first three columns of the V matrix are then fitted using these functions in eq. 4 and the results of these least-square determinations are shown in fig. 5. The fit for the first two components is very good and the third is adequate in view of the noise of the data. Thus, this model provides a suitable representation of our observations. However, in view of the presence of linear coefficients in eq. 4, one cannot determine the parameters δ and $\Delta\bar{A}_4$.

The question remains as to the magnitude and wavelength dependence of the nonlinearity parameters defined in section 3. The matrix A was fitted globally such that each of the 81 wavelengths was a complete binding curve, with data-set-specific optical parameters and the equilibrium binding



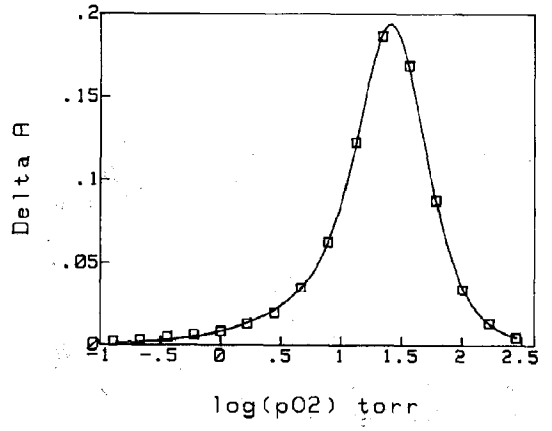


Fig. 7. Fit of data set 1 at a single wavelength (430 nm) for the linear case and for $\delta = -0.20$. The two curves are superimposable to within the width of the line. Residuals (top of figure) are normalized to $\sigma = 0.001$.

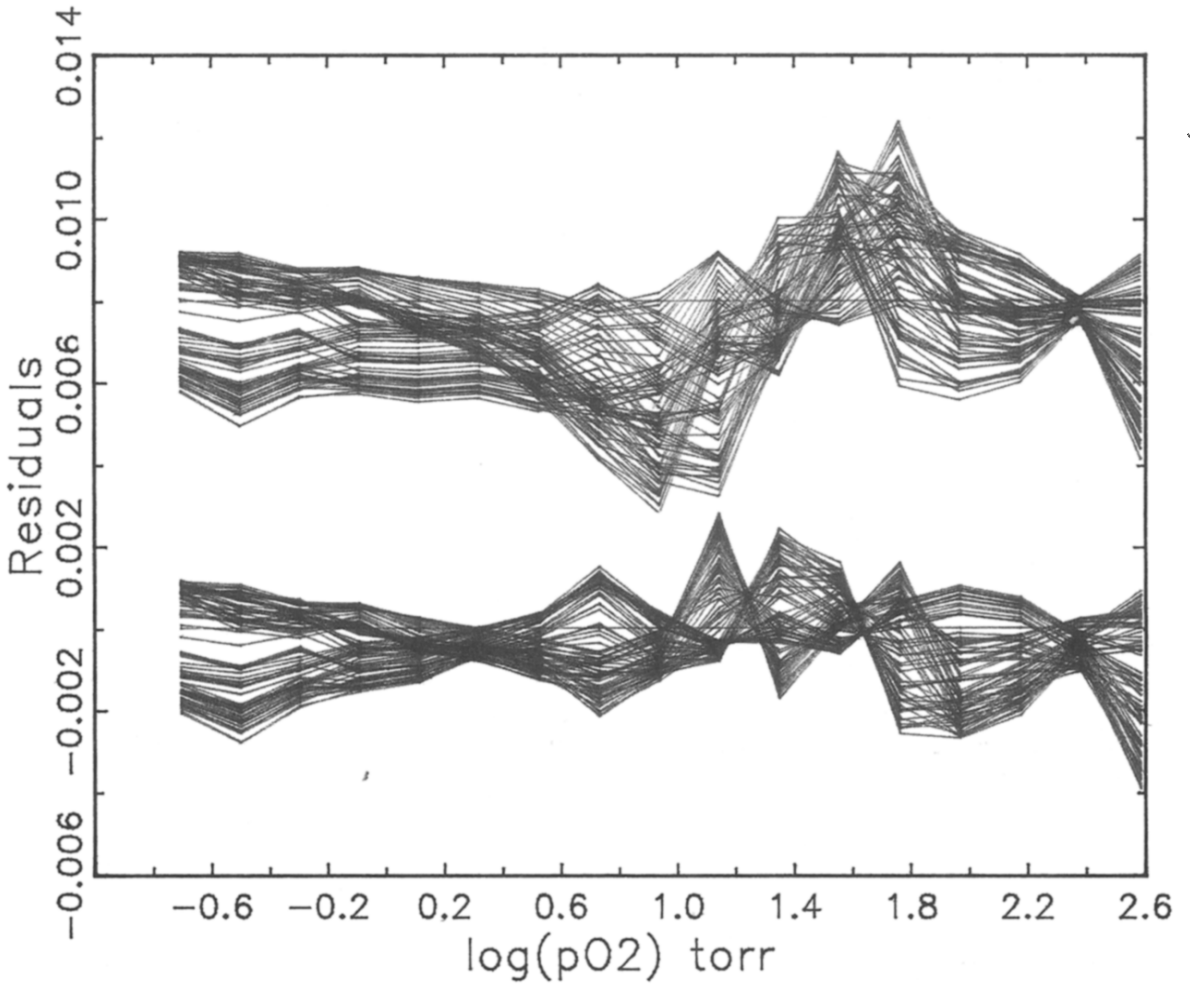


Fig. 8. Comparison of the residuals of the global fit of data set 1 (81 different wavelengths as a function of 16 oxygen partial pressure changes) to eq. 6 with linearity assumed ($\delta = 0$) and with non-linearity allowed as $\delta = \delta_1 = \delta_2$ and $\delta_3 = 0$. Residual offset 0.008 in linear case for clarity.

constants, the βs, common to all data sets. Several modelling situations were examined. The three most informative cases are: (1) where linearity is assumed ($\delta = 0$); (2) where a wavelength-independent nonlinearity parameter, determined by $\delta = \delta_1 = \delta_2$ and $\delta_3 = 0$, is examined; and (3) where a wavelength-dependent parameter δ for the last situation is explored. More elaborate situations did not reveal meaningful resolution of parameters. The results are summarized in table 2 for the three data sets. In the case where δ was allowed to depend upon wavelength we obtained the result shown in fig. 6 for data set 1. In fig. 7 we show the best fit to a single-wavelength data set from set 1 of absorbance changes as a function of geometric mean partial pressure for two situations: (1) linear optical case and (2) nonlinear optical case using the fixed parameter of $\delta = -0.20$. Either function accommodates the data well at a single wavelength, and any deviation from linearity is not readily apparent. In fig. 8 the residuals of the complete global fit for the same two cases are illustrated for data set 1. When all wavelengths are fitted with common equilibrium constants, and

Table 2

Fitted parameters with least-square confidence intervals (67%) for multiple wavelength oxygenation studies of hemoglobin

	Parameters	Linear assumption	Nonlinear, δ constant	Nonlinear, δ varies as λ
Data set 1	β_1	0.051 ± 0.001	0.079 ± 0.003	0.081 ± 0.003
	β_2	$1.03\times10^{-3} \pm 1.8\times10^{-5}$	$2.38\times10^{-3} \pm 2.6\times10^{-4}$	$2.53\times10^{-3} \pm 2.4\times10^{-4}$
	β_3	$1.16\times10^{-5} \pm 4.6\times10^{-7}$	$1.25\times10^{-5} \pm 1.0\times10^{-6}$	$1.25\times10^{-5} \pm 1.0\times10^{-6}$
	β_4	$7.51\times10^{-7} \pm 7.6\times10^{-9}$	$1.67\times10^{-6} \pm 1.6\times10^{-7}$	$1.77\times10^{-6} \pm 1.5\times10^{-7}$
	δ	0	-0.28 ± 0.03	see fig. 6
	σ	0.00132	0.00128	0.00117
Data set 2	β_1	0.054 ± 0.001	0.076 ± 0.003	0.071 ± 0.003
	β_2	$1.22\times10^{-3} \pm 2.1\times10^{-5}$	$2.60\times10^{-3} \pm 2.8\times10^{-4}$	$2.56\times10^{-3} \pm 2.6\times10^{-4}$
	β_3	$8.60\times10^{-6} \pm 5.6\times10^{-7}$	$4.96\times10^{-6} \pm 1.8\times10^{-6}$	$5.51\times10^{-6} \pm 1.7\times10^{-6}$
	β_4	$1.03\times10^{-6} \pm 1.0\times10^{-8}$	$2.01\times10^{-6} \pm 1.9\times10^{-7}$	$1.99\times10^{-6} \pm 1.7\times10^{-7}$
	δ	0	-0.24 ± 0.03	–
	σ	0.00128	0.00124	0.00113
Data set 3	β_1	0.045 ± 0.001	0.054 ± 0.002	0.053 ± 0.001
	β_2	$1.40\times10^{-3} \pm 1.7\times10^{-5}$	$1.91\times10^{-3} \pm 1.7\times10^{-4}$	$1.88\times10^{-3} \pm 1.0\times10^{-4}$
	β_3	$1.01\times10^{-5} \pm 4.6\times10^{-7}$	$1.00\times10^{-5} \pm 6.8\times10^{-7}$	$1.01\times10^{-5} \pm 4.6\times10^{-7}$
	β_4	$1.03\times10^{-6} \pm 8.2\times10^{-9}$	$1.43\times10^{-6} \pm 1.3\times10^{-7}$	$1.41\times10^{-6} \pm 7.6\times10^{-8}$
	δ	0	-0.14 ± 0.04	–
	σ	0.00102	0.00100	0.00070

linear optical response is assumed, systematic deviations between the data and the best fit function are seen to be present.

5. Discussion

As stated in section 3, the solution conditions using inositol hexaphosphate (IHP) were chosen to maximize sample stability against oxidation and to provide lower oxygen affinities that can be studied to the highest level of precision by means of the thin-layer optical cell methodology. However, under these conditions, small changes in the HbA_0 spectrum are known to be induced by IHP [24]. Thus, the second component in the SVD analysis might then be considered as due to linkage of IHP and O_2 binding. Imaizumi et al. [8] found that the effect of IHP on the oxy-HbA_0 extinction coefficients, ϵ, at 413 and 430 nm were changed by only 0.26 and 0.11%, respectively, upon deoxygenation. This clearly does not account for the 20% optical effect found in the best-fit global analysis of the optical response data. Furthermore, the binding constants for IHP to HbA_0, determined previously [25], show that only a small fraction (0.06) of IHP is dissociated upon oxygenation under the solution conditions employed, and thus dissociation of IHP must make only a minor contribution to the changes suggested by the model-based data analysis. Preliminary experiments in the absence of IHP at high pH (pH 9, 0.2 M borate) have also yielded essentially similar SVD results, although Hb samples under these conditions have so far proven to be much less stable.

The results of the SVD analysis indicate that the assumption of linear optical response to degree of saturation is invalid and that eq. 7 with all $\Delta\bar{A}_i$ equal (i.e., $\delta = 0$) is not an adequate description of the system. In terms of the model-based analysis inclusion of a wavelength-independent δ is significant to a 67% confidence level by the F-test ratio, although the absence of a strict isosbestic point indicates this is not the best representation either. Indeed, if all intermediate species had optical spectra that were strictly proportional to each other, then only a single transition would be found in the SVD analysis. In all data sets, however, the F-test ratio warrants inclusion of a wavelength-dependent δ to a statistically significant level of greater than 99% certainty. This

observation agrees with studies of deviations which occur at the isosbestic points as a function of the degree of oxygenation [6,7].

In light of the close agreement of the SVD analysis of each of the triplicate experiments, the range of fitted δs (see table 2) led us to examine the parameter error surface curvature using the method of Bates and Watts [26]. This procedure allows characterization of the error surface in a nonlinear fitting problem (and thus the magnitude of the true uncertainty) in terms of the linear error surface approximation [21]. The deviation is separated into intrinsic curvature and parameter effects, i.e., nonlinearity introduced by the formulation of the fitting equation. It was found that in the case of eq. 6 the intrinsic curvature was small, but that there was a large parameter effect due to the form of the parameterization in the fitting equation. This implies that the fitted parameters would have very large associated uncertainties and that a reparameterization of eq. 7 might improve the situation [27]. Unfortunately, we have been unable to reformulate the fitting equation in a manner that improves parameter estimation. For example, recasting the βs in terms of free energies of binding yields a larger parameter effect than eq. 7 as written. It should be noted that this problem becomes more profound the greater the correlation between parameters, so that when each stoichiometric species has an independently assigned optical parameter neither the optical parameter nor the overall equilibrium constants are resolved.

In order to explore the question of how the nature of the nonlinear model affects the ability to resolve the defining parameters, a simulation study was constructed from eq. 7 using $\Delta A_j = A(x_j) - A(x_{j-1})$ where $x_j = x_{j-1}D$ ($D = 0.600$ gave 16 values of x_i used in generating the simulated data). It was assumed that the optical scaling parameters $\delta_1 = \delta_2 = \delta = 0.10$, $\delta_3 = 0$, were wavelength-independent and that the overall optical effect $\Delta\bar{A}_4$ varied linearly from $+1$ to -1 over a range of 100 wavelengths. 1500 data sets were generated with an added random noise ($\sigma = 0.001$) to each point of a given set. This standard error is consistent with the experimental observations. Each 'data' set was then fitted by the nonlinear fitting program to the model where the optical scaling parameter (δ) was allowed to depend upon wavelength, and the average parameter values at several selected wavelengths and their standard deviations were computed from the 1500 samples. The recovered parameters and their distribution of values were found to be unskewed with an approximate normal distribution. As expected, the standard deviation of the recovered δs did not depend upon wavelength, but unexpectedly the magnitude of the standard deviation was much larger than found in fitting the usual linear parameters that are found in a nonlinear fitting problem, and is, for example, much larger than the uncertainty for $\Delta\bar{A}_4$ in the case of $\delta = 0$.

In conclusion, we find from both the SVD- and model-based analysis of optical spectra measurements in the Soret region for HbA_0 in the presence of IHP and under precisely imposed oxygen partial pressures that more than one optical transition is needed to represent the data. The model-dependent nonlinear least-squares analysis is found to be quite sensitive to the number of optical parameters chosen to describe the system but fitting the data to a first-order model leads to the suggestion that the optical parameters describing the oxygen binding to the first and second stoichiometric species are approx. 20% smaller than those of the third and fourth stoichiometric species. This analysis suggests that significant deviations from the usually assumed optical linearity in oxygen binding by HbA_0 may be a general feature. However, before such a general conclusion can be reached it will be necessary (1) to improve the conditions for obtaining highly stable materials over reasonably long (several hours) exposure to different ligand activities, and/or (2) to employ an independent, direct optical titration method that does not depend so crucially upon the detailed least-squares analysis of binding curves from optical changes revealed in the present study.

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