ABSORBANCE SPECTROSCOPY AND LIGAND BINDING: SEEING IS BELIEVING

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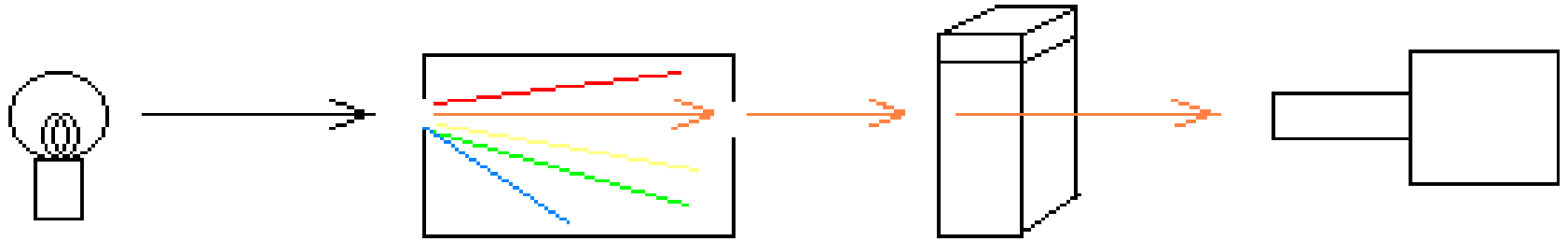
Nature of UV/Vis absorbance changes

UV/Vis photons have energy enough to cause quantum transitions in the electrons present in bonding orbitals: e.g. $\pi \rightarrow \pi^*$ quantum jumps. If the binding of a ligand to a protein occurs via formation of a true chemical bond, it may be directly associated to a change of the absorbance spectrum (with respect to the free, unbound couple of the protein and the ligand). Given the energies of photons in the near UV/Vis wavelength range, absorption is essentially confined to the quantum transitions of delocalized π electrons of aromatic chromophores (i.e. aromatic residues or co-factors) and d orbitals of transition metals (in metalloproteins).

Unfortunately only a handful of protein ligands form true bonding orbitals with their characteristic absorbance spectra. In many cases the protein-ligand complex forms because of weak chemical interactions (H-bonds, hydrophobic contacts, etc.) that are spectroscopically silent; even in these cases, however, the presence of the ligand in the binding site may perturb the absorbance spectra of some protein chromophore (e.g. a Trp residue in the binding site), and be associated to a detectable UV/Vis absorbance change.

Scheme of an Absorption Spectrophotometer



Lamp monochromator sample detector

To carry out a measurement using the simplest instrument schematized above, the researcher puts in the sample compartment a spectrophotometric cuvette containing the "blank" (i.e. the buffer he will use during actual the measurements) and records the light energy transmitted at the appropriate wavelength. He then replaces the blank with the sample and takes a new reading. The following definitions apply:

Intensity at wavelength λ I_λ = (photon) reading of the photodetector

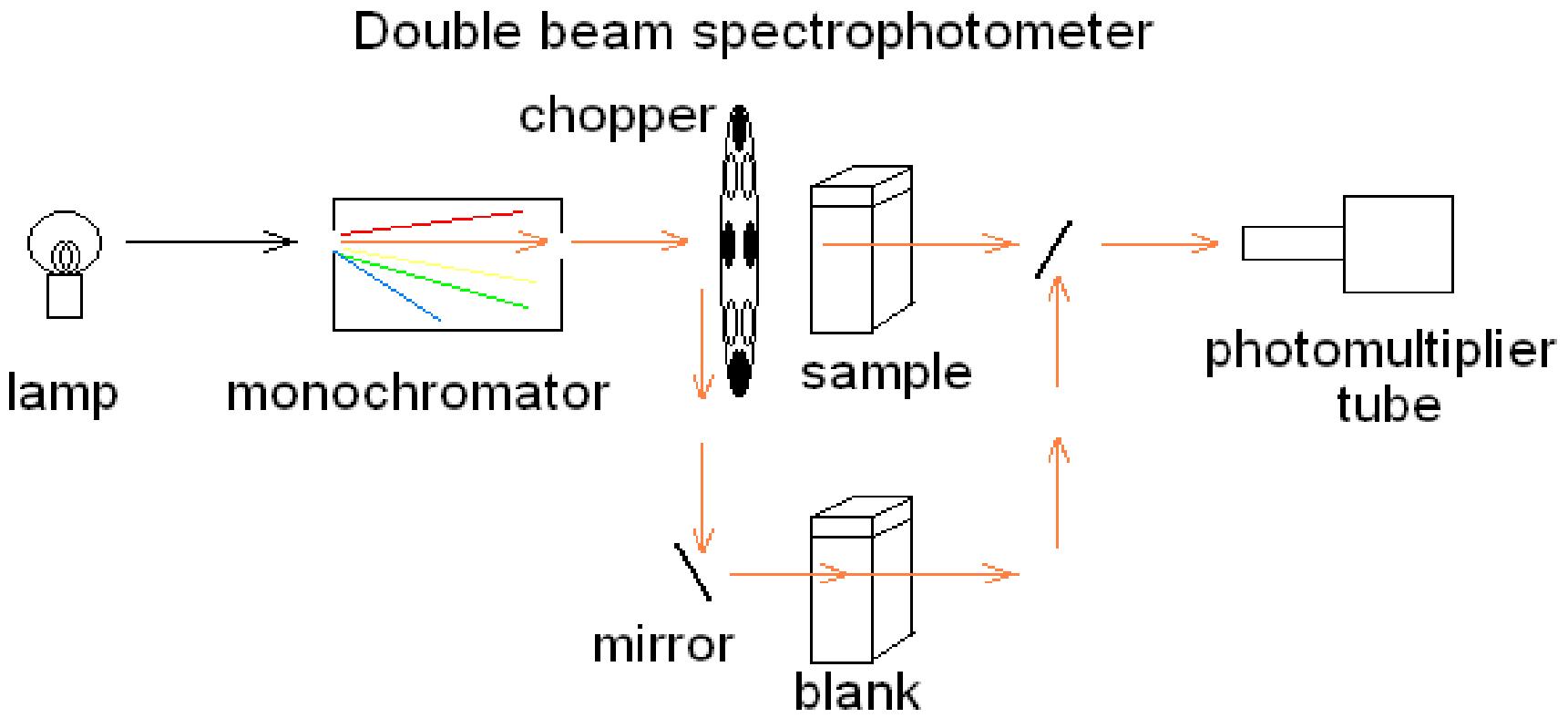
Transmittance at wavelength λ $T_\lambda = I_{trans,\lambda}/I_{inc,\lambda}$ $(0 < T < 1)$

Corrected Transmittance of the sample at wavelength λ

$$T_\lambda' = T_{sample,\lambda}/T_{blank,\lambda} \qquad (0 < T' < 1)$$

Absorbance of the sample at wavelength λ $A_\lambda = - \log_{10} T_\lambda'$ $(A > 0)$

In order to correct for lamp intensity oscillations, most research spectrophotometers adopt a double beam design in which a sample and a matched blank are read alternatively; moreover, a motorized monochromator allows the researcher to record the full absorbance spectrum of his/her sample, i.e. to scan the absorbance over an arbitrarily defined range of wavelengths.



UV/Vis absorption is governed by the law discovered by Lambert and Beer:

$A_\lambda = \varepsilon_\lambda C L$

where A_λ represents the absorbance at wavelength λ, ε_λ the molar extinction coefficient of the chromophore at wavelength λ per cm pathlength, C its molar concentration, and L the pathlength (usually 1 cm).

Lambert's law essentially states that **absorbance is linearly correlate (directly proportional) to concentration**, i.e. that the measure of the former allows the researcher to calculate the latter, not a small feat for complex biological macromolecules that do not lend themselves to easy direct chemical determinations.

A very important point to take into account is that the measurement of light intensity (i.e. of absorbance) occurs essentially in real time, at least down to the ns regime (corresponding to GHz frequences of the photmultiplier tube / amplifier), implying that **the method is suitable for time-resolved (kinetic) measurements**.

Application of absorbance measurements to ligand binding equilibria (and kinetics).

If we record absorbance spectra of the same protein solution equilibrated with different concentrations of its ligand X, and if formation of the complex is associated to an absorbance change, we obtain a series of spectra, whose absorbance values are linear combinations of those of the ligand, the unliganded protein and the protein-ligand complex:

$$A_{\lambda,i} = (\varepsilon_{\lambda,P}\,[P]_i + \varepsilon_{\lambda,X}\,[X]_i + \varepsilon_{\lambda,PX}\,[PX]_i)\,L$$

Most often the extinction coefficient of the ligand is negligible, thus the free ligand concentration can be neglected in the Asorbance expression.

Moreover, the terms [P] and [PL] sum to the total protein concentration $[P]_{tot}$, and can be expressed as a function of the fractional ligand saturation Y, defined as $Y = [PL] / [P]_{tot}$; thus we obtain:

$$A_{\lambda,i} = [P]_{tot}\, L\, [\varepsilon_{\lambda,P} (1 - Y_i) + \varepsilon_{\lambda,PX}\, Y_i] = [P]_{tot}\, L\, (\varepsilon_{\lambda,P} + \Delta\, \varepsilon_{\lambda,PX-P}\, Y_i)$$

Taking in consideration that:

$[P]_{tot}\, L\, \varepsilon_{\lambda,P}$ = absorbance of the unliganded protein;

$[P]_{tot}\, L\, \Delta\, \varepsilon_{\lambda,PL-P}$ = absorbance difference between the fully liganded and fully unliganded protein.

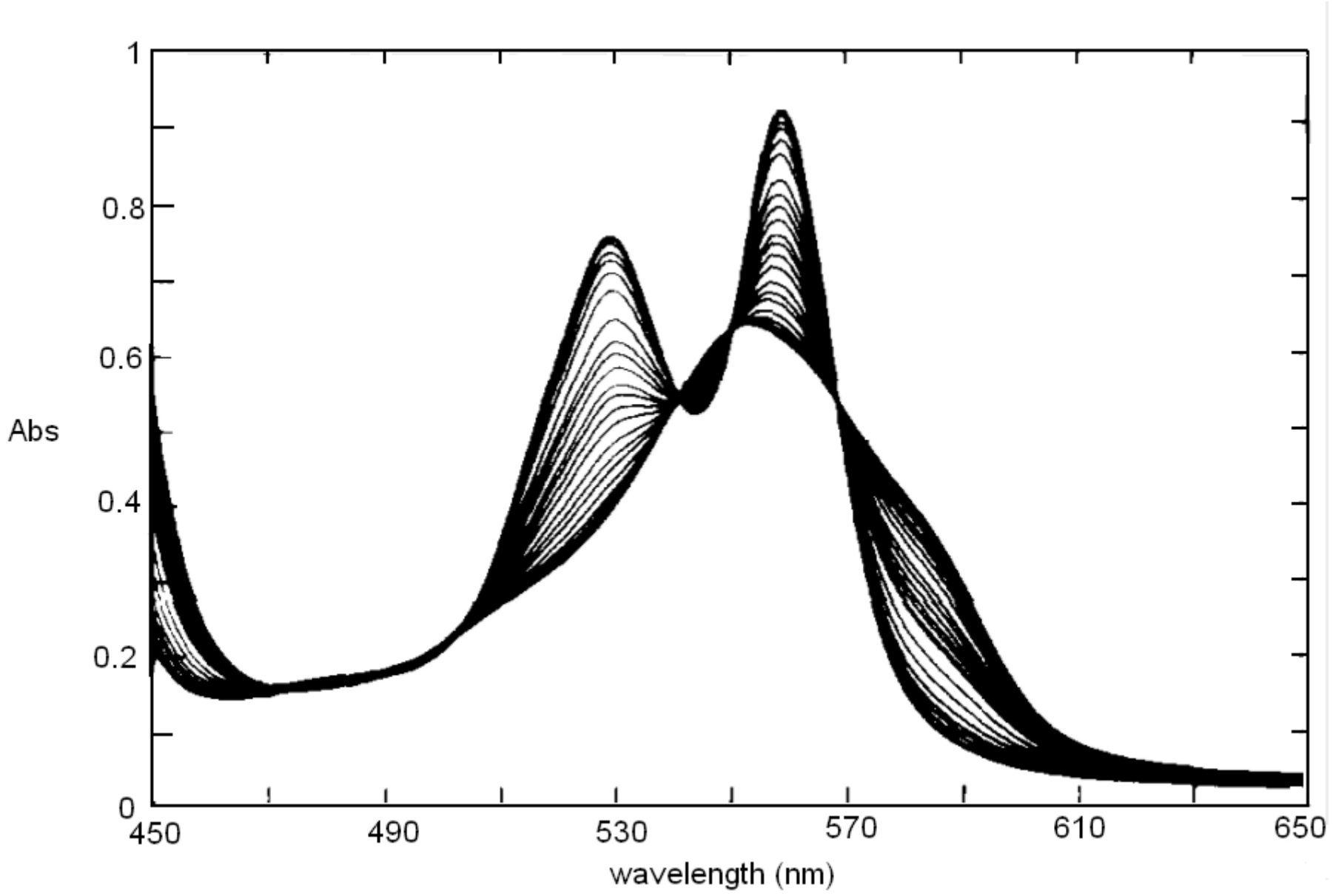
we simplify:

$$A_{\lambda,i} = A_{\lambda,P} + \Delta\, A_{\lambda,PL-P}\, Y_i$$

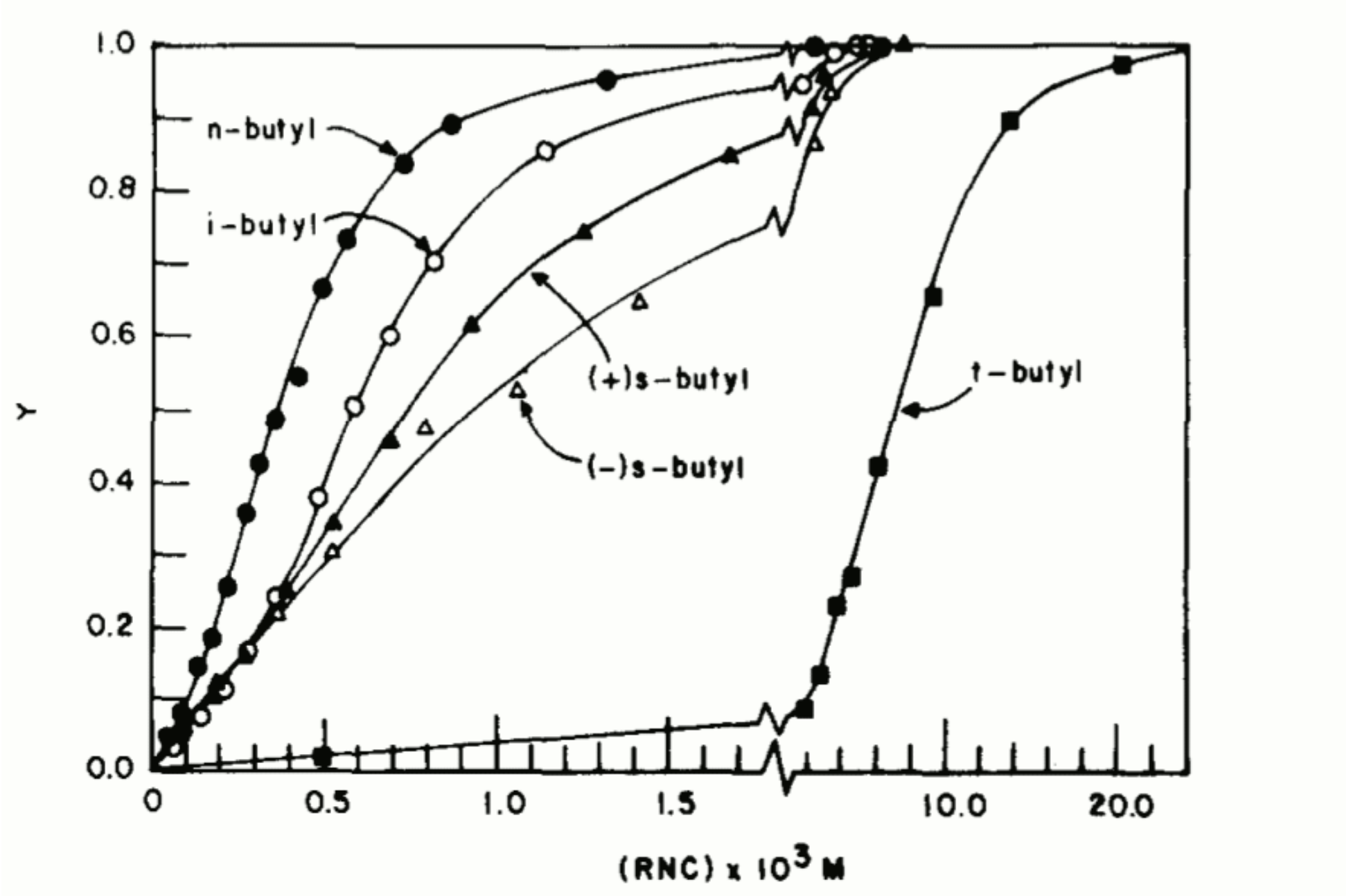
which easily yields the fractional saturation for experimental condition i as:

$$Y_i = (A_{\lambda,i} - A_{\lambda,P}) / (A_{\lambda,PL+} - A_{\lambda,P})$$

Absorbance spectra of the binding isotherm of n-propyl isocyanide and human hemoglobin (Reisberg P. & Olson J.S., J. Biol. Chem. 1980, 255, 4144-4150).



Analysis of the experiment presented in the preceding slide (full circles)



PRINCIPLES OF DATA ANALYSIS

Calculating an equilibrium constant from a set of absorbance data is in principle quite straightforward:

(i) one needs a reasonable chemical hypothesis, in the simplest possible case the single site equilibrium binding:

$$P + L \Longleftrightarrow PL$$

governed by the dissociation constant:

$$Kd = [P]\,[L] / [PL]$$

(ii) From the above hypothesis, the researcher should derive an equation capable of predicting the expected signal. E.g. for the single site binding we have that the fraction of bound sites is:

$$Y_i = [L]_i / (Kd + [L]_i)$$

which predicts:

$$A_{calc,\,i} = A_P + (A_{PL} - A_P)\,[L]_i / (Kd + [L]_i)$$

(iii) The researcher has to guess an initial estimate for the parameters of the equation he has derived. In our example the parameters are Kd, AP and APL, which can be reasonably guessed as: the free ligand concentration required to achieve half the total absorbance change; the absorbance at wavelength λ in the absence of ligand, the absorbance at the same wavelength in the presence of excess ligand, respectively.

(iv) The initial guess allows the researcher to calculate the error function (usually the deviance) of the ligand binding isotherm:

$$D_0 = \Sigma (A_{sper,\, i,\, \lambda} - A_{calc,\, i,\, \lambda})^2$$

(v) The calculus is moved to a computer which employs a non-linear minimization routine to calculate a new parameter set such that the deviance at the end of the calculus is lower than the initial one, i.e.:

$$D_1 < D_0$$

(vi) Step v is iterated till the decrease in the error function between two successive iteration is less that a selected value; when this happens we say that the procedure has *converged*.

GOODNESS OF FIT

If the data analysis procedure described above is straightforward, the evaluation of the results one obtains is not. The problem is that a more complex chemical hypothesis will always produce a better fit and one needs absolute (rather than comparative) criteria to establish when the error function is good enough.

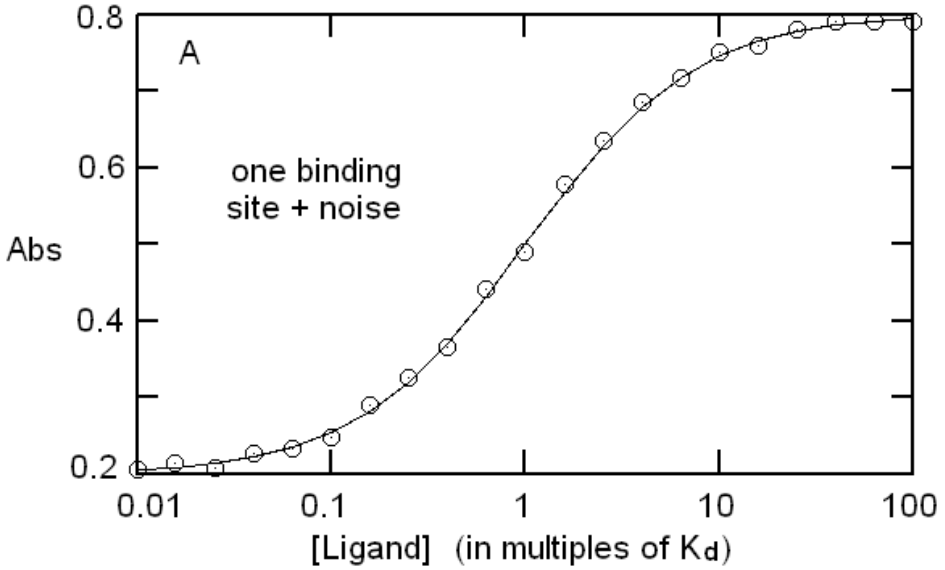
Commonly adopted criteria are:

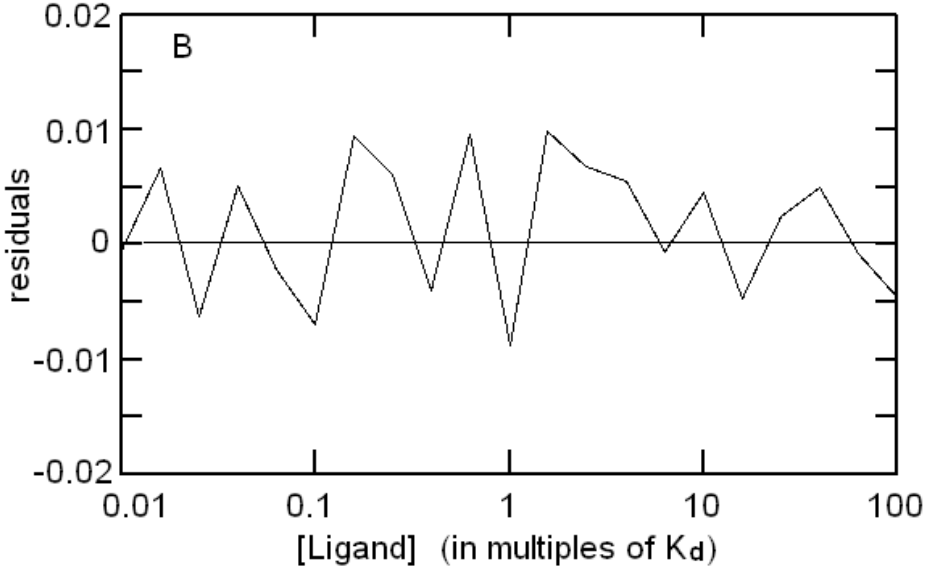
(i) the plot of the residuals ($A_{calc,\, i,\, \lambda} - A_{sper,\, i,\, \lambda}$) should be randomly distributed, and should cross the zero line at much higher frequency than the signal.

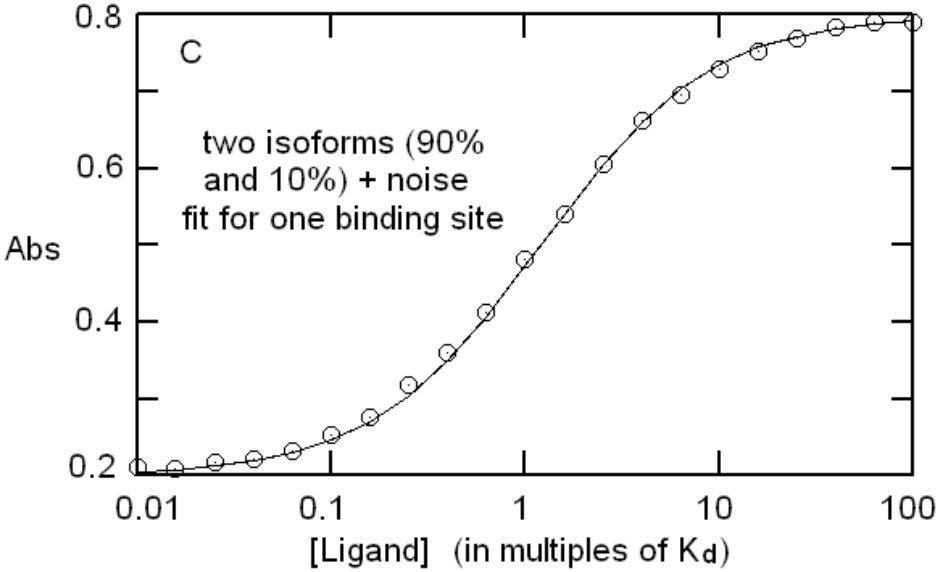
(ii) The standard deviation should be of the same order of magnitude as that one would obtain by repeating the same experimental point over and over.

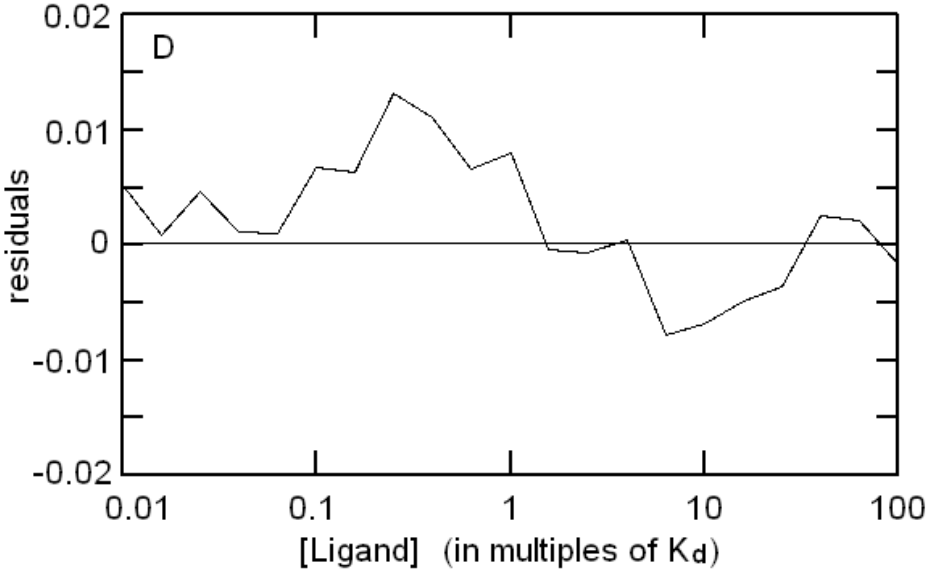
(iii) Adoption of a more complex chemical model should provide only a modest improvement with respect to the above criteria.

Distribution of the fit residuals for two simulated binding curves: A for a pure one-site protein, B for a mixture of two isoforms (90+10%). Random noise added to both. Fit for a single binding site in both cases.







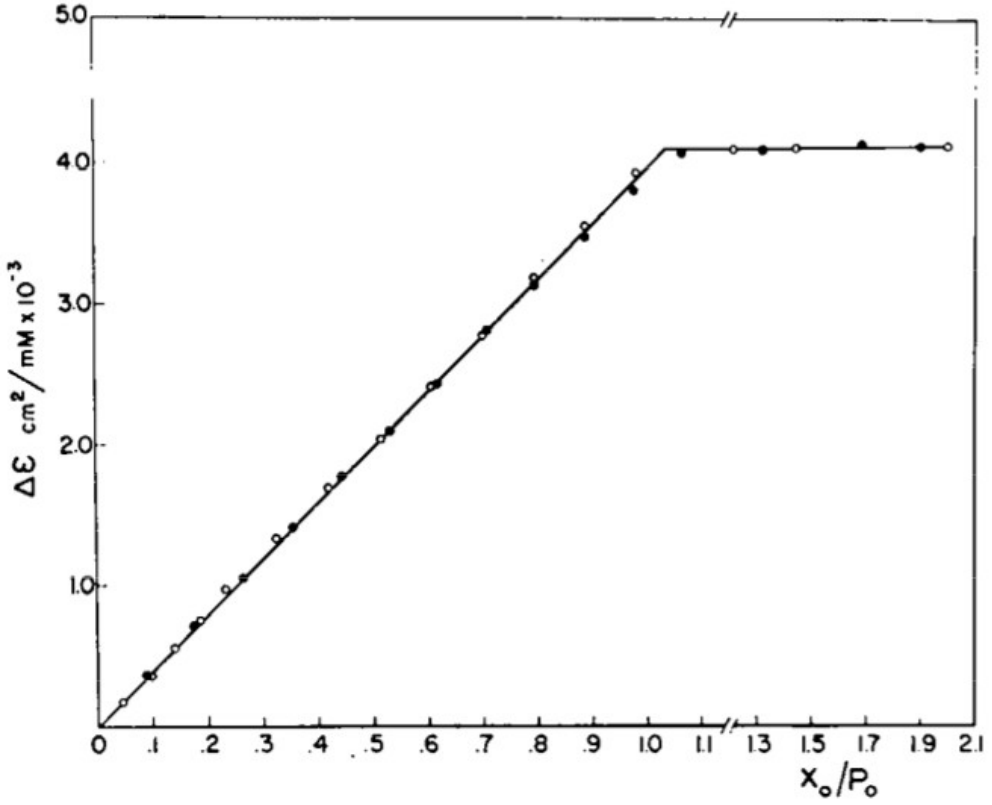


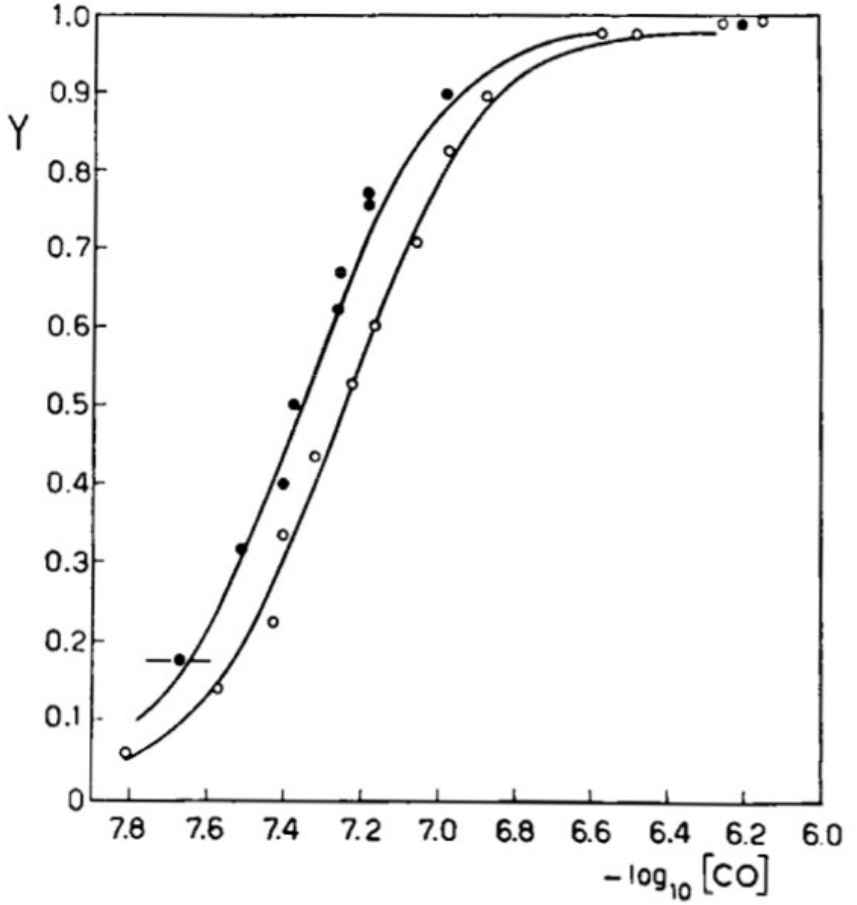
Linearity

Absorbance spectroscopy, thanks to Lambert's law is so powerful and so useful a technique for the study of ligand binding that its limitations and difficulties are often overlooked. ***The most important problem with absorbance spectroscopy is to make sure that the absorbance signal is indeed linear with binding***. One should not confuse the linear relationship of absorbance to concentration (Lambert's law) with linearity to binding: indeed the absorbance change may be due to a ligand-dependent structural rearrangement of the protein, and the relationship between the two phenomena of ligation and structural change may be non-linear. Linearity should be tested by experiment, under conditions in which the fraction of liganded sites Y is known independently. E.g. E. Antonini and S. Anderson demonstrated the linearity of absorbance changes and ligand binding in HbCO, taking advantage of the high affinity of the ligand, whose free concentration, under suitable experimental conditions is negligible.

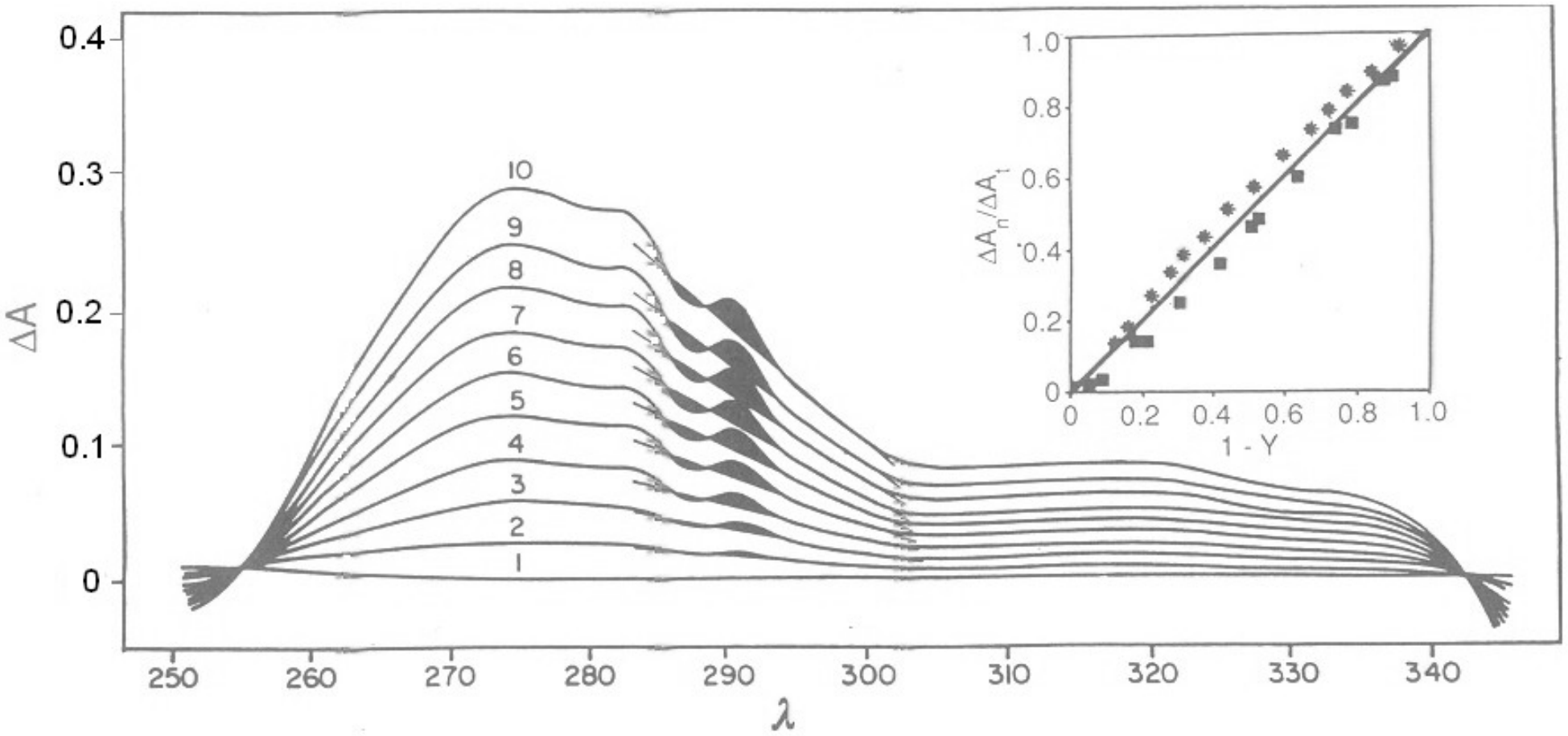
The experiment devised by S. Anderson and E. Antonini to test the linearity of the absorbance signal to fractional saturation (reaction: Hb + CO → HbCO).

Left panel: high Hb concentration – $[CO]_{free} = 0$;
Right panel: low Hb concentration, and non-zero $[CO]_{free}$.





A spectroscopic feature of the reaction of Hb with O_2 in the near UV region, studied by Imai and Yonetani and attributed to the solvent exposure of a Trp residue as a consequence of ligand-linked structure changes of the protein, was demonstrated to be non-linear with ligand saturation.



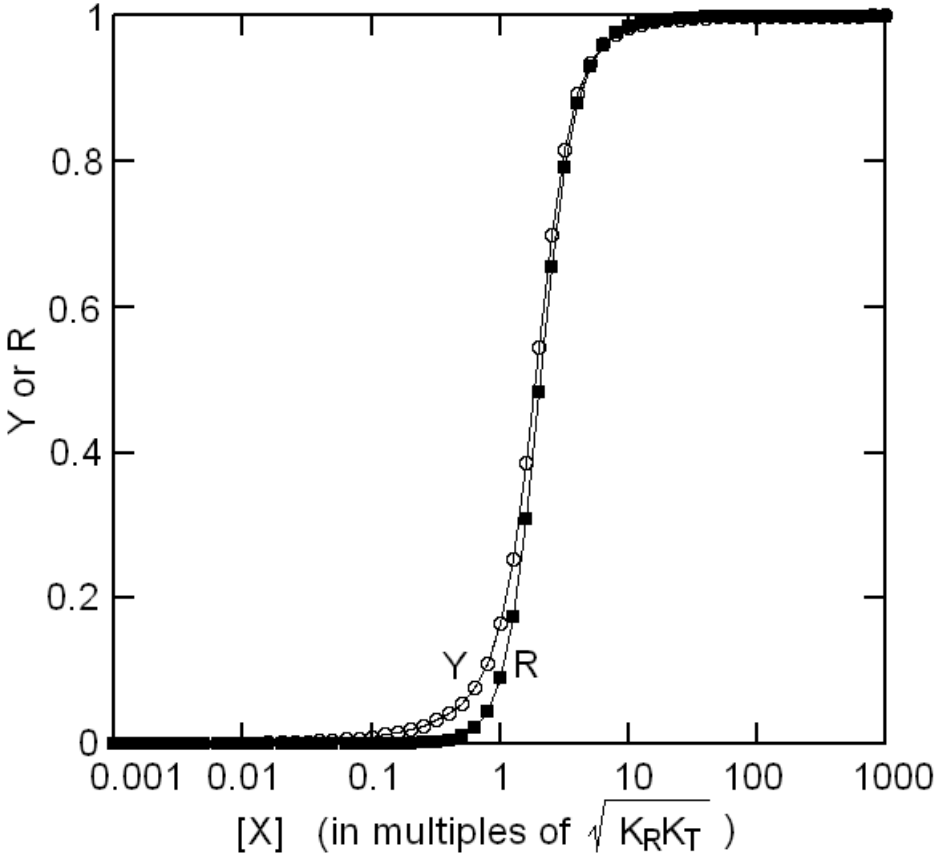
Explanation of non-linearity

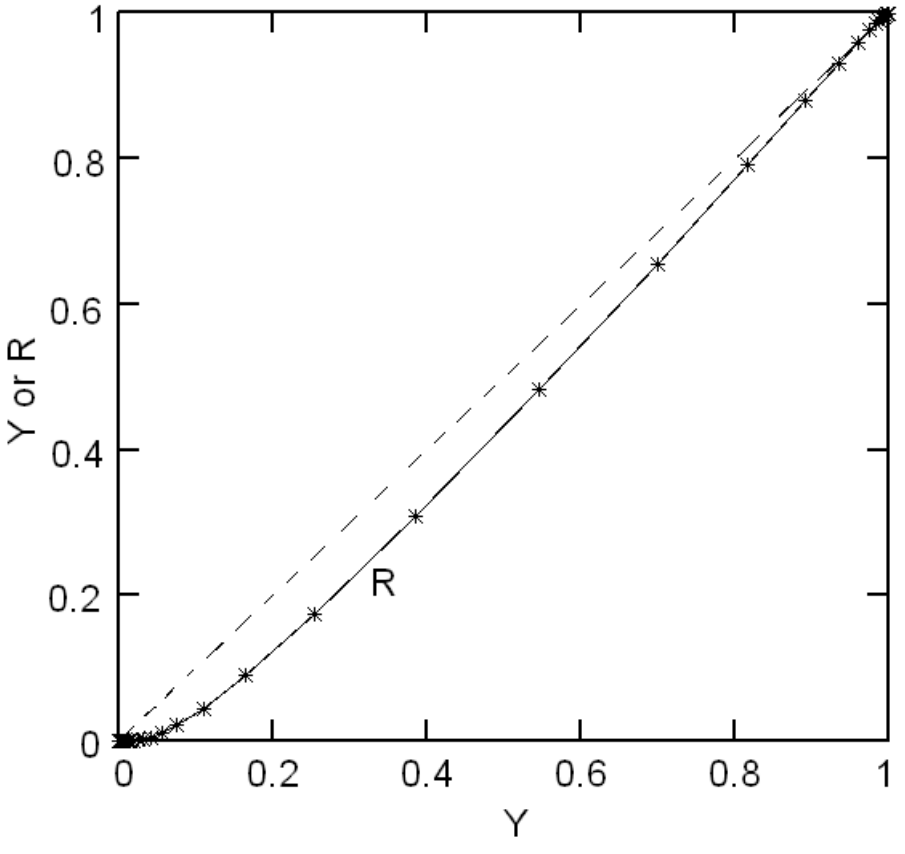
Lambert's law guarantees that absorbance is linearly related to concentration, but in an oligomeric protein an absorbance change may be linearly related to a structural change, which is induced by the ligand but not linearly related to ligand saturation.

The most straighforward case is that of an allosteric protein, like hemoglobin, which is stable in two structural conformations. The classical allosteric model of Monod, Wyman and Changeux (1965) postulates that the two structural conformations, called R and T, are in equilibrium irrespective of the presence of the ligand, and their relative population is biased by ligation.

Both structures/states bind ligands, with different affinity, and the protein switches from the T to the R structure as ligation proceeds. A signal which monitors the structure (either R or T) evolves differently from one which monitors ligation (to both R and T).

In this system, the ligand binds to both the T and R state, at low concentrations (and low degrees of saturation) prevalently to the T state of the protein; at high concentrations prevalently to the R state. It follows that a spectroscopic feature directly related to binding and one related to the protein structure will both present a dependence on ligand concentration, but not the same dependence. This would explain the non-linearity of the signal studied by Imai and Yonetani.





Isosbestic points

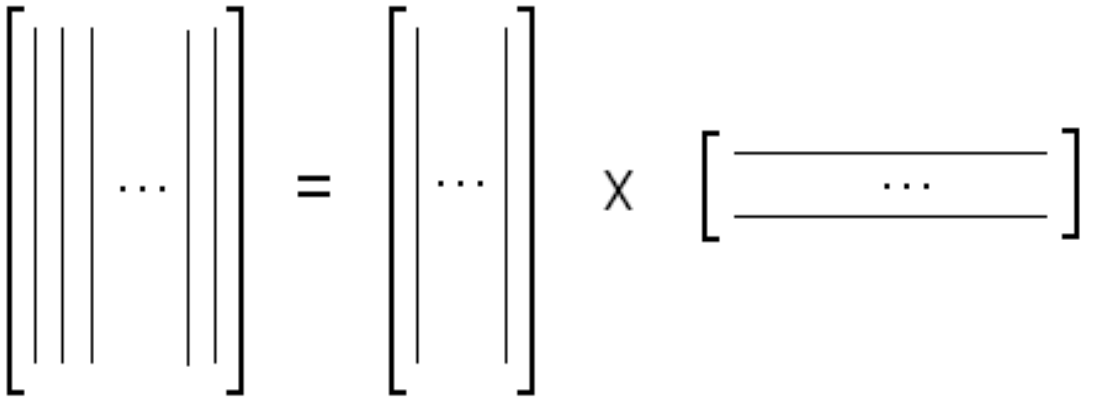
Isosbestic points are wavelengths at which the extinction coefficients of the liganded and unliganded (or any couple of) derivatives have the same extinction coefficients. If the chemical reaction one is studying only involves two chemical species, one finds perfect isosbestic points. The finding of isosbestic points does not guarantee linearity of the absorbance signal to binding, nor does lack of them demonstrate absence of linearity; however *lack of isosbestic points demonstrates that the reaction has at least three species (e.g. reactant, intermediate and product) and implies that the absorbance signal is non-linear to binding at least at some wavelengths*.

Important cases of lack of linearity in the presence of isosbestic points occur when the reaction we study has intermediates which can be spectroscopically resolved only in limited regions of the spectrum (e.g. the UV feature identified by Imai and Yonetani in the UV spectrum of Hb occurs in a region which has isosbestic points at 255 and 343 nm).

Multiple components analysis

If the reaction of interest involves multiple spectroscopically distinct intermediates, its analysis is usually complex and model-dependent. The essential features of this system are: (i) signal may not be linear with ligation; and (ii) the spectroscopic transition may lack true isosbestic points. Matrices are a powerful instrument to deal with these conditions, since matrix operations allow the researcher to analyze full spectra at once, rathre than single wavelengths.

If we collect the spectra of our experiment in a matrix **A** in which each column is a spectrum and each row a time course or equilibrium profile at a single wavelength, we can express Lambert's law as the product of matrices **ε** (the matrix of the extinction coefficients of the relevant chemical species) and **C** (the matrix of the concentrations of the relevant chemical species):



$$A = \varepsilon \times C$$

The important advantage we gain from matrix representation, is that matrix backdivision of **A** by **ε** directly yields **C**, i.e.:

$$\mathbf{C} = \mathbf{A} \setminus \boldsymbol{\varepsilon}$$

and this relationship holds also if a noise component is present in **A**.

The main limit of this approach is that we must provide the matrix of extinction coefficients for all the intermediate species involved, and some of these may not be available (e.g. some ligation intermediates cannot be prepared, and their absorbance spectrum collected, in a purified state). Matrix algebra offers an elegant solution to this problem, called the Singular Value Decomposition (SVD).

SVD decomposes matrix **A** into the product of three matrices, called **U**, **S**, and **V**, such that:

$$\mathbf{A} = \mathbf{U} \times \mathbf{S} \times \mathbf{V}^{\mathbf{T}}$$

The SVD solution is unique because of the following internal constraints: (i) **U** and **V** are orthogonal matrices, i.e. their transpose is also their inverse; (ii) **S** is a diagonal matrix whose nonzero elements are positive and arrenged in decreasing order. If the dimensions of **A** are **m** x **n** (i.e. n spectra of m wavelength readings have been collected), the dimensions of **U** are **m** x **n**, those of **S** and **V** are **n** x **n**. The **m** x **n** **U**x**S** matrix is formally analogous a matrix of extinction coefficients **ε**, and given the decreasing order of the elements of **S**, only the first few columns are significant, the remaining ones containing mostly noise.

The columns of matrix **V** contain the ligand-dependent amplitudes of the spectroscopic components **U**x**S**, i.e. they are analogous to the concentrations of the chemical species (as one would record in kinetic time courses or equilibrium ligand binding).

Difference absorption spectra of the rebinding of CO after photolysis of HbCO (from Hofrichter et al. 1983, PNAS, 80: 2235-9).

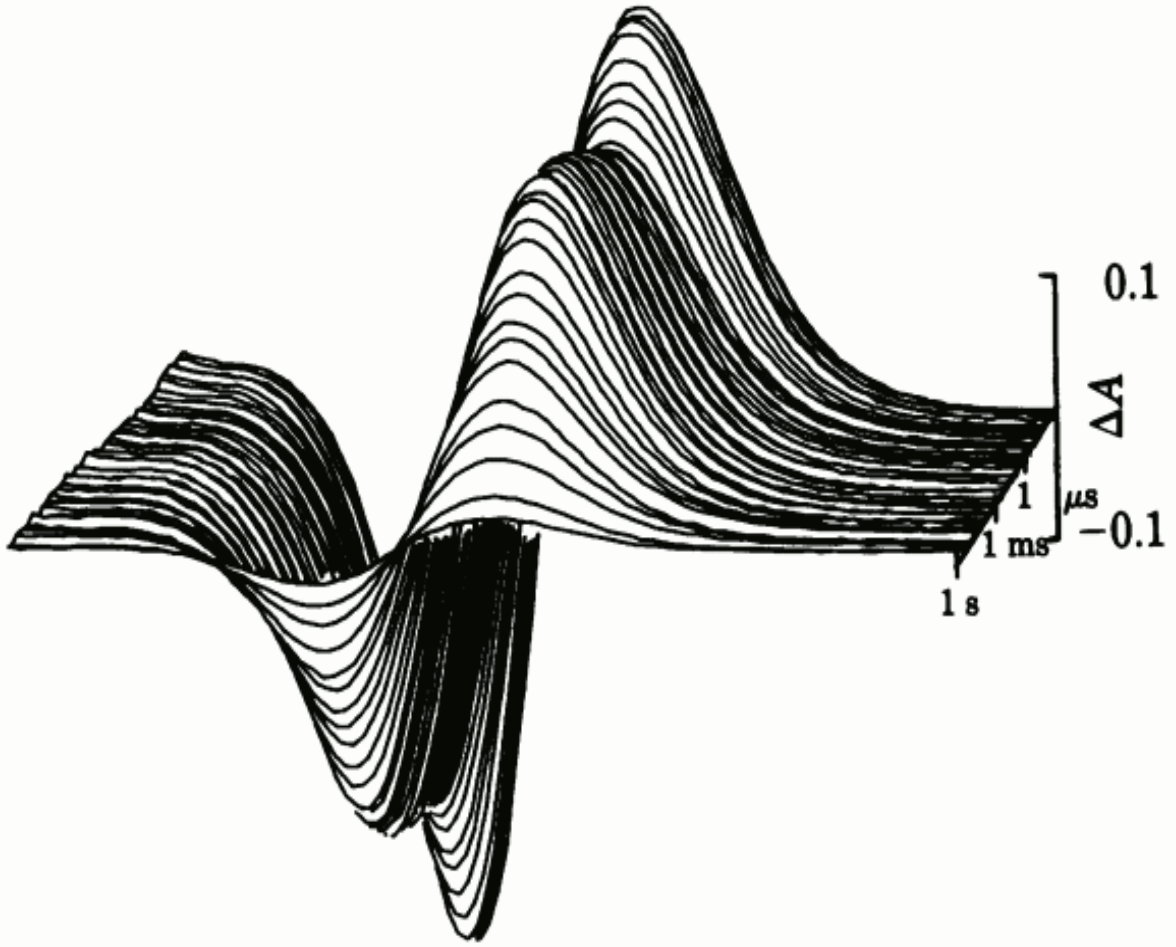


FIG. 1. Spectra after photolysis of HbCO. A representative subset of the transient difference spectra collected for sample b, in which 100% saturated HbCO was fully photolyzed (90%) at 0.1 atm CO pressure (1 atm = 101.3 kPa) is shown. Each curve represents the difference in A between the deoxy photoproduct and the HbCO reference on the vertical (y) axis as a function of wavelength (x). The logarithm of the time is plotted on the z axis with the earliest times in the background. The data span eight decades of time (1 ns to 0.1 s) with five spectra per decade. The sample A at the CO peak (419 nm) was 0.81 and the peak (430 nm) A in a fully deoxygenated reference spectrum was 0.575.

SVD analysis of the experiment presented in the preceding slide.

Panel a: the three most important components of the UxS matrix.

Panel b: enlargement of the third UxS component.

Panels c,d and e: time courses of the corresponding three columns of V.

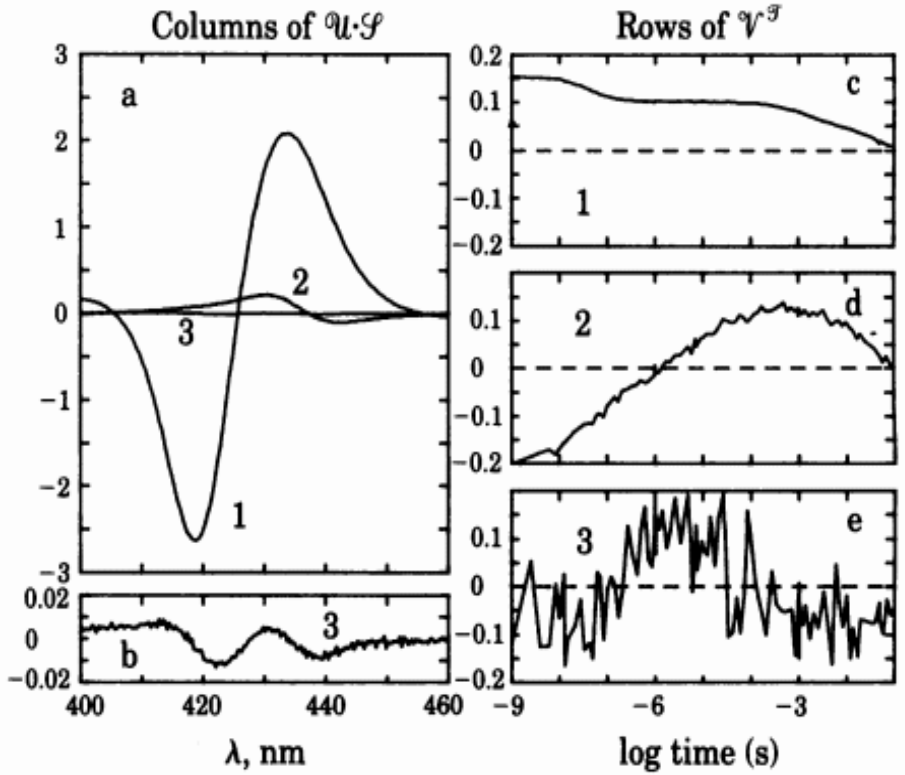


FIG. 2. SVD of observed spectra. The SVD of $\mathcal{A}$ is defined as $\mathcal{A} = \mathcal{U}\mathcal{S}\mathcal{V}^{\mathcal{T}}$ (16, 17), where $\mathcal{A}$ is the $m \times n$ matrix of spectra measured at n different time delays between photolysis and probe pulses and each of the n columns contains absorbance values at m wavelengths; $\mathcal{U}$ is an $m \times n$ matrix in which the columns of $\mathcal{U}$ are an orthonormal set of m-dimensional vectors, which we call the basis spectra; $\mathcal{S}$ is an $n \times n$ diagonal matrix with all nonnegative elements, called the singular values of $\mathcal{A}$; and $\mathcal{V}$ is an $n \times n$ unitary matrix in which the rows of $\mathcal{V}^{\mathcal{T}}$ (columns of $\mathcal{V}$) are the time courses of the amplitudes of each of these basis spectra. (*a*) The three basis spectra obtained by SVD from the 103 spectra observed in the experiment shown in Fig. 1. The basis spectra are scaled by their singular values, $S_1 = 17.3$, $S_2 = 1.27$, $S_3 = 0.082$, to display their relative contributions to the observed data. (*b*) Basis spectrum 3 displayed on an expanded scale. (*c–e*) Normalized time dependences of the three basis spectra. The increasing noise in each of the successive components results from the decreased amplitude of each basis spectrum in the observed spectra. In fitting the time dependence, these data were weighted by the singular value associated with each basis spectrum.

Time courses of V columns.

Panel a: the first V column from the experiment ipresented in the preceding slides.

Panels b,c and d: same as a but under different experimental conditions

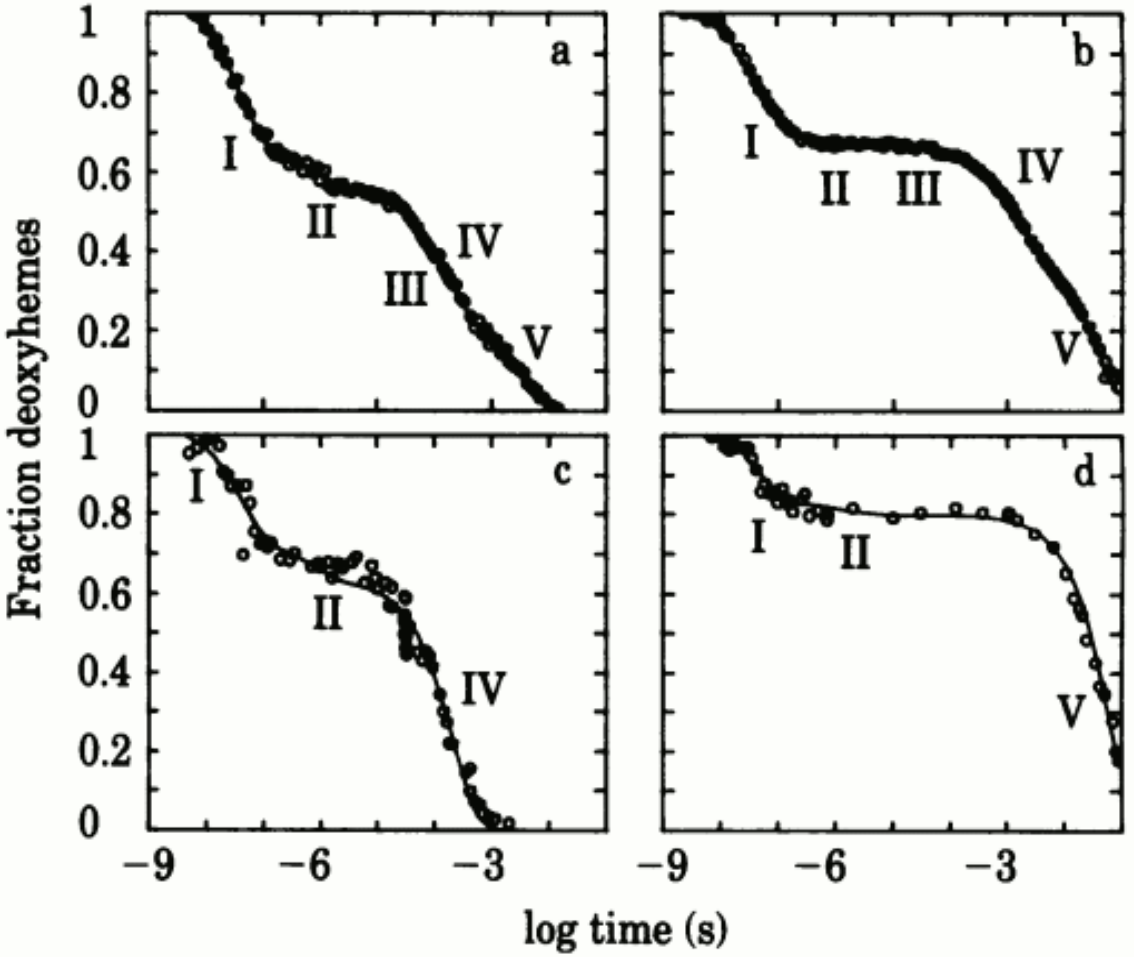


FIG. 3. CO recombination kinetics. The kinetics of CO rebinding to photolyzed HbCO are shown. (*a*) 1.0 atm CO, 100% saturation, full photolysis. (*b*) 0.1 atm CO, 100% saturation, full photolysis. (*c*) 1.0 atm CO, 100% saturation, 22% photolysis. (*d*) 3×10^{-6} atm CO, 10% saturation, full photolysis. The fraction of deoxyhemes was obtained by dividing the amplitude of the first SVD basis spectrum at each time by the amplitude at the peak of photolysis. The lines through the data show the fit to the amplitude of the first basis spectrum obtained from the simultaneous least-squares fit to the time dependence of all of the significant basis spectra obtained from the SVD analysis of each data set.

Interpretation of the SVD analysis

The interpretation of a SVD analysis is not completely straighforward, and cannot be carried out in a model-independent way.

The outline of the procedure is as follows:
(i) the reseracher, by inspection of the S matrix (whose elements are in decreasing order), of the UxS matrix (whose first columns have the appearance of spectra, while the last ones contain pure noise), and of the V matrix (whose columns should resemble titration curves or kinetic time courses) decides how many components are significant and deserve quantitative analysis. It is uncommon that more than 5 columns of UxS and V qualify.

(ii) The researcher defines a model that quantitatively describes the reaction and applies the usual least squares non linear minimization routine to the first *n* columns of V.

(iii) The researcher obtains from step (ii) constants and amplitudes (spectroscopic parameters equivalent to concentrations of the pure chemical species); let us call these Ks and $\mathbf{V}_{\mathbf{amps}}$.

(iv) A matrix of $\mathbf{V}_{\mathbf{amps}}$ is constructed; the product $\mathbf{U}\mathrm{x}\mathbf{S}\mathrm{x}\mathbf{V}_{\mathbf{amps}}$ yields the model dependent matrix $\boldsymbol{\varepsilon}\mathrm{x}\mathbf{C}_{\mathbf{tot}}$ as corresponding to the chosen chemical model.

(v) The matrix $\boldsymbol{\varepsilon}\mathrm{x}\mathbf{C}_{\mathbf{tot}}$ should conform to some requisites: it should give the correct spectra for all chemical species that can be obtained in the pure state, and reasonable (e.g. non-negative) spectra for chemical species which cannot be obtained in their pure state and whose spectrum is unknown.

(vi) The chemical variance between the fitted and experimental data should be low (since discarding the last columns of $\mathbf{U}\mathrm{x}\mathbf{S}$ and $\mathbf{V}$ discards mostly noise, one expects the fits to be more accurate).