Kinetic and spectroscopic properties of the cyanide complexes of ferrous haemoglobins I and IV from trout blood

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The cyanide ion is a ligand of ferrous as well as ferric haemoproteins and this study presents a kinetic characterization of the dissociation of its complexes with the two main haemoglobin components from trout blood. Both these haemoglobins bind oxygen co-operatively at neutral or alkaline pH values but one of them is insensitive to pH and allosteric effectors (haemoglobin I, HbI) while the other (haemoglobin IV, HbIV) is strongly sensitive and shows the so-called Root effect (i.e. the incomplete oxygen saturation in air-equilibrated solutions at pH values of < 6.5). Comparison of the kinetics of dissociation of cyanide from ferrous forms of HbI and HbIV reveals that: (i) cyanide dissociates in both cases by a complex reaction, and, at least in the case of HbIV, this may be attributed to functional differences between the α and β subunits; (ii) the reaction is only scarcely co-operative in HbI and not at all so in HbIV; and (iii) the Bohr and Root effects are not manifested in this reaction. The functional heterogeneity of ferrous α and β chains of trout HbI has not been observed for any other ligand; moreover, the observation that co-operativity for cyanide dissociation is expressed by human haemoglobin but not by trout HbIV is surprising.

INTRODUCTION

Co-operative ligand binding by haemoglobin has been the subject of a cohort of studies, and although the details remain controversial, the following general picture of the molecular phenomena underlying co-operativity and allostery is widely accepted. Liganded and unliganded haemoglobins have different geometries of haem, planar in the hexa-co-ordinated liganded derivatives and domed in the penta-co-ordinated unliganded derivatives, and different quaternary structures [1,2]. The transfer of information from the haem iron to the protein matrix is mediated by a histidine residue (His F8, the proximal histidine), whose Nϵ is co-ordinated to the metal and follows its movements in and out of the porphyrin ring.

The co-operative properties of ferrous haemoglobin have been traditionally investigated using different ligands, most often oxygen and CO, and useful information has been derived also from the study of less usual ligands such as isocyanides and NO. The cyanide complexes of ferrous haemoglobins and myoglobins have been described since 1930 [3–6], but, probably because of the low affinity and the insufficient technological background, their characterization has been largely incomplete. In particular it was unclear whether or not the binding of cyanide to ferrous haemoglobin was co-operative.

Cyanomethaemoglobins or myoglobins are reduced by dithionite while the ligand is bound, and yield the ferrous iron–cyanide complex, which, due to the very low affinity ($K = 1\ M^{-1}$, according to [3,4]), dissociate to cyanide and the unliganded ferrous haemoprotein [5,6]; thus significant information can be obtained from the time course of the dissociation reaction.

It was demonstrated [7] that this reaction is pH-dependent in wild-type horse and sperm whale myoglobins, as well as in site-directed mutants of sperm whale myoglobin which bind oxygen and CO in a pH-insensitive mode. A crucial role has been attributed to the distal histidine, which would determine the rate constants and their pH dependence by donating a proton to the bound anion [7,8]. The kinetic approach has also provided the conclusive demonstration that binding of cyanide is co-operative in human haemoglobin [8], and non-co-operative in the haemoglobin from the enteric parasitic worm *Parascaris equorum* [9]; since the latter haemoglobin binds oxygen non-co-operatively and with high affinity, it seemed to us that some parallelism could be identified in the reaction of oxygen and cyanide with ferrous haemoglobins and myoglobins.

Once we had demonstrated that the analysis of cyanide dissociation may provide new information on the mechanism of co-operativity and ligand recognition under mild experimental conditions (i.e. neutral pH and low ligand concentrations), we undertook a study of this reaction in the two main haemoglobin components from trout blood, haemoglobin I (HbI) and haemoglobin IV (HbIV) [10]. These proteins display different functional properties, and play complementary roles in the physiology of oxygen delivery *in vivo*.

Trout HbI binds oxygen co-operatively and is insensitive to pH and other allosteric effectors [10,11]; its main function is that of assuring the basic level of oxygen to the active tissues. On the other hand, co-operativity and oxygen affinity of HbIV display dramatic sensitivity to allosteric effectors, most notably to the hydrogen ion; indeed, while oxygen binding is co-operative at pH 7–7.5, it becomes non-co-operative at pH 6.5 or less, and the affinity of one type of polypeptide chain for this ligand drops so drastically that a partial oxygen pressure of several atmospheres is required to achieve complete saturation. This phenomenon, called the Root effect [12], allows trout HbIV to release oxygen against a high hydrostatic pressure into the swim bladder [13] and the tissues of the eye ([14]; for a review see [15]).

The molecular basis of the Root effect has been much investigated [16,17]; briefly, we may say that at least two factors contribute to production of the full expression of the Root effect, namely the pH-induced stabilization of the low-affinity T allosteric state in liganded haemoglobin (i.e. the inhibition of the

Abbreviations used: HbI, haemoglobin I; HbIV, haemoglobin IV; R, relaxed; T, tense; SVD, singular value decomposition.

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allosteric transition) and the functional inequivalence of the two chains. The former factor has been correlated to the presence of a serine residue at position β 93 (F9) [16]; using site-directed mutants of human haemoglobin and comparative analysis, this substitution has been successively proven to be important but not sufficient to stabilize the T state at low pH values [18].

A study of cyanide dissociation from the ferrous derivatives of trout HbI and HbIV is expected to clarify some questions, i.e. whether or not cyanide dissociation discriminates between the two haemoglobin chains and is able to express the Bohr and Root effects; the results presented below demonstrate some unforeseen properties of these haemoglobins.

MATERIALS AND METHODS

Experimental techniques

HbI and HbIV from trout blood were prepared as described previously [11,19]. Reagents were of analytical grade.

The instrument used to record transient spectroscopic changes is a Tracor TN6500 photodiode array spectrophotometer coupled to the observation chamber of a Gibson Durrum stopped-flow apparatus [7]; each experiment was output as an MS-DOS file containing up to 80 uncorrected transmittance spectra recorded over an arbitrarily decided time scale ranging from 5 ms to several minutes; rescaling to a separately recorded baseline spectrum of the buffer solution yielded the true transmittance spectra. The spectral range recorded by the 1024-element photodiode array may be, depending on the grating monochromator chosen, either 300 or 150 nm, with a nominal resolution of 0.3–0.15 nm/diode (the actual resolution is slightly poorer due to the finite width of the light beam which passes though the 0.25 mm slit).

Methaemoglobin was obtained by a 10 min incubation of the corresponding oxygenated derivative with a 5-fold stoichiometric excess of potassium ferricyanide and 1 mM sodium cyanide at room temperature; ferricyanide was removed by gel filtration on a Sephadex G25 column equilibrated in the desired buffer (containing 1 mM cyanide) and the protein was either used immediately or stored at -20 °C as the cyanomethaemoglobin derivative.

In a typical experiment a 1–5 μM solution of purified methaemoglobin, dissolved in the desired buffer (containing 1 mM sodium cyanide), was mixed in the stopped-flow apparatus (thermostatically maintained at 20 °C) with a 0.1 M solution of sodium dithionite containing, if desired, 1 mM CO and 0.1–10 μM Methyl Viologen (a redox indicator which catalyses the electron transfer from dithionite to cyanomethaemoglobin [20]). The initial derivative is thus cyanomethaemoglobin and the resulting one is either deoxy- or carbonmonoxyhaemoglobin. Since liganded haemoglobin has the high-affinity, relaxed (R) allosteric conformation, while unliganded ferrous haemoglobin has the low-affinity, tense (T) one, in the absence of CO ^{R}cyanohaemoglobin decays to ^{T}haemoglobin and undergoes the allosteric transition, while in the presence of the gas it decays to ^{R}carbonmonoxyhaemoglobin without undergoing the allosteric transition (a replacement reaction, see [21] for a review).

Usually 60 spectra of 1024 transmittance readings were collected over a logarithmically spaced time scale ranging from 5 ms to 60 s and the wavelength range explored was 390 to 690 nm, which comprises the Soret and visible regions [21].

The experimental data were analysed on a Intel 486-based IBM PC-compatible personal computer using commercial software (mostly MATLA by The Math Works Inc., Natick, MA, U.S.A. and FACSIMILE by EAEA, U.K.); a few routines to transfer data between different programs were developed using the Borland Turbo Pascal compiler.

Data reduction and analysis

The 1024 light intensity readings constituting each spectrum were averaged in groups of four and transformed into absorbance values, to yield 256 wavelength spectra. Each experiment was reduced to a $256 \times n$ matrix (the **A** matrix), where n, the number of spectra collected, ranges between 20 and 80, and is usually 60. The columns of **A** are difference spectra, their baseline spectrum being that of the species populated at the end of the reaction (i.e. either haemoglobin or carbonmonoxyhaemoglobin); the use of difference spectra forces one of the spectroscopic components to zero and reduces the degrees of uncertainty of the subsequent analysis. The **A** matrix represents the convolution of three chemically meaningful matrices, i.e. those of the absorption coefficients (**E**), of the concentrations of the m chemical species (**C**) involved in the reaction, and of the error associated with each measurement:

$$\mathbf{A} = \mathbf{E} \times \mathbf{C} + \text{noise} \quad (1)$$

E is (in this case) $256 \times m$ and **C** is $m \times n$. To unequivocally obtain **E** and **C** is impossible because the problem is underdetermined (the number of chemical species involved, m, is an unknown; many couples of **E** and **C** may yield **A** and the elements of the matrix noise have to be minimized).

Since **E** and **C** cannot be calculated, we applied the Singular Value Decomposition (SVD), a matrix deconvolution algorithm which yields the three matrices, **U**, **S** and **V** [22,23]:

$$\mathbf{A} = \mathbf{U} \times \mathbf{S} \times \mathbf{V}^{T} \quad (2)$$

where $\mathbf{V}^{T}$ is the transpose of matrix **V**. This deconvolution is unequivocal because of the following internal constraints: the $256 \times n$ **U** and the $n \times n$ **V** matrices are orthogonal matrices, while the $n \times n$ **S** is a diagonal matrix whose elements are positive and arranged in decreasing order.

U, **S** and **V** have no immediate physicochemical meaning, i.e. there is no model independent procedure to reduce them to the desired matrices **E** and **C**. However, a number of points should be appreciated. (i) Each column of **U** represents the pseudo-spectrum of one of the spectroscopic components detected by the algorithm. (ii) **S** represents a relative 'scaling factor' of **U**, and therefore the product $\mathbf{U} \times \mathbf{S}$ corresponds to the absorbance of the spectroscopic components, which do not necessarily correspond to pure chemical components. (iii) Because of the decreasing value of the elements of **S** only the first few columns of $\mathbf{U} \times \mathbf{S}$ are significant; indeed our data are usually described by the first two (or, at the most, three) components of **U** (that is, all the pertinent information is contained in a 256×2 **U** matrix, a 2×2 **S** matrix and a 60×2 **V** matrix; we call these the 'reduced' matrices ^{r}**U**, ^{r}**S** and ^{r}**V**). (iv) The columns of the **V** matrix describe the time courses of each spectroscopic component; **V** is the only numerical structure containing time-dependent information.

Point (iii) is important and deserves further discussion. As the SVD is not an 'exact' procedure, the product $\mathbf{U} \times \mathbf{S} \times \mathbf{V}^{T}$ yields an approximation of **A**, thus the more 'components' (i.e. **U** and **V** columns) selected, the better the approximation obtained; therefore the choice of number of significant components is both crucial and somewhat arbitrary. We assumed that the correct number of components is the lowest required to satisfy the condition that the average absolute difference between the

original data (elements of **A**) and the reconstructed data (elements of the product ${}^{r}\mathbf{U} \times {}^{r}\mathbf{S} \times {}^{r}\mathbf{V}^{T}$) is less than 1 % of the mean of the absolute original data:

${}^{t}\mathbf{A}(i,j)$ is defined as $\Sigma\ {}^{r}\mathbf{U}(i,h) \times {}^{r}\mathbf{S}(i,i) \times {}^{r}\mathbf{V}^{T}(h,j)$

so that the number of spectroscopic components (h) satisfies the condition:

$$\Sigma\,|\mathbf{A}(i,j) - {}^{t}\mathbf{A}(i,j)| < \Sigma\,|\mathbf{A}(i,j)|\,/100 \qquad (3)$$

This condition is in keeping with a reasonable estimate of the accuracy of the instrument (approx. 5 parts in 1000), and holds only in experiments where no significant baseline fluctuations have occurred (baseline fluctuations may be corrected by selectively removing the $\mathbf{U} \times \mathbf{S}$ and **V** columns in which they are contained, to yield a noiseless approximation of the original data [23]; this procedure was unnecessary in the present study).

Further analysis consists of the fitting of the columns of ${}^{r}\mathbf{V}$ to the desired kinetic scheme. All the kinetic schemes used in this work represent variations of the two-step mechanism:

$$\mathrm{Hb^{+}CN^{-}} \xrightarrow{\mathrm{Na_2SO_4}} \mathrm{HbCN^{-}} \xrightarrow{\mathrm{H^{+}}} \mathrm{Hb + HCN}$$

Scheme 1

already used in previous work [7,8]. The fitting procedure yields the rate constants of the two processes (reduction and dissociation) and the amplitudes of the **V** components, i.e. the values that the elements of **V** would assume if each spectroscopic component were populated to 100 %; these may be collected in the matrix **V fit** (which, for the fitting of a two-column **V** to the above Scheme 1, has the size 3×2, the last column being an array of zeroes); thus the matrix of the differential absorption coefficients of the actual chemical species involved in the reaction is obtained from:

$$\mathbf{E} = ({}^{r}\mathbf{U} \times {}^{r}\mathbf{S} \times \mathbf{V}\ \mathbf{fit}^{T})/C_{tot} \qquad (4)$$

(where C_{tot} represents the scalar value of the total protein concentration, the number of chemical species (m) is 3 for Scheme 1 and **E** is the $256 \times m$ matrix of absorption coefficients defined in eqn. (1). Absolute absorbance values are obtained by summing the reference spectrum (scaled by C_{tot}). Notice that **E** obtained with this method is model-dependent (as are the values of **V fit**).

Experiments carried out at the same pH in the presence and absence of CO should have identical reduction phases, but may differ in the cyanide release phase, since in the absence of the gas the end product is unliganded T-state haemoglobin while in its presence the end product is R-state carbonmonoxyhaemoglobin. For this reason the appropriate **V** columns of such experiments were fitted together to yield common reduction rate constants, while allowing the dissociation rate constants to depend on CO.

RESULTS

Spectroscopic transitions upon mixing cyanomethaemoglobin with dithionite

Two typical sets of difference absorbance spectra are shown in Figure 1 (upper panel: HbI; lower panel: HbIV); the experiments presented were carried out in 0.1 M Tris/HCl buffer, pH 8.3, containing 1 mM sodium cyanide and the reduction of the cyanomethaemoglobins was achieved by mixing with 0.1 M dithionite; at this pH both haemoglobins would bind oxygen co-

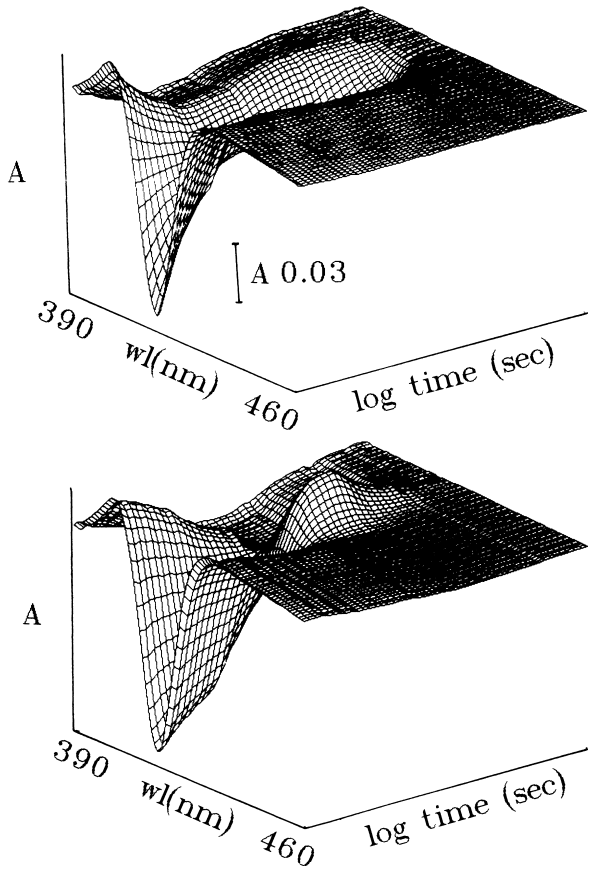


Figure 1 Difference spectra recorded in the course of a typical experiment

Upper panel: trout HbI; lower panel: trout HbIV. Experimental conditions (both panels): 0.1 M Tris/HCl buffer, pH 8.3, 2 μM haemoglobin, 50 mM sodium dithionite (concentrations after mixing). Spectra were collected over the wavelength range 390–690 nm (the range reported in this Figure is only 390–460 nm) and over the time range 5 ms–20 s (see also Figure 2).

operatively and neither of them would show any evidence for intramolecular heterogeneity with this ligand. It is clear that the two proteins behave differently, since HbIV displays, at intermediate times, a positive peak centred around 435 nm, whereas HbI does not. This difference has been attributed with confidence to a large heterogeneity in the reduction of the iron atom of the α and β chains of cyanomethaemoglobin I by dithionite (as shown below).

SVD analysis

The spectra recorded at different times after mixing were collected in a rectangular matrix and subjected to the SVD procedure as described in the Materials and methods section.

It will be appreciated that while the singular spectroscopic components (i.e. the columns of **U**) are very similar for the two haemoglobins (Figures 2A and 2B), their time evolutions (i.e. the columns of **V**) are significantly different (Figures 2C and 2D), confirming the expectation that HbI and HbIV are spectroscopically similar but functionally different.

All our experiments are described by at most three spectroscopic components, judging mainly from the values of the elements of matrix **S** and the amplitude of the columns of $\mathbf{U} \times \mathbf{S}$, and most often two of them satisfy the condition defined by eqn. (3). Taking as examples the two experiments reported in Figures

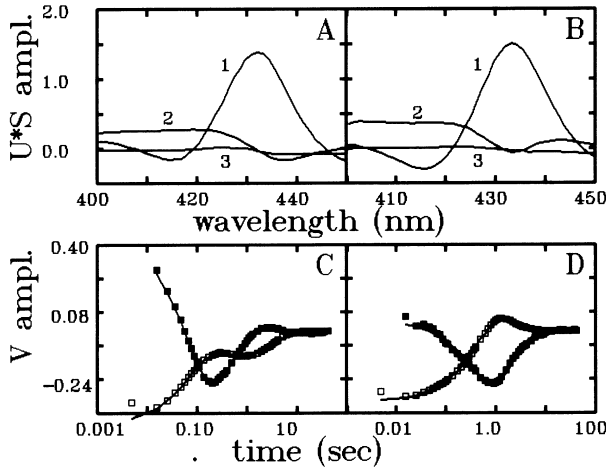


Figure 2 SVD analysis of the experiments presented in Figure 1

(**A**) and (**B**): columns 1–3 of the $\mathbf{U} \times \mathbf{S}$ matrices as obtained from the SVD of the spectra reported in Figure 1. (**A**) HbI; (**B**) HbIV. (**C**) and (**D**): columns 1 (open squares) and 2 (closed squares) of **V** (from the SVD of the spectra reported in Figure 1). (**C**) HbI; (**D**) HbIV. The lines represent the best fit according to kinetic Scheme 3 for HbI or to Scheme 1 for HbIV (see text).

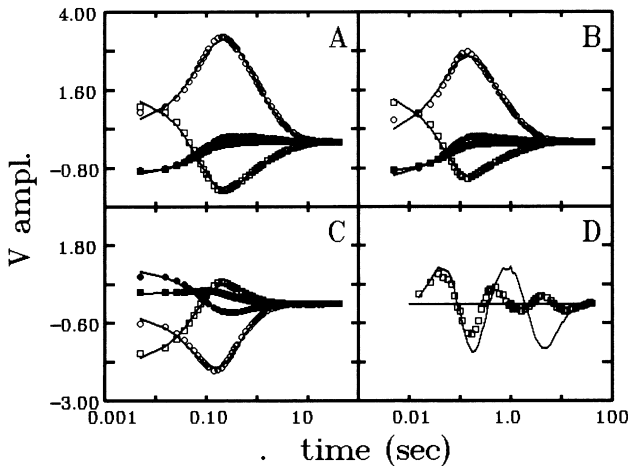


Figure 4 Time course of the amplitude of V for HbIV

Same as Figure 3; (**A**) pH 8.3; (**B**) pH 7.2; (**C**) pH 6.0; (**D**) residuals of the fit of column 1 of **V** at pH 8.3 in the presence of CO. Solid line: Scheme 2; squares: Scheme 2.

2(A) and 2(B), which report the amplitude of the first three columns of $\mathbf{U} \times \mathbf{S}$, it may be noticed that the values of the diagonal elements of **S** are 4.27, 1.37 and 0.47 (HbI, Figure 2A) and 4.64, 1.84 and 0.49 (HbIV, Figure 2B); subsequent elements of **S** further decrease to approx. 0.07 and 0.03 respectively and so on (in both cases). Accordingly, the amplitude of columns 3 to 60 of $\mathbf{U} \times \mathbf{S}$ is very close to zero. Since the minimum number of spectroscopic components is three (for the three chemical species involved, see Schemes 1, 2 and 3) and the spectrum of the third species is forced to zero by the use of difference spectra, this result suggests that the system is simple, i.e. that either (i) the two haemoglobin chains are spectroscopically equivalent or (ii) the chains are spectroscopically inequivalent but the time course of their reaction is closely superimposable (so that at any given time the relative amount of each derivative of the α and β chain is the same). The analysis reported below shows that the time courses of the amplitude of **V** columns are satisfactorily described assuming that the two chains are functionally inequivalent while spectroscopically similar; it is interesting that the ferrous cyanide derivatives of the α and β chains of human haemoglobin are spectroscopically inequivalent [8].

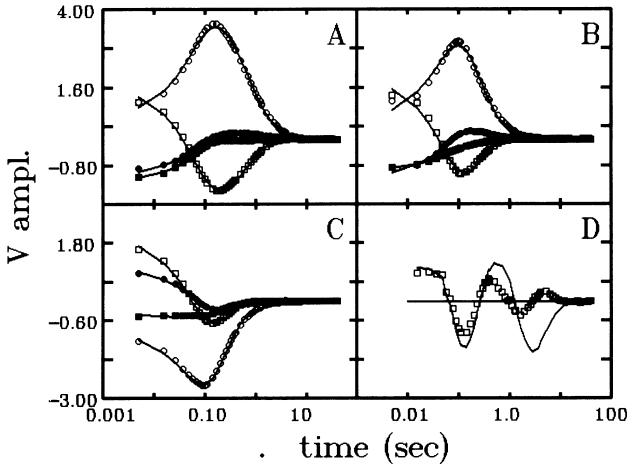


Figure 3 Time course of the amplitude of V for HbI

Amplitude of columns 1 and 2 of **V** obtained from the SVD of a set of cyanide dissociation experiments carried out in the presence of Methyl Viologen (the original spectra and the columns of **U** are not reported). Experimental conditions, all panels: 0.1 M phosphate or Tris/HCl buffer, 2 μM Hb, 50 mM sodium dithionite, 0.5 μM Methyl Viologen. Symbols: squares: no CO; circles 0.5 mM CO; open symbols: **V** column 1; closed symbols **V** column 2. Continuous lines represent the best fit according to Scheme 2 (**A**, pH 8.3; **B**, pH 7.2; **C**, pH 6.0; **D**, residuals of the fit of column 1 of **V** for pH 8.3 in the presence of CO amplified 10-fold; solid line: Scheme 1; squares: scheme 2); dotted lines: the best fit according to Scheme 1.

The reduction of cyanomethaemoglobin I by dithionite is complex and several kinetic schemes may be applied; thus we carried out a set of experiments in which the reduction of the two cyanomethaemoglobins was achieved in the presence of different concentrations of Methyl Viologen, a redox indicator which reacts rapidly with dithionite and haemoglobin and thereby catalyses the reduction of the haem iron [20]. As shown below, the additional information collected in these experiments allows an unequivocal interpretation of the kinetic behaviour of both haemoglobins.

Kinetics of cyanide dissociation in the presence of Methyl Viologen

As shown in Figure 3 for HbI and in Figure 4 for HbIV, in the presence of Methyl Viologen the reduction of the haem iron and the dissociation of cyanide are widely separated in time, and the reaction profile assumes the characteristic rise and fall shape [the half-time for the reduction phase being approx. 0.04 s independent of pH, while that for the dissociation phase ranges between 0.15 and 1 s (Table 1)]. Since Methyl Viologen absorbs light in the spectral range 500–700 nm and below 400 nm, we limited our observations to a spectral window covering the Soret region (410–460 nm).

Experiments were also carried out in the presence of 0.5 mM CO (after mixing); in this case the reaction product is carbonmonoxyhaemoglobin and the gas effectively replaces cyanide [21]. In fact, since the pseudo-first-order rate constant for CO combination under these conditions is high (over 100 s^{-1}) and

Table 1 Rate constants for the reduction and dissociation steps obtained from the best fit of the first two columns of the V × S matrices to kinetic Scheme 1 (as described in the Materials and methods section)

Experimental conditions were as described in Figures 3 and 4. k_r is the rate constant for the reduction of cyanomethaemoglobin, k_d that for cyanide dissociation in the absence of CO and k_{dCO} the one for cyanide dissociation in the presence of 0.5 mM CO. (Note: fits were carried out on sets of four time courses, namely **V**1 and **V**2 for experiments carried out in the presence of CO and **V**1 and **V**2 for experiments carried out in the absence of the gas; the same procedure was adopted for the fit to more complex models, as reported in Table 2 and therefore the values of the sum of squared residuals may be compared directly in the two tables.)

Hb	pH	k_r (s^{-1})	k_d (s^{-1})	k_{dCO} (s^{-1})	Sum of squared residuals
I	8.3	16.5	1.40	1.04	0.63
I	7.2	18.5	5.43	3.35	0.92
I	6.0	19.1	4.77	3.48	0.45
IV	8.3	14.4	0.67	0.67	0.95
IV	7.2	20.9	1.24	1.07	1.01
IV	6.0	13.2	2.39	1.93	0.48

exceeds that of the dissociation of cyanide by a factor of 20 or more (Tables 1 and 2), haemoglobin remains liganded throughout the reaction time course, and partially liganded intermediates are never populated [all the molecules being described by the formula $Hb(CN^-)_n(CO)_{4-n}$, with n ranging from 0 to 4]; consequently the allosteric transition does not occur. On the other hand, in the absence of CO unliganded intermediates accumulate, the allosteric transition occurs and the reaction rate is expected to increase as the reaction proceeds.

The simplest Scheme 1 was applied to describe the reaction of both haemoglobins under all the experimental conditions (Figures 3 and 4, dotted lines in all panels, and Table 1), with the assumption that the reduction step is independent of CO. The description offered by Scheme 1 is useful as a gross screening of the data but is often unsatisfactory after a deeper analysis, as detailed below (see the residuals of the fit at pH 8.3, reported as a continuous line in Figures 3D and 4D).

In the presence of CO in both haemoglobins (and in the case of HbIV also in its absence), the dissociation of cyanide is better described by two exponentials (see the residuals shown as open squares in Figures 3D and 4D). A plausible hypothesis to explain the observed time courses is that in both haemoglobins the α and β chains in the R state release cyanide at different rates; this accounts for the biphasic reaction observed in these cases and suggests the following kinetic scheme:

$$\text{Alpha } (Fe^{3+})\text{–CN} \xrightarrow{k_r} \text{alpha } (Fe^{2+})\text{–CN} \xrightarrow{k_a} \text{alpha } (Fe^{2+})\text{–CO}$$
$$\text{Beta } (Fe^{3+})\text{–CN} \xrightarrow{k_r} \text{beta } (Fe^{2+})\text{–CN} \xrightarrow{k_b} \text{beta } (Fe^{2+})\text{–CO}$$

Scheme 2

An implicit assumption underlying Scheme 2 is that the possible heterogeneity of the reduction of the two chains in the presence of Methyl Viologen and dithionite is negligible (i.e. k_r is the same); this is consistent with the experimental results.

Since we cannot unequivocally attribute the fast and slow phases to either the α or β chains, in the following description and in Table 2 the two dissociation rate constants have been referred to as k_{d1} and k_{d2}, instead of k_a and k_b.

The time courses of all the experiments carried out on HbIV, and those on HbI in the presence of CO, were fitted to Scheme 2 with the additional assumption that the α and β chains may not share the same absorption coefficients (as discussed above this assumption is expected to be redundant and proved indeed unnecessary in HbIV); moreover, since the original spectra are difference spectra, the absorption coefficients of α-CO and β-CO were fixed to zero (reference spectrum). The results of this fit are reported in Table 2, and as continuous lines in Figures 3 and 4; the two kinetic schemes may be compared from the sum of the squared residuals (SSR in Tables 1 and 2) and from the plot of the residuals of the two fits at pH 8.3 in the presence of CO (Figures 3D and 4D).

Trout HbIV dissociates cyanide at approximately the same rate in the presence and in the absence of CO, suggesting minor co-operativity in the rate of dissociation of the anion (see Table 1); accordingly the time course is always heterogeneous. Moreover when the reaction of HbIV was fitted to Scheme 2 the level of each of the slow- and fast-reaction components approaches almost 50 %, confirming that they correspond to the two chains,

Table 2 Rate constants for the reduction and dissociation steps obtained from the best fit of the first two columns of the V × S matrices to kinetic Schemes 1 or 2 (as described in the text)

Experimental conditions were as described in Figures 3 and 4. (**A**) Experiments on HbI fitted to Scheme 1 in the absence of CO and to Scheme 2 in its presence; the values of k_r and k_{d1} were forced to be independent of CO, but in the presence of this ligand a fraction of the optical transition is governed by the slower rate k_{d2}. (**B**) Experiments on HbIV fitted to Scheme 2 in the absence and in the presence of CO; the values of k_r and the fraction of the slowly dissociating component were forced to be independent of CO and those of k_{d2} were independent of CO (hence only one value is quoted).

(A) Experiments on HbI

pH	k_r	k_{d1}	k_{d2} (CO only)	Fraction SRF	Sum of squared residuals
8.3	15.6	1.47	0.338	0.191	0.298
7.2	16.7	5.65	0.98	0.195	0.46
6.0	15.6	5.97	0.98	0.170	0.124

(B) Experiments on HbIV

pH	k_r	k_{d1} (no CO)	k_{d1CO}	k_{d2}	Fraction SRF	Sum of squared residuals
8.3	12.0	2.99	1.39	0.345	0.46	0.270
7.2	16.9	6.03	2.65	0.553	0.41	0.357
6.0	12.1	6.03	4.37	1.32	0.50	0.266

and a good fit was obtained by fixing this parameter to 50 %, i.e. imposing perfect spectroscopic equivalence of the two chains.

As for the rate constants for dissociation exhibited by HbIV, we show that the faster component is sensitive to CO, while the slower is not: indeed, although the fitting routine was devised to allow the values of both the dissociation rate constants to be dependent on CO (in order to simulate the possible co-operativity), the value of k_{d2} was estimated to be the same in the presence and in the absence of the gas. It is evident that co-operativity (if present) should be expressed as an increase of the slower (rather than the faster) rate constant in the absence of CO and thus this result would not be explained by implying that the reaction is co-operative. These data, however, would fit nearly as well by imposing the condition that the two rate constants k_{d1} and k_{d2} are both independent of CO, and exactly as well by allowing the rate constant for the reduction step to vary by a factor of 20 % in the presence and absence of CO, while keeping k_{d1} and k_{d2} independent of CO. In view of these observations we consider the difference between k_{d1} and k_{d1CO} close to the limit of resolution of the technique, and hence not worthy of further analysis at present.

In the absence of CO the dissociation of cyanide from HbI is described adequately by a single exponential, as in Scheme 1 (Figure 3, all panels: squares and dotted lines), while it requires two exponentials in the presence of CO (Figure 3, circles and continuous lines); moreover, as is evident from Tables 1 and 2, in the presence of the gas the dissociation of CN^- is slower, a clear cut indication of kinetic co-operativity. It was demonstrated in an earlier contribution that the dissociation of cyanide from human ferrous haemoglobin in the absence of CO is co-operative [8]; thus, in this respect, the functional behaviour of trout HbI parallels that of human HbA.

That the time course of the dissociation of CN^- is exponential in the absence of CO and biphasic in its presence suggests intramolecular heterogeneity in the R state (as in the case of HbIV). The increase in the reaction speed associated with the allosteric transition cancels out the kinetic complexity and thus in the absence of CO the time course of dissociation appears to be exponential; hence the results obtained in the absence of the gas may be described by Scheme 1, which is oversimplified since its agreement with the experimental data derives from the opposite effects of intramolecular heterogeneity and co-operativity.

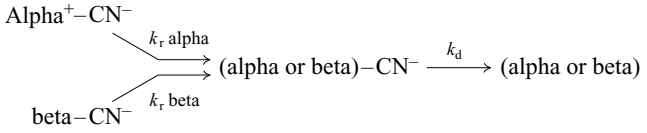
It is unclear why the amplitudes of the slowly and quickly dissociating components are so different (Table 2: Slowly Reacting Form (SRF) of haemoglobin < 20 %), also, in view of the fact mentioned above, that gross spectroscopic heterogeneity is to be ruled out on the basis of the SVD analysis. This result casts doubt on the identification of the heterogeneity of the reaction with a difference between the α and β chains. An alternative hypothesis is therefore suggested in the Discussion section.

Analysis of the time courses recorded in the absence of Methyl Viologen

On the basis of the information obtained in the experiments carried out in the presence of Methyl Viologen, we returned to the reaction of dithionite alone (Figures 2C and 2D).

Completion of the overall reaction of trout HbI with dithionite takes much longer than would be expected on the basis of the rate of cyanide dissociation determined in the reaction with dithionite plus Methyl Viologen. Because the dissociation is independent of the reductant employed, the rate-limiting step is to be identified with the reduction of one type of chain (the other being reduced much faster).

A quantitative description of this reaction demands a modified version of Scheme 1 to allow different rate constants for the reduction and dissociation of the α and β chains (plus a co-operativity factor). Since this model requires the determination of at least four rate constants it is subjected to large uncertainties and we did not even attempt to use it; we used instead a simplified scheme to test quantitatively this hypothesis in the case of trout HbI (Figure 2C), under the assumption that the chemical inequivalence of the two chains in the dissociation of cyanide in the absence of CO can be neglected, because of co-operativity (see above). The kinetic scheme employed may be summarized as follows:



Scheme 3.

Scheme 3 requires the determination of three rate constants ($k_{r\alpha}$, $k_{r\beta}$ and k_d) and implies spectroscopic equivalence of the two chains; as for the dissociation rate constants in Scheme 2, we are not able to decide whether the faster reduction rate refers to the α or the β chain and hence in the following discussion the two reduction constants are referred to as k_{r1} and k_{r2}. The relevance of k_d in the description of the time course, according to this Scheme, depends on the ratios k_{r1}/k_d and k_{r2}/k_d, and, in the case of HbI, is very small because one of the two reduction rate constants is smaller than k_d (Figure 2C). For this reason the value of k_d is poorly determined in this kind of experiment and the only conclusion to draw is that the value of k_d obtained in the experiments carried out in the presence of Methyl Viologen can also be satisfactorily used to describe the time course of the reaction in the absence of the dye. In spite of its limitations Scheme 3 offers a satisfactory description of the experimental data (Figure 2C).

The reaction of cyanomethaemoglobin IV with dithionite generates the intermediate only slightly, and the simplest kinetic scheme (Scheme 1) is sufficient for a qualitative description (Figure 2D). From the results presented in this and the above section, it was possible to calculate the spectrum of the intermediate species, which shows a Soret peak at 433 nm and is very similar to that already reported for HbA [8].

Effect of pH on the dissociation of cyanide

The most interesting functional feature of trout HbIV is the Root effect (see the Introduction); thus we studied the pH-dependence of cyanide dissociation from this haemoglobin and from HbI, whose reaction with oxygen and CO is pH-insensitive instead. As evident from Tables 1 and 2, the effect of pH on the dissociation of cyanide from ferrous HbI and HbIV is not only small, but is the same for the two haemoglobins. Since this reaction is pH-sensitive even in myoglobins devoid of Bohr effect for oxygen, presumably due to the protonation state of the distal histidine (which interacts with the bound anion and may catalyse its protonation to hydrocyanic acid, thus promoting its dissociation

[7]), we conclude that the Root effect characteristic of HbIV is not manifested in the rate of dissociation of cyanide. This conclusion is in good agreement with other results presented in this work, as discussed below.

DISCUSSION

The results presented above reveal some unexpected properties of the two main haemoglobin components from trout blood; indeed these two haemoglobins display marked differences in their oxygen-binding properties, while the dissociation of cyanide is quite similar. The obvious difference in the functional behaviour of the two haemoglobins observed in Figures 1 and 2 has been ascribed to the reduction reaction, which depends also on factors other than the reactivity of the haem iron (such as the charge on the surface of the protein in the reductant binding site), and will not be considered further.

Properties of HbI

Trout HbI binds oxygen co-operatively and its affinity is independent of allosteric effectors (pH, organic phosphates, etc.); moreover the reactivities of its α and β subunits with this ligand are equivalent. By contrast, the dissociation of cyanide shows a marked intramolecular heterogeneity and a significant pH-dependence which, in the absence of Bohr groups and on the basis of our previous work on myoglobins [7], is attributed to the interaction and the possible proton transfer from the protonated distal His (E7) to the bound anion (or to the anion in the haem pocket). Moreover the dissociation of cyanide from this haemoglobin expresses a detectable kinetic co-operativity (responsible for the overall faster and apparently homogeneous dissociation of cyanide in the absence of CO) in good agreement with the co-operativity observed with oxygen.

Properties of HbIV

The reaction of trout HbIV with oxygen is exquisitely sensitive to pH and below pH 6.5 co-operativity disappears, the protein being frozen in the low-affinity T state; moreover the two chains display marked functional inequivalence, the partial pressure of O_2 required to achieve half saturation of the α- and β-chain ($p_{\frac{1}{2}}$) differing by a factor of 1000 (the Root effect [12–14,24]). Since this protein undergoes an allosteric transition upon oxygen binding in neutral or alkaline environments (and since the two chains are almost equivalent in the R state), under these experimental conditions co-operativity overcomes the functional inequivalence of the two chains. Thus the oxygen-binding properties of trout HbIV show a marked discontinuity over the physiological pH range, allowing the protein to upload oxygen at relatively low tension in the gills and to release oxygen against high partial pressures of this gas in the swimbladder and the choroid rete in the eye, two organs which are able to lower significantly the blood pH [13–15]. It is also pertinent to observe that the Root effect is much smaller when CO is chosen as the ligand, mainly because the affinities of the α and β chains for this ligand are almost equivalent, both in the R and the T state.

The results presented here show a number of effects. (i) Trout HbIV displays minor or no co-operativity in the rate constant for cyanide dissociation, even when the experiment is carried out in an alkaline buffer (i.e. where oxygen and CO are co-operative). This is puzzling, since human haemoglobin [8] and trout HbI are both co-operative in this reaction.

(ii) This haemoglobin displays almost no effect of pH over and above that already observed in the case of trout HbI and most myoglobins, which has been attributed to the protonation of the bound anion assisted by the distal His [7]. Human haemoglobin, on the other hand, has a much more marked effect of pH, because of the additional contribution of the quaternary effect linked to the protonation of the Bohr residues [8]. This observation is consistent with the non-co-operative dissociation of cyanide, since the most important contribution to the Bohr effect is linked to the allosteric transition.

(iii) There is no evidence of the Root effect.

These three experimental observations would be explained assuming that the dissociation rate of cyanide is the same in the T and R states.

Concluding remarks

This paper adds some information to the relevance of the protein–ligand interactions in determining the co-operativity of haemoglobin; it is indeed uncommon that the same ligand is bound co-operatively by one haemoglobin and non-co-operatively by another (provided that both are capable of co-operative oxygen or CO binding).

Co-operative ligand binding by haemoglobin is the result of the presence of two allosteric states with different ligand affinities, and demands that the low-affinity conformation is energetically favoured in the absence of ligand. In the case of HbI the above conditions are fulfilled, although with the complication of the intramolecular heterogeneity, while in the case of HbIV the results presented above do not warrant a conclusive statement, but would be consistent with a minor difference in the rate constant of cyanide dissociation in the T and R states.

As anticipated in the Results section, the unequal amplitudes of the quickly and slowly dissociating forms of HbI apparently contrast with the attribution of the two kinetic phases to the two types of chain. Since the starting protein is pure and the reaction is homogeneous in the absence of CO, this result is puzzling; as a working hypothesis, we may suggest that this might be due to a difference in co-operativity between CO and CN^-. In this case one might expect that $Hb(CN)_4$ behaves differently from $Hb(CO)_3CN$. This is by no means the only possible explanation, but we suggest it over all others in view of the fact that it is in keeping with the low kinetic co-operativity of ferrous HbI-CN^-.

It is also important to remark that our observations, being limited to the dissociation reaction, do not exclude the possibility that significant co-operativity may be expressed in the rate constant(s) for the combination of cyanide, similar to what is observed in the case of CO. From this perspective it is surprising that co-operativity for the binding of the same ligand should be expressed differently in the two trout haemoglobins. According to the relationship between kinetic co-operativity and the properties of the transition state developed by Szabo [25], this finding implies that in the case of the reaction of cyanide, the transition state differs in the two haemoglobins, being closer to the deoxy T state in HbIV than in HbI.

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