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Kinetics of oxygen binding and subunit assembly for the hemoglobin alpha subunit

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Abstract

A thorough kinetic characterization of the O_2-binding and self-association reactions of α-subunits of human hemoglobin A has been performed. All of the rate constants for a five step reaction model linking the monomer–dimer reaction to the O_2-binding steps have been determined for the first time. Our analysis of the ligand binding reaction shows that both monomer and dimer have nearly identical intrinsic O_2-association and dissociation rate constants and therefore identical affinities for oxygen. During this investigation we discovered a small absorbance difference between the oxy-monomer and oxy-dimer alpha-subunits. This difference spectrum enabled direct measurements of the αO_2 self-association reaction. We find an association rate constant of, 2.0 10^5 $M^{-1}s^{-1}$, similar to that for other subunit assembly processes in the hemoglobin system. Our results also suggest that the deoxy-subunit assembly kinetics must be similar to that for the oxy-subunit. These kinetic results together with the equilibrium constants obtained for these solution conditions by Ackers and coworkers provides, for the first time, a complete kinetic and thermodynamic description of all the intrinsic ligand binding and association reactions for alpha-subunits.

Keywords: Hemoglobin A; Oxygen binding kinetics; α-Subunit self-association

1. Introduction

The properties of the isolated α- and β-subunits of hemoglobin serve as a basis set and framework for understanding the more complex behavior of the hemoglobin A tetramer. Therefore, since the pioneering studies by Bucci et al. [1,2], there have been numerous investigations of the kinetic and thermodynamic behavior of the α- and β-subunits, and inter-comparisons of these data with those for the 'R'- and 'T'-states of hemoglobin. However, such quantitative comparisons can be unreliable when made between data for different solution conditions.

In the late 1970's it was discovered by Gary Ackers and coworkers that the properties of the isolated subunits are more complex than previously thought. For example, their studies showed that the extent of association of β-subunits into

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β-tetramers is linked to oxygenation, and therefore the O_2 affinities of β_4 is significantly higher than that of β-monomers [3,4]. Thus, intersubunit interactions in the β-tetramers act to raise the affinity, rather than lowering it as in the T state of hemoglobin A, a phenomenon they named "quaternary enhancement". Similar studies with α-subunits showed that self-association lead to homogeneous α_2-dimers, but the extent of association did not appear to be ligand-linked which implies there is no O_2-affinity difference between α and α_2.

Because of this new complexity in the behavior of the subunits, and because Ackers and coworkers have provided an extensive data base of equilibrium and thermodynamic data for a single set of solution conditions, in our laboratory we have been re-investigating the kinetic behavior of the isolated subunits and hemoglobin A under these same solution conditions (0.1 *M* Tris-HCl, 0.1 *M* NaCl, 1.0 m*M* EDTA, pH 7.4). We have previously reported a kinetic analysis of the self-association and ligand-binding reactions for the β-subunits [5], and have recently reported O_2-binding kinetics for the $\alpha\beta$-dimer and the fourth O_2-molecule on the hemoglobin tetramer [6]. In this study, we complete this series of kinetic studies with an investigation into the kinetics of oxygen binding and self-association reactions for the hemoglobin alpha subunit. A unique feature of our study was the ability to measure, directly, the ligand binding rate constants for the dimer and to directly measure the self-association reaction of the oxygenated alpha-subunit.

2. Materials and methods

The α-subunits of hemoglobin A were prepared as described previously [7], stored under liquid nitrogen, and dialyzed against "Ackers' buffer" (0.1 *M* Tris-HCl, 0.1 *M* NaCl, 1 m*M* EDTA, pH 7.4) prior to use. Greater than 95% of the cysteine residues were in the reduced state. Protein absorption spectra were measured with a computer interfaced CARY 118C spectrophotometer at 15°C using methods described previously [7]. The protein concentration of homogeneous oxy-, deoxy-, and met-α-samples were calculated from the absorption spectrum using molar extinction coefficients determined previously for each species [8]. Alpha-subunit samples were deoxygenated initially in a tonometer with flowing hydrated nitrogen gas at 5°C and then transferred under positive nitrogen pressure to a cooled temperature-jump (T-jump) cell. Increasing oxygen saturations in the cell were obtained by titrating small amounts of oxy-α-solution into the T-jump cell. The absorption spectrum of a sample in the T-jump cell was measured using a specially adapted cell holder inside the CARY 118C spectrophotometer. The protein concentration of each alpha-subunit species (oxy-α, deoxy-α, and met-α) within the T-jump cell were calculated from the sample's visible absorption spectrum using a recursive multiple linear least squares computer program and the known extinction coefficient spectrum fo each species [8,9]. This calculation was performed on spectra taken before and after each temperature jump. Molar extinction spectra and tables containing characteristic maximum and isobestic points in the visible and Soret wavelength region for oxy-, deoxy-, and met-alpha-subunits were available from previous studies [8]. The temperature jump instrument, data acquisition and analysis have been described previously [8,10]. Temperature jump relaxation spectra were simulated using a computer program based on that written by Ilgenfritz [11]. The computer program was written to describe the relaxation rates and amplitudes presented in Scheme I in Section 3 [8]. The temperature jump magnitude was set to 5°C with a final temperature of 15°C (a final temperature of 15°C was used to limit oxidation and denaturation of the protein). The small equilibrium perturbation for both the monomer–dimer and ligand binding reactions, induced by an increase in temperature, resulted in a less than 2% change in species concentration. Due to the magnitude of the kinetic and thermodynamic parameters, as well as the small size of the perturbation, linearization of the rate equations for analysis was valid [12]. Temperature jump experiments were recorded at 366 nm in either a 1-cm or 0.2-cm path length cell. The oxygen concentration and extent of

Table 1

Kinetic, thermodynamic and extinction coefficient parameters used for the kinetic analysis of the hemoglobin alpha-subunit reactions in Scheme 1 [a]

Parameter	Reaction step in Scheme 1		
	R1 and R3	R2	R4 and R5
K_i (M^{-1}) [b,c]	5,900	1.78×10^6	1.78×10^5
ΔH_i (kcal/mol) [b,c]	+4.3	−14.2	−14.2
k_i (M^{-1} s^{-1}) [c,d,e]	$2.0\pm0.16\times10^5$	$2.4\pm0.19\times10^7$	$2.7\pm0.1\times10^7$
k_{-i} (s^{-1}) [c,d,e,]	33.6	13.5	15.0
Extinction coefficients (M^{-1} cm^{-1}) (366 nm) [d,f]			
αO_2	α_2O_4	α_2O_2	α	α_2
$2.22\ 10^4$	$4.53\ 10^4$	$5.61\ 10^4$	$3.35\ 10^4$	$6.7\ 10^4$

[a] Conditions are: 0.1 *M* Tris-HCl, 0.1 *M* NaCl, 1 m*M* EDTA, pH 7.4, 15°C.

[b] K_i and ΔH_i are the equilibrium binding constant and enthalpy of reaction, respectively, for reactions $i = 1, 2, 3, 4$ and 5. These thermodynamic parameters were obtained from Valdes and Ackers [3,4]; Mills et al. [15].

[c] Intrinsic values are reported for the kinetic and thermodynamic parameters of reactions 4 and 5.

[d] Values in rows 3 to 5 were determined by this work.

[e] k_i and k_{-i} are the association and dissociation rate constants for reactions $i = 1, 2, 3, 4$ and 5. The dissociation rate constant, k_{-i}, was calculated from K_i and k_i. The value for k_2 was determined from an analysis of Fig. 2. The value for k_4 and k_5 is a concensus value determined from; (1) a weighted linear least squares fit to the data in both Figs. 2 and 3, and (2) The best fit to the data in Figs. 2 and 3 using the kinetic simulation program and values given in this table.

[f] Extinction coefficients were determined as described in the Section 2.0, except the value for α_2O_2 is the average value of α_2 and α_2O_4 extinction coefficients.

dimer formation were calculated using equilibrium binding constants determined previously by Ackers and coworkers (see Table 1).

2.1 Relaxation amplitude analysis

The relaxation amplitude, i.e., the change in optical signal, (ΔA, Abs/cm) of a bimolecular reaction can be calculated according to the equation:

$$\Delta A = l\,\Delta\epsilon\,\Delta c, \qquad \Delta c = \Gamma\left(\Delta H/RT^2\right)\Delta T,$$

$$\Gamma = \left(1/[\alpha] + 1/[\alpha O_2] + 1/[O_2]\right)^{-1}$$

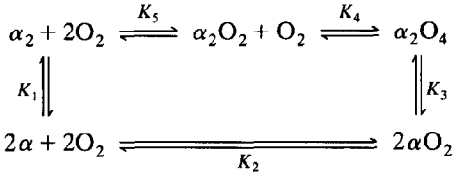
$$\Delta\epsilon = \epsilon_{oxy} - \epsilon_{deoxy}$$

where ϵ_{oxy} and ϵ_{deoxy} are the molar extinction coefficients of the oxy- and deoxy-monomer alpha-subunits (Table 1), l is the path length, Δc is the change in concentration of the deoxy species, ΔH is the enthalpy of reaction (Table 1), ΔT is the size of the temperature jump, T is the absolute temperature and R is the universal gas constant [12]. $[\alpha]$, $[\alpha O_2]$ and $[O_2]$ are the equilibrium concentrations of deoxy- and oxy-alpha-subunit and free oxygen, respectively.

3. Results

3.1 Ligand-binding and self-association reactions

The temperature-jump relaxation method was used to examine the kinetics of oxygen binding to the hemoglobin alpha-subunit. We were particularly interested in determining if there was a difference in the binding kinetics between the monomer (α) and dimer (α_2) species. Potential differences were sought by examining the kinetics



Scheme 1.

over a protein concentration range in which 5 to 55% of the subunits were in the form of dimer, and with oxygen saturations between 20 and 95%.

The simplest model describing the coupled ligand binding and self association reactions is shown in Scheme 1.

For each of these reactions Ackers and coworkers have determined both the equilibrium O_2-binding and self-association constants as well as the enthalpy of reaction (see Table 1). The current kinetic analysis uses these equilibrium constants for determining the concentration of different species.

Scheme 1 contains five elementary reactions which interact to form a closed, circular system. The number of chemical relaxations for this system is four, i.e., the number of independent rate equations. In general, each of the four relaxations represents the complex re-equilibration of the five elementary reactions. The complexity of Scheme 1 requires that one study it under conditions which simplify and reduce the number of observable relaxations.

3.2 Monomer ligand-binding kinetics

Our kinetic investigation of the alpha-subunit began by studying the oxygen binding reaction of samples containing primarily the monomer subunit. T-jump experiments were performed with partially oxygen-saturated protein solutions containing 3 μM to 16 μM heme. The percent heme in the form of dimer was sufficiently low (3 to 14%) that reaction two in Scheme 1 adequately described the major ligand binding reaction.

T-jump experiments consistently showed two phases when the optical signal was measured at 366 nm. The first was a very fast (time unresolved) phase which has been shown to always be present in partially saturated hemoglobin T-jump studies [13,14]. This phase was not a ligand binding reaction, but involved a fast change in the extinction coefficient due to the increase in temperature. The second phase could be described by a single exponential with a relaxation time varying between 2.5 and 15 ms. Data acquisition out to three seconds showed no additional phases other than cooling.

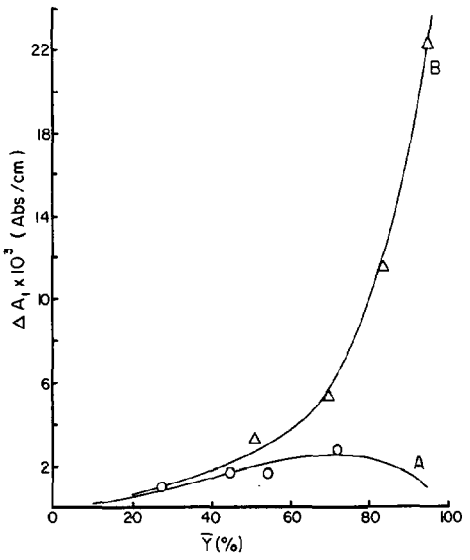


Fig. 1. Relaxation amplitude dependence on the degree of αO_2-saturation. Measured amplitudes for a sample at 5 μM heme (5.3% dimer, heme) are shown as circles. Curve A is the theoretical amplitude dependence on saturation for a bimolecular reaction according to the amplitude equation, given in Section 2, using the parameters for Reaction 2 in Table 1, and for 5 μM heme. Amplitudes measured for samples containing 192 μM (52% dimer, heme) are shown as triangles. Curve B is the O_2-binding amplitude dependence for Scheme 1 determined using the relaxation kinetics computer simulation program, parameters given in Table 1, and 192 μM heme. The following sample conditions were used for all experiments: 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM Na_2EDTA, pH 7.4, 15°C.

The millisecond exponential phase was the oxygen-binding reaction to the monomer subunit. This was confirmed by an analysis of both the relaxation amplitude and relaxation time. Curve A in Fig. 1 describes the theoretical change in relaxation amplitude with respect to saturation for the bimolecular reaction of reaction two. The experimental data, represented by circles, conforms well to this theoretical prediction for a bimolecular reaction.

The concentration dependence of the inverse relaxation time for reaction two is described by the following equation:

$$1/\tau_2 = k_2(\alpha + [O_2]) + k_{-2} \qquad (1)$$

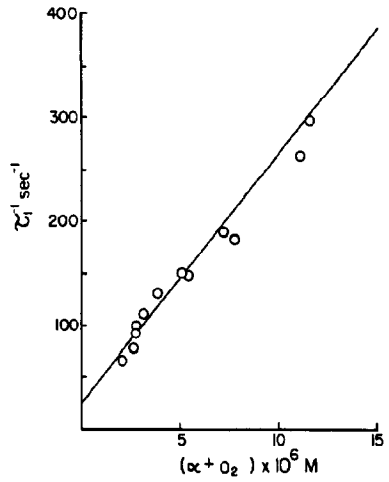


Fig. 2. Concentration dependence of the O_2-binding relaxation rate at low concentrations of alpha-subunits. The x-axis is the sum of the concentrations of deoxy-alpha-subunits and free O_2. Protein concentrations range from 3.0 to 16 μM heme and O_2-saturations are between 28 and 92%. The solid line is a weighted linear least squares fit of eq. (1) using a binding constant of 1.78 10^6 M^{-1}. The fitted values for k_2 and k_{-2} are 2.4 ± 0.18 10^7 M^{-1} s^{-1} and 24.2 ± 7.3 s^{-2}. Sample conditions are as in Fig. 1.

where α and $[O_2]$ are the deoxy-alpha-subunit and free oxygen concentrations at equilibrium. The straight line shown in Fig. 2 represents the best fit from a weighted linear least squares analysis of the experimental data. The inverse relaxation time is linear with respect to $(\alpha + [O_2])$ as expected for a bimolecular reaction. The slope of the fit, and therefore O_2-association rate constant, k_2, is 2.4 ± 0.18 10^7 M^{-1} s^{-1}. The intercept or O_2-dissociation rate constant, k_{-2}, is 24.2 ± 7.3 s^{-1}. Using these rate constants the kinetically determined equilibrium binding constant, ${}^kK_2 = k_2/k_{-2}$, is equal to 0.99 10^6 M^{-1} (0.70 to 1.53 10^6 M^{-1}) (the estimated error was calculated from the combined uncertainty of the rate constants).

3.3 Dimer ligand-binding kinetics

Kinetic experiments were also performed at higher protein concentrations (38 μM to 217 μM heme) to determine if there were kinetic ligand-binding differences between alpha-monomers and dimers. At the highest protein concentration, 55% of the protein was in the form of dimer based on the published monomer–dimer association constants [3]. At a wavelength of 366 nm there were always three relaxation phases observed. Again there was a very fast, time unresolved, phase due to temperature difference spectra which was not analyzed. The second phase was exponential in form and its relaxation time varied from 0.4 to 5.0 ms, depending on the protein concentration and saturation. The third and final phase was also exponential, with relaxation times of 5 to 30 ms and an amplitude that was 5 to 50% of the total amplitude. Data were recorded out to three seconds and no other relaxations were observed. Analysis of the high concentration experiments began with the interpretation of the fast resolved ms phase which involved the oxygen binding reaction.

The principal question we wished to address was whether the O_2-binding kinetics or equilibria of the alpha dimers was significantly different from those of the monomers. Therefore, we analyzed the data for the case in which the two alpha species had identical intrinsic rate constants and asked whether the data differed significantly from this case. Below are written the equilibrium binding constants for the monomer and dimer ligand-binding reactions in terms of both their apparent rate constants and intrinsic O_2-association and dissociation rate constants (k_1, k_{-1}) under the assumption that the intrinsic rate constants are the same for each reaction process, i.e.:

$$K_2 = k_2/k_{-2}, \quad K_2 = K_1 = k_1/k_{-1}$$
$$K_4 = k_4/k_{-4}, \quad K_4 = \tfrac{1}{2}K_1 = k_1/(2k_{-1})$$
$$K_5 = k_5/k_{-5}, \quad K_5 = 2K_1 = 2k_1/k_{-1}$$

Under these conditions oxygen binding for reactions two, four and five in Scheme 1 can be described by the following single reaction:

$$\Sigma\alpha + O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (\Sigma\alpha)O_2$$

where

$$\Sigma\alpha = [\alpha] + [\alpha_2] + [\alpha_2 O_2]$$

For this case only a single relaxation is expected to be observed for the ligand-binding reaction and the inverse relaxation time is linear throughout the protein concentration range as shown in eq. (2).

$$1/\tau_I = k_1(\Sigma\alpha + [O_2]) + k_{-1} \quad (2)$$

The association rate constant, k_1, and the dissociation rate constant, k_{-1}, are easily determined from the slope and intercept respectively [12].

A plot of $1/\tau_I$ vs. $(\Sigma\alpha + [O_2])$ for the high protein concentration experiments is shown in Fig. 3. The indicated points are for the experiments with protein concentrations of 38 to 217 μM heme and oxygen saturations of 34 to 95%. The best weighted linear least squares fit to both the low and high protein concentration data (data points from Fig. 2 not shown in this figure) is shown by curve B in Fig. 3. The intrinsic rate constants determined from this fit are $k_1 = 2.54 \pm 0.07\ 10^7\ M^{-1}\ s^{-1}$ and $k_{-1} = 22.1 \pm 2.2\ s^{-1}$. The kinetically determined binding constant, ${}^kK_1$, for this fit is $1.15\ 10^6\ M^{-1}$ ($1.02\ 10^6$, $1.31\ 10^6\ M^{-1}$). These values fall within the confidence intervals of the rate constants calculated for the monomer (low protein concentration) ligand-binding reaction. Curve A in Fig. 3 is the extrapolated line for the best fit to only the low concentration data (shown in Fig. 2). A weighted fit to only the high protein concentration data gave $k_1 = 2.7 \pm 0.15\ 10^7\ M^{-1}\ s^{-1}$ and $k_{-1} = 10.6 \pm 21.5\ s^{-1}$ (similar to curve C, Fig. 3).

An amplitude analysis of the fast phase further confirmed that this phase was consistent with identical ligand-binding properties for both monomers and dimers. Curve B in Fig. 1 shows the theoretical amplitude dependence of the ligand binding reactions in Scheme 1 with respect to oxygen saturation. This calculation was performed using a small perturbation relaxation kinetics simulation program (Section 2) using various oxygen saturations, a protein concentration of 192 μM and assuming identical intrinsic O_2-affinities for the monomer and dimer (the values used are shown in Table 1). The experimental data (triangles in Fig. 1) behave exactly like that expected for monomer and dimer subunits with identical affinities.

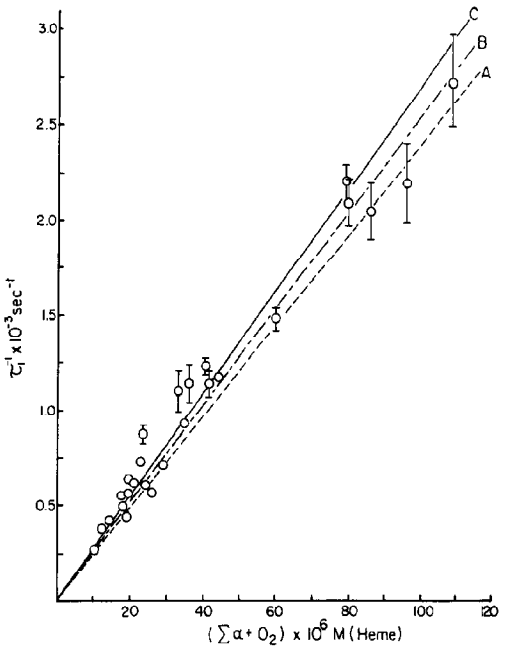


Fig. 3. Concentration dependence of the O_2-binding relaxation rate at high concentrations of alpha-subunits. Data are plotted for protein concentrations of 38 to 217 μM heme (25 to 55% dimer, heme) and O_2-saturations of 34 to 95%. Curves A and B are weighted linear least squares fits to the data according to the following analysis: Curve A is the extrapolated fit for the low protein concentration data shown in Fig. 2 (data not shown here). Curve B is the best fit to data in both Fig. 2 and Fig. 3 according to eq. (2). The slope, k_1, is $2.54 \pm 0.07\ 10^7\ M^{-1}\ s^{-1}$ and the intercept, k_{-1}, is $22.1 \pm 2.2\ s^{-1}$. An equilibrium binding constant of $1.78\ 10^6\ M^{-1}$ was used to calculate the concentrations for deoxy-alpha-subunits and O_2. Curve C is the best fit of the data in Fig. 2 and Fig. 3 for Scheme 1 using the relaxation kinetics computer simulation program. The parameters used for this fit are shown in Table 1 except the value for k_2 was $2.6\ 10^7\ M^{-1}\ s^{-1}$ and k_4, k_5 were $2.8\ 10^7\ M^{-1}\ s^{-1}$. Curve C is also similar to the least squares fit of only the high concentration data shown in Fig. 3. The error bars on the data represent a one standard deviation confidence range (i.e. ± 1 S.D.), determined from the exponential analysis computer program.

3.4 *Self-association kinetics*

The slow relaxation phase observed in the partial saturation, high protein concentration, ex-

periments was more difficult to interpret than the fast ligand-binding phase for two reasons. First, the known thermodynamic [3,4,15,16], kinetic [17] and spectral properties [7] of the alpha-subunit species in Scheme 1, when used in the simulation program, indicated that the amplitudes of all the slow phases should be negligible. Second, the relaxation rate of the slow phase depend only weakly on the protein concentration or O_2-saturation, so this phase could not be assigned readily to one of the elementary reactions.

Suspecting that the slow phase was due to an absorption difference between monomer and dimer, unresolved by previous experiments, we performed T-jump experiments on fully oxygenated alpha-subunit solutions. Experiments were performed on oxy-alpha-subunit solutions, exposed to air to give 99.8% O_2-saturation, with protein concentrations of 46 μM to 242 μM heme. The fraction of dimer in these solutions varied from 28% to 56% (heme). At 366 nm three phases were observed. The first phase was again a very fast, time unresolved, phase arising from temperature dependent absorption spectra. The faster time resolved phase had a small amplitude and a characteristic relaxation time of less than 1 ms, and was due to the oxygen binding reactions. The slowest phase had a relaxation time that varied from 8 to 20 ms, depending on the protein concentration. We believe this slow phase arises from the oxy-monomer–dimer association reaction (Reaction 3 in Scheme 1).

A monomer–dimer association reaction such as that for the oxy alpha subunit (Reaction 3 in Scheme 1) has a relaxation rate described by:

$$1/\tau_3 = k_3(4[\alpha O_2]) + k_{-3} \qquad (3)$$

The slow phase of the oxy-alpha-subunit experiments was analyzed using eq. (3) and the data are shown in Fig. 4 (the concentration of oxygenated monomer alpha-subunits was calculated assuming $K_3 = 5{,}900\ M^{-1}$, [3]).

The linear relationship of the data confirmed our assignment of this phase as the self-association reaction for the oxy-alpha-subunits. A weighted linear least squares fit of this data gave an association rate constant, k_3, equal to $2.0 \pm 0.16\ 10^5\ M^{-1}\ s^{-1}$ and a dissociation rate constant, k_{-3}, equal to $39.0 \pm 3.5\ s^{-1}$. Using the association and dissociation rate constants a kinetically determined association constant was calculated, ${}^kK_3 = k_3/k_{-3} = 5{,}100\ M^{-1}$ (4,300–6,000 M^{-1}). This value agrees quite well with the equilibrium determined association constant reported by Valdes and Ackers [3], Table 1.

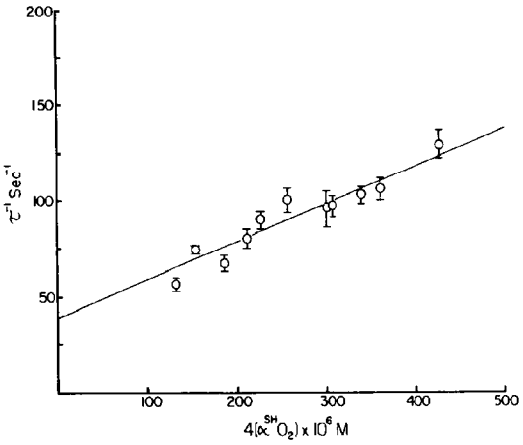


Fig. 4. Concentration dependence of the self-association relaxation rate for oxy-alpha-subunits. The solid line is a weighted linear least squares fit to the data to eq. (3). The slope of this fit, k_3, is $2.0\ 10^5\ M^{-1}\ s^{-1} \pm 0.16\ 10^5\ M^{-1}\ s^{-1}$ and the intercept, k_{-3}, is $39.0\ s^{-1} \pm 3.5\ s^{-1}$. Sample conditions are as described in Fig. 1.

The slow phase amplitude appeared to originate from a small extinction change between the oxy-monomer and oxy-dimer. This conclusion has been substantiated by computer simulations of the relaxations present at high O_2-saturation according to Scheme 1. These relaxation kinetics simulations showed that there are three overlapping fast relaxations involving primarily Reactions 2, 4, and 5 in Scheme 1. A fourth, much slower, relaxation was also predicted involving only the re-equilibration of the oxy-self-association reaction, and it was found to be uncoupled from all the other elementary reactions. That is, the relaxation rate of this slow phase was not dependent on the kinetics of any of the other reactions. Thus, the analysis and interpretation presented above is valid.

The relaxation amplitudes observed for the slow phase are shown in Fig. 5. From the results of the simulation program it was determined that an extinction difference at 366 nm of 2% between the oxy-monomer —$\epsilon(\alpha O_2) = 22.2$ mM-heme^{-1} cm^{-1}— and dimer —$\epsilon(\alpha_2 O_4) = 22.6$ (mM-heme^{-1} cm^{-1}— was sufficient to account for the observed T-jump slow phase amplitude. The solid curve in Fig. 5 is the simulated behavior of the slow phase amplitude, plotted with respect to the total protein concentration, C^T, for this extinction difference of 2% (see Table 1 for the parameters used in the simulation). The agreement between the data (circles) and the theoretical curve is quite good. No slow relaxation phase could be detected for the fully deoxygenated alpha-subunits which suggested that no significant extinction difference exists between these two species.

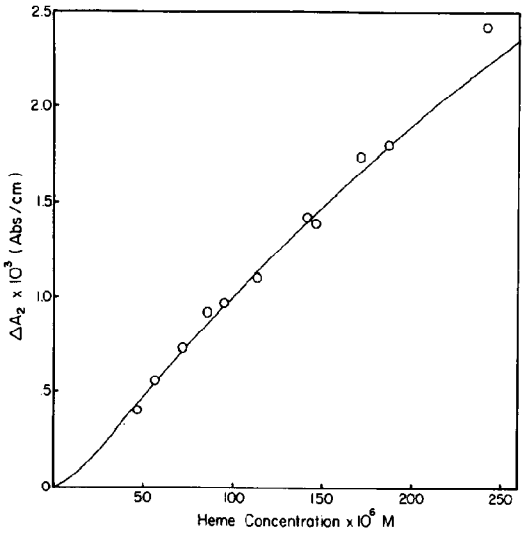


Fig. 5. Protein concentration dependence of the self-association relaxation amplitude for oxy-alpha-subunits. The solid line was calculated using the relaxation kinetics computer program. Parameters used in the simulation program are given in Table 1. The measured amplitude dependence agrees well with the simulation calculations. The simulation calculations used a 2% extinction difference between αO_2 and $\alpha_2 O_4$. Sample conditions are as described in Fig. 1.

3.5 Analysis of the slow phase in oxygen-binding kinetics

The discovery of a 2% extinction difference between oxy-monomer and dimer made it possible to interpret the slow phase present in the partially saturated, high protein concentration, experiments. For these sample conditions, relaxation kinetics computer simulations were run using the parameters given in Table 1 to calculate the relaxation times and amplitudes for the four relaxations occurring in Scheme 1. Simulations that did not include an extinction difference between the oxy-monomer and dimer, but assigned equal intrinsic affinities for each of the ligand-binding on self-association reactions generated one large amplitude corresponding to O_2-binding reactions and negligible amplitudes for the three slowest relaxations which involved coupled oxygen binding and monomer–dimer association reactions. When a 2% extinction difference between the oxy-monomer and dimer was included in the simulation program the amplitude of the slowest phase, A_4, became quite large, as expected since this phase involves predominately the protein association reactions. The amplitudes of the other two slow phases, A_2 and A_3, also become resolvable, though smaller than A_4. The best fit to all the experimental relaxation amplitude and rate data occurred when the values of the equilibrium, kinetic and extinction coefficient paramenters shown in Table 1, were used in the simulation program. Small O_2-affinity differences between the monomer and dimer did not lead to significant amplitudes for the slower phases. All of these results suggest that the origin of the slow phase amplitude in the partially oxygenated experiments performed at high protein concentration was due to a small 2% extinction difference between the oxygenated monomer and dimer.

4. Discussion

4.1 Ligand binding kinetics

We have measured the kinetics of oxygen binding to isolated hemoglobin alpha-subunits under conditions which, for the first time, would resolve

any kinetic differences between the monomer and dimer. The intrinsic O_2-association rate constant for both the monomer and the dimer species is approximately 2.4–2.7 10^7 M^{-1} s^{-1}. This is consistent with the monomer and dimer species having identical O_2-binding kinetic properties. The O_2-association rate constant measured previously by Noble et al. [18] and Brunori and Schuster [17] for the alpha-monomer species was 5 10^7 M^{-1} s^{-1} (phosphate buffer, pH 7, 20°C). This rate constant would be 3.9 10^7 M^{-1} s^{-1} at 15°C (assuming an activation energy, E_a, of 8.1 kcal/mol, Noble et al. [18]), and is similar to our value with differences possibly due to different solvents. Kinetic analysis of the O_2-binding data indicates the O_2-dissociation rate constant for monomer and dimer species is between 10 and 24 s^{-1}. It is more appropriate, however, to calculate the O_2-dissociation rate constant indirectly from the more accurately determined O_2-association rate constant, determined in this study, and the equilibrium binding constant determined by Ackers and coworkers (see Table 1). The O_2-dissociation rate constant calculated from these values is 14.6 s^{-1} a value which is similar to that reported by Brunori and Schuster [17].

Another aim of this investigation was to determine kinetically whether the dimer has an oxygen affinity different from that of the monomer. Although oxygen binding rate constants have been obtained from equilibrium binding studies by the Ackers' group, these studies were performed with solutions that contained primarily the monomer species [15]. Thus, the characteristics of the dimer species under these buffer conditions was never thoroughly examined. Our study examined the ligand binding behavior of the alpha-subunits under conditions in which 50% of the heme was in the form of dimer. The kinetically determined oxygen affinity, calculated using both the high and low protein concentration data is 1.15 10^6 M^{-1}. The kinetically determined oxygen affinity for samples containing primarily monomer was only 16% lower than this value. The similarity in these kinetically determined affinities and rate constants, suggests that the oxygen binding properties of the monomer and dimer are essentially identical.

While self-association does not alter the O_2 kinetic and equilibrium binding properties of the α-subunit, its association with the β-subunit to form either the $\alpha\beta$-dimer or the R-state tetramer $(\alpha_2\beta_2(O_2)_3)$ leads to a ligand binding quaternary enhancement of 700–800 cal/mol for both chains. What is the kinetic explanation for this enhanced affinity? A recent study by Philo and Lary [6] which examined the O_2-binding kinetics of the $\alpha\beta$-dimer and R-state tetramer indicated that the α-subunit in each state has identical O_2-association rate constant, viz. 3.05 ± 0.25 10^7 M^{-1} s^{-1} and 2.8 ± 0.1 10^7 M^{-1} s^{-1} (Ackers buffer, 21.5°C), respectively. It becomes clear after correcting for temperature differences [18] that the enhanced affinity is not mediated through the O_2-association rate constant since the α-monomer and dimer have a comparable value of 3.3 ± 0.5 10^7 M^{-1} s^{-1} at 21.5°C. On the other hand, comparisons of the O_2-dissociation rate constant indicate that the kinetic basis for the decrease in O_2-affinity in the isolated α-subunit (21.5°C) arises from a three- to four-fold increase in its O_2-dissociation rate, 32 ± 5 s^{-1} (calculated using the above given O_2-association rate constant and the equilibrium constant measured at 21.5°C by Mills et al. [15]), relative to that of the $\alpha\beta$-dimer, 8.7 ± 0.5 s^{-1}, or R-state tretramer, 9.0 ± 0.2 s^{-1} [6].

4.2 Self-association kinetics

In addition to characterizing the oxygen binding of the alpha-subunits we were also able to examine the oxy-alpha-subunit self-association reaction. The oxy-monomer–dimer association rate constant measured in this study is 2.0 ± 0.16 10^5 M^{-1} s^{-1} and the dissociation constant is 39 ± 3.5 s^{-1}. Based on these rate constants the kinetically determined oxy-alpha-association rate constant is 5,100 M^{-1} (4,300–6,000 M^{-1}). This kinetically determined association constant is very similar to that obtained from equilibrium measurements, 5,900 M^{-1} [3]. Since no significant, kinetically determined, difference in oxygen affinity could be resolved between the monomer and dimer species one must conclude that the deoxy-monomer–dimer association and dissociation rate constants

are most likely the same as those for the oxy-species.

The association rate constant has a value similar to that measured for the hemoglobin oxy-beta-subunit monomer–dimer and dimer–tetramer reactions, 2.5. 10^5 M^{-1} s^{-1} [5]. The association rate constant for the hemoglobin *deoxy-*$\alpha\beta$-dimer–tetramer reaction is 7.3 10^5 M^{-1} s^{-1} [19] and that for the hemoglobin *oxy-*$\alpha\beta$-dimer–tetramer reaction is approximately 2.9 10^5 M^{-1} s^{-1} (calculated using an equilibrium dissociation constant of 3.4 10^{-6} M, and a dissociation rate constant of 1.0 s^{-1}, [20]). These four association reactions have similar association rate constants, yet, they differ greatly in their extent of association (the equilibrium constants vary from 5,900 M^{-1} to 4 10^{10} M^{-1}) due to large differences in their dissociation rate constants. Thus, our new data regarding the alpha-hemoglobin self-association kinetics further emphasizes the fact that association rates are rather non-specific, and the stability of hemoglobin subunit complexes is determined almost entirely by the dissociation kinetics.

4.3 Extinction difference

We have been able to resolve a small extinction difference at 366 nm between the oxy-alpha-monomer and dimer. The extinction difference calculated from the fully saturated experiments is approximately 2% at 366 nm, where the dimer species has the larger extinction coefficient. This is the first time an extinction difference between the oxy-monomer and dimer alpha-subunits species has been reported. Earlier equilibrium spectral studies in our laboratory did not show any difference in extinction between the oxy-monomer and dimer alpha-subunits [7]. The small extinction difference found here, however, would not have been detected by our earlier techniques.

We were surprised to observe a spectral difference between species with essentially identical oxygen affinity, since it is widely believed that affinity differences and heme spectra are strongly correlated. This correlation had already been broken in one direction by the finding that species with affinities differing by a significant ~800 cal/mol can have identical spectra [7]. The present results violate this correlation in a new way, by demonstrating for the first time a significant spectral difference between species which have essentially identical (±75 cal/mol) affinities. Thus, it is now clear that oxy-heme spectra are unreliable guides to differences in affinity, and *vice versa*.

5. Summary and conclusions

Combining the present kinetic studies on the alpha-subunit with earlier kinetic and thermodynamic data for alpha- and beta-subunits [3,4,5,15], we now have an essentially complete set of equilibrium and kinetic parameters describing ligand binding and subunit association, for both alpha- and beta-subunits, under the same solution conditions. This complete data set will enable accurate comparisons of the changes in kinetic and thermodynamic behavior of these subunits when they are combined into hemoglobin tetramers and $\alpha\beta$-dimers, and is a necessary first step toward a kinetic description of hemoglobin ligand binding and protein association reactions which is as thorough and detailed as the equilibrium description provided by Ackers and coworkers.

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