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Laser Kinetic Spectroscopy Studies of the Bimolecular Oxygenation of α- and β-Subunits within the R-State of Human Hemoglobin

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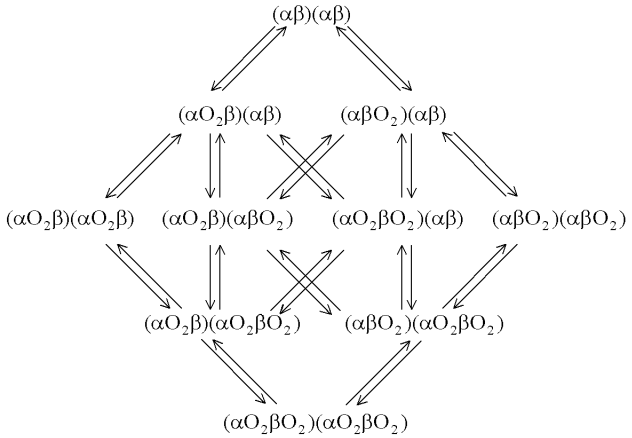
Abstract—Bimolecular oxygenation of tri-liganded R-state human hemoglobin (HbA) is described by bi-exponential kinetics with association rate constants $k_\alpha = 27.2 \pm 1.3$ $(\mu M \cdot sec)^{-1}$ and $k_\beta = 62.9 \pm 1.6$ $(\mu M \cdot sec)^{-1}$. Both the observed processes have been assigned to the bimolecular oxygenation of α- and β-subunits of the native tetrameric protein by molecular oxygen. The quantum yields of photodissociation within the completely oxygenated R-state HbA are $\gamma_\alpha = 0.0120 \pm 0.0017$ and $\gamma_\beta = 0.044 \pm 0.005$ for α- and β-subunits, respectively. The oxygenation reactions of isolated α^{PCMB}- and β^{PCMB}-hemoglobin chains are described by mono-exponential kinetics with the association rate constants $k_\alpha = 44 \pm 2$ $(\mu M \cdot sec)^{-1}$ and $k_\beta = 51 \pm 1$ $(\mu M \cdot sec)^{-1}$, respectively. The quantum yields of photodissociation of isolated α^{PCMB}- and β^{PCMB}-chains (0.056 ± 0.006 and 0.065 ± 0.006, respectively) are greater than that observed for appropriate subunits within the R-state of oxygenated HbA.

Key words: human hemoglobin, oxyhemoglobin, α- and β-subunits, molecular oxygen, kinetic absorption spectroscopy, oxygenation, bimolecular reaction, rate constant

Hemoglobin is an allosteric tetrameric protein performing two major biological functions: it transports molecular oxygen (O_2) from the lungs to peripheral tissues and transports carbon dioxide (CO_2) and protons from peripheral tissues to respiratory organs for subsequent elimination from the organism. Hemoglobin is the intriguing object because it not only plays a cardinal role in the physiology of respiration, but due to the relative simplicity of its structure it is also an excellent model for studies on nonlinear and cooperative interactions in proteins composed of several subunits. Tetrameric human hemoglobin (HbA) is an assembly of two dimers composed by α- and β-subunits, each containing heme *b* (Fe-protoporphyrin IX). This tetrameric protein is known to possess the ability to bind four oxygen molecules, one per the heme of each subunit. X-Ray structure analysis has revealed two conformations of HbA quaternary structure. The oxygenated HbA form corresponds to the state with high affinity to the ligand (R-state of the quaternary structure), whereas the deoxygenated one corresponds to the state with low affinity (T-state) [1]. The affinity of HbA to O_2 increases with saturation with oxygen (positive cooperativity), so that the protein self-controls its affinity to the ligand in functional connection with physiological conditions [1, 2].

Numerous studies have addressed the nature of structural changes in the protein and correlation between its structure and function, often demonstrating opposing views on the subject of study. The oxygenation of hemoglobin can be conceptually described using various allosteric models. Among them, the extended Adair model (see [3]) seems to be best suited for the detailed description of this reaction. Based on a difference between α- and β-subunits of the HbA tetramer, this model operates with 10 diverse states of HbA and 32 rate constants for bimolecular interactions of O_2 with the HbA molecule. The most illustrative scheme for the oxygenation of HbA is as follows:



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where $(\alpha\beta)(\alpha\beta)$ and $(\alpha O_2\beta O_2)(\alpha O_2\beta O_2)$ are deoxy- and oxyhemoglobin and $(\alpha\beta)$ and $(\alpha O_2\beta O_2)$ are deoxygenated and oxygenated HbA dimer, respectively. Oxygenated subunits are shown together with O_2 on this scheme. Each reaction stage i (i = 1-16) has a corresponding equilibrium constant (affinity) K_i that is the ratio of the bimolecular association constant k_i to the monomolecular dissociation constant l_i:

$$K_i = \frac{k_i}{l_i} . \quad (1)$$

Since α- and β-subunits differ from each other in their structure, for detailed understanding of the molecular mechanism of cooperative HbA oxygenation it is necessary to know what the functional properties of each subunit type are, both in the individual state and in the composition of the native tetramer [2, 4] in its ten possible conformational forms.

To draw in details of the mechanism by which O_2 cooperatively binds to HbA, Perutz [5] hypothesized from the data of X-ray structural analysis that the binding of the ligand with the β-subunit of the T-state HbA is sterically hampered by amino acid residues situated in close vicinity to the iron atom of heme. As a result, the ligands are supposed to bind first α-subunits of HbA in the T-state. This binding, in turn, initiates the switch from the quaternary structure of low affinity to the quaternary structure of high affinity in which β-subunits have a more broadly opened distal pocket and so they can easily accept oxygen. Perutz's proposed order of attachment of oxygen to hemoglobin contradicts the results of Gibson and coworkers [6, 7]. Their experimental data suggest an absence of significant difference between HbA subunits in their oxygen binding kinetics. Thus, despite multiple experimental and theoretical issues for the last 30 years, there is yet no distinct consensus on different subunit reactivity in the R- and T-states of hemoglobin.

Thus, it must be emphasized that some problems, such as the direct detection of a bimolecular stage of O_2 interaction with different conformational states of HbA in completely oxygenated, partially oxygenated, and completely deoxygenated native forms, as well as determination of these reaction parameters, are not yet certainly solved. It is not trivial to determine the bimolecular rate constants of subunit oxygenation in the state of the native tetramer, because sequential binding of four oxygen molecules to hemoglobin being initially in the T-state might be described, as mentioned above, by 16 association constants, and by the same number of dissociation constants.

The goal of this study was to measure, using laser kinetic spectroscopy with nanosecond resolution, the oxygenation kinetics for tri-liganded R-state HbA and its isolated α- and β-chains.

MATERIALS AND METHODS

Our experiments are based on the photodissociation of oxygenated protein forms [8-11]. After the act of induced dissociation, i.e., the cleavage of the heme–O_2 bond, the oxygen molecule either diffuses from the intraprotein area to the outer medium (solvent) or rebinds to the iron atom of heme not yet leaving the protein. The latter process is monomolecular, whereas the reaction of repeated binding is bimolecular (non-geminal) for the HbA subunits in which O_2 has left the protein. The efficacy of geminal recombination δ determines the productivity of O_2 escape from the protein matrix to the environment ($\rho = 1 - \delta$), the quantitative measure of which may be presented as follows:

$$\rho = \frac{\gamma}{\gamma_0} , \quad (2)$$

where γ_0 is the primary quantum yield of photodissociation and γ is the quantum yield of photodissociation, which represents the ratio of the number of deoxygenated heme molecules left after a completion of the geminal stage of reversible binding (equal to the number of O_2 molecules dissociated and departed from the protein) to the number of absorbed light quanta. Monomolecular dissociation constant (l) is in direct proportion to the efficacy of O_2 escape from the protein matrix and the number of dark splitting of heme–O_2 bonds per unity of time. It was noted previously [11, 12] that the primary quantum yields of photodissociation $\gamma_0 = 0.23 \pm 0.03$ are equal for all the protein species listed above, thus the photodissociation quantum yield γ is a quantitative measure of O_2 escape from the protein.

Kinetic parameters of the reaction of reversible oxygen binding were measured using equipment for laser kinetic spectroscopy. Excitation light impulse of the second-harmonic emission from a Nd:YAG laser (532 nm) was directed by a mirror system to the sample compartment. The pulse duration was ~20 nsec, the pulse energy was 1.5-6.0 mJ, and the exposed sample area was 6-12 mm^2. A KGM12-100 halogen lamp with stabilized power supply was used as a probing light source; the exciting light pulse was directed against the probing beam at an angle of $9 \pm 1°$. Lamp radiation was focused on the sample using a condenser and further on the entrance slit of the recording monochromator with a FEU-84 photoelectron multiplier behind its output slit. The signal from the photodetector was digitized, displayed, and measured using a BORDO 110 digital oscillograph (Unitekhprom BSU, Belarus). Kinetic measurements for each protein sample were conducted in 3-10 sets of experiments.

The time response of the registration system was 850 nsec. Kinetic curves were recorded in the 430-435 nm spectral region. The recording system of the absorption spectrometer with nanosecond time reso-

lution used in previous studies enabled the detection of change in absorption as small as $5 \cdot 10^{-4}$ [13]. System component optimization was performed, thus allowing the detection of the induced absorption in the considered range up to 10^{-5} and, hence, far more detailed consideration on the reactions studied. The experimental kinetic data were fitted by least squares using a computer. The kinetic data processing was based on the assumption that the photoinduced absorption kinetics can be represented as a sum of decaying exponentials:

$$A(t) = \sum_{i=1}^{n} a_i \exp(-t/\tau_i). \quad (3)$$

The fit quality was estimated from the Durbin–Watson parameter, from the Student coefficients for approximated parameters of functions, and from the magnitude and regularity of the fit residual distribution.

Oxyhemoglobin was isolated from fresh blood by the method described previously [14]. Further purification was carried out on a column (65 × 1.5 cm) with DEAE-Sephadex A-50 to produce organic phosphate-free hemoglobin [15]. Hemoglobin solutions in 10 mM Tris-HCl, pH 7.4, were prepared for experiments.

Oxyhemoglobin was divided into α- and β-subunits by the treatment of the $5 \cdot 10^{-4}$ M protein solution with tenfold excess of *p*-chloromercuribenzoic acid (PCMB) in 0.02 M potassium phosphate, pH 6.7 [16]. Isolated α- and β-subunits were prepared by ion-exchange chromatography of the PCMB-treated oxyhemoglobin on CM-cellulose (Servacel CM-32) in a gradient of both pH (6.0-8.5) and ion strength (0.01-0.02 M potassium-phosphate buffer) at 4°C [16].

The quantum yield of photodissociation γ was determined from the maximum amplitude of photoinduced absorption of deoxygenated heme forms and from the kinetics of the following disappearance of this absorption. The quantum yield γ was calculated from the equation:

$$\gamma = \left(\frac{\Delta A_0}{\Delta A_0^{st}} \right) \gamma^{st}, \quad (4)$$

where ΔA_0 and ΔA_0^{st} are the amplitudes in the curve maxima for the tested and standard compounds, respectively, and γ^{st} is the quantum yield for the standard compound. HbA solution in 10 mM Tris-HCl, pH 7.4, was used as a standard earlier characterized by the value of $\gamma^{st} = 0.028 \pm 0.003$ [12].

Dissociation of hemoglobin tetramer into dimers is negligible at the protein concentration of 10^{-4} M in 10 mM Tris-HCl, pH 7.4. The rate constants for the association of tri-liganded HbA with O_2 were determined from the disappearance kinetics of the photoinduced absorption of the native HbA deoxy subunits under partial, no more than 1%, photodissociation of O_2 from the tetramer completely saturated with oxygen molecules. Considering the contribution of the geminal stage to the recombination we anticipate that the initial level of photodissociation is no more than 10% [11]. These conditions of photoexcitation correspond to the event in which each hemoglobin molecule statistically loses a single oxygen molecule, and the tetrameric protein is in the R-conformation [17]. One of two sequences of events can be realized properly in an experiment for the individual HbA molecule:

$$(\alpha O_2 \beta O_2)(\alpha O_2 \beta O_2) \xrightarrow{h\nu} (\alpha \beta O_2)(\alpha O_2 \beta O_2) +$$
$$+ O_2 \xrightarrow{k_\alpha} (\alpha O_2 \beta O_2)(\alpha O_2 \beta O_2),$$

$$(\alpha O_2 \beta O_2)(\alpha O_2 \beta O_2) \xrightarrow{h\nu} (\alpha O_2 \beta)(\alpha O_2 \beta O_2) +$$
$$+ O_2 \xrightarrow{k_\beta} (\alpha O_2 \beta O_2)(\alpha O_2 \beta O_2),$$

where k_α and k_β are, respectively, the rate constants for the association of α- and β-subunits of tri-liganded HbA with O_2.

Independent measurements were performed with isolated α- and β-HbA chains modified with PCMB (α^{PCMB}- and β^{PCMB}-chains of HbA). Concentrations of the chains varied in the range 2-100 μM. The number of chains subjected to photodissociation was no more than 6% of their overall number. The following sequences of events were realized in experiments:

$$\alpha^{PCMB} O_2 \xrightarrow{h\nu} \alpha^{PCMB} + O_2 \xrightarrow{k_\alpha} \alpha^{PCMB} O_2,$$
$$\beta^{PCMB} O_2 \xrightarrow{h\nu} \beta^{PCMB} + O_2 \xrightarrow{k_\beta} \beta^{PCMB} O_2,$$

where k_α and k_β are, respectively, the rate constants for the association of α^{PCMB}- and β^{PCMB}-chains of HbA with O_2.

The rate constant k was calculated according to the equation:

$$k = 1/(\tau [O_2] \varphi), \quad (5)$$

where τ is the binding time of deoxygenated subunit with O_2 calculated from the experimental curve of photoinduced absorption, $[O_2]$ is the concentration of oxygen dissolved in buffer at standard atmospheric pressure and at the test temperature t (°C), and φ is the ratio of the test ambient pressure to the standard pressure. All the experiments were done at room temperature and monitored atmospheric pressure.

The absorption spectra were recorded using SF10 and SF14 spectrophotometers (LOMO, USSR). The absorption values for the tested solutions were equalized

at the excitation wavelength if required for quantitative comparison.

RESULTS

Analysis of the data represented in Table 1 indicates that the oxygenation kinetics of tri-liganded R-state HbA can be approximated by the following bi-exponential function type:

$$\Delta A = a_\beta \cdot \exp(-t/\tau_\beta) + a_\alpha \cdot \exp(-t/\tau_\alpha), \qquad (6)$$

where ΔA is the change in sample absorption (Fig. 1) and a and τ are, respectively, the amplitude and characteristic time of the exponentials. The mean values and the 95% confidence intervals for the recombination rate constants k calculated from the two characteristic times are k_β = 62.9 ± 1.6 $\mu M^{-1} \cdot sec^{-1}$ and k_α = 27.2 ± 1.3 $\mu M^{-1} \cdot sec^{-1}$ (Table 1). The amplitudes of the corresponding components are 79 ± 2 and 21 ± 2%. Based on the considerations given below, both the observed processes were compared with the oxygenation processes involving β- and α-subunits of HbA in tri-liganded tetrameric R-form. Using the γ^{st} value and component amplitudes in the reaction kinetics, we calculated the quantum yields of photodissociation for β- and α-subunits of the native completely oxygenated HbA in R-conformation. As indicated by the amplitude ratio a_β to a_α, the quantum yields for these processes differ by a factor of 3.8 and are 0.044 ± 0.005 and 0.0120 ± 0.0017, respectively (Table 1). Decreasing the HbA concentration from 100 to 4.7 μM heme increases the percentage of HbA dimer in solution. This fact results in a relatively small decrease in the rate constants k: for β-subunits k_β changes from 62.9 ± 1.6 to 57 ± 3 $\mu M^{-1} \cdot sec^{-1}$ and for α-subunits k_α changes from 27.2 ± 1.3 to 22 ± 2 $\mu M^{-1} \cdot sec^{-1}$. The amplitudes of the two processes at HbA concentration of 4.7 μM heme are 81 ± 3 and 19 ± 3%, thus agreeing within the experimental error with the amplitudes calculated for tetrameric HbA. We can conclude from these data that the presence of no more than 20% dimers in HbA solution results in the increase by no more than 20% of the rate constants k for the native HbA subunits and, within the experimental error, does not alter the contributions of the subunits into the apparent reaction kinetics. It is of interest to determine in the future the characteristics of α- and β-subunits composing HbA dimer that, unlike the tetramer, is known to be devoid of cooperative properties on O_2 binding.

Now let us consider the data on the bimolecular stages of oxygenation of individual HbA subunits. As noted above, we have chosen the α- and β-HbA chains modified by PCMB as the objects of our investigation. The choice of α^{PCMB}- and β^{PCMB}-chains of HbA was not accidental. It is due to some difficulties in preparation of β^{SH} monomer form, because this protein exists as a dimer–tetramer mixture over a wide range of its concentration [18-20]. Measurements within the 0.1-μM con-

Table 1. Kinetic parameters of oxygen binding to tri-liganded R-state HbA (the data are taken from reports in which the studied oxygenation reaction was considered as a sum of two exponential processes)

Buffer	[HbA], μM	k_β, $\mu M^{-1} \cdot sec^{-1}$	k_α, $\mu M^{-1} \cdot sec^{-1}$	a_β, %	a_α, %	γ_β	γ_α	Reference
10 mM Tris-HCl, pH 7.4	100	62.9 ± 1.6	27.2 ± 1.3	79 ± 2	21 ± 2	0.044 ± 0.005	0.0120 ± 0.0017	our data
10 mM Tris-HCl, pH 7.4	4.7	57 ± 3	22 ± 2	81 ± 3	19 ± 3	—	—	our data
50 mM Tris or Bis-Tris, 100 mM Cl^-, pH 6.5-8.4	200	76 ± 8	36 ± 3	—	—	—	—	[21]
50 mM Bis-Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4	100	146 ± 29	42 ± 8	50	50	—	—	[22]
0.1 M Bis-Tris, 0.1 M KCl, pH 7.0	20-50	100 ± 13	28 ± 9	50	50	—	—	[9, 23]
0.1 M Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4	55	64 ± 1	28 ± 1	~66	~34	—	—	[28]

Note: The experimental data are given as means with 95% confidence intervals.

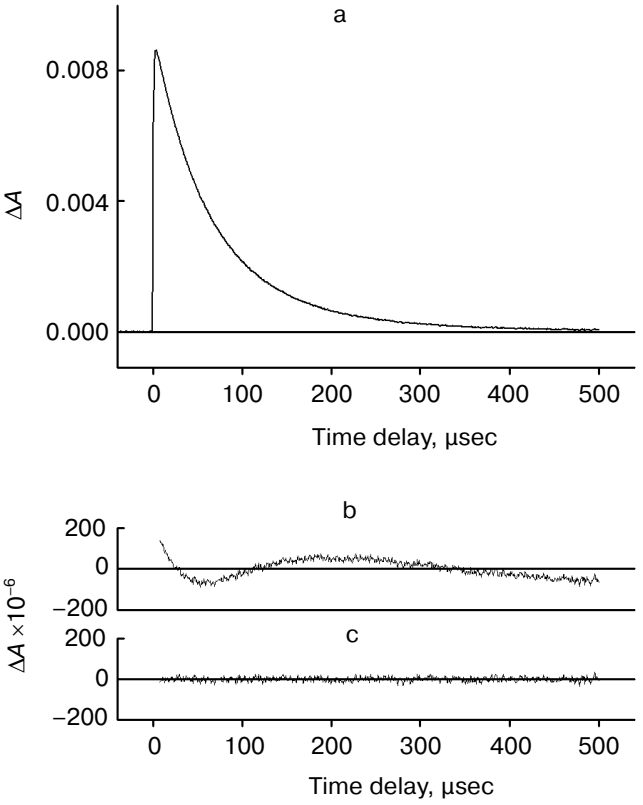

Fig. 1. a) Kinetics of the recombination of O_2 with tri-liganded R-state HbA. Conditions: 10 mM Tris-HCl, pH 7.4; 20°C; HbA concentration is 100 μM by heme; optical path length is 2 mm; wavelengths of excitation and detection are 532 and 430 nm, respectively. b, c) Residual deviations from one-exponential (b) and two-exponential (c) models approximating these kinetics.

the measurements, does not result within the experimental error in the change in rate constant for the association between O_2 and deoxygenated α^{PCMB}-chain. The mean value and confidence interval for the rate constant were determined as $k_\alpha = 44 \pm 2\ \mu M^{-1}\cdot sec^{-1}$ (Table 2 and Fig. 2, curve *1*). Invariance of the rate constant k_α value at varying protein concentration is explicable based on preferentially monomeric form of α^{PCMB}-chain within the considered concentration range. As for β^{PCMB}-chains, we also could not detect a significant change in the association rate constant k_β (from 49 ± 1 to $51 \pm 1\ \mu M^{-1}\cdot sec^{-1}$) (Table 2 and Fig. 2, curve *2*) at varying concentration within the range from 100 to 2 μM heme. β^{PCMB}-chains preferably exist in dimeric form at concentrations ~100 μM [18]. The decrease in protein concentration to 2 μM heme induces the decay of dimers and transition of the protein into preferably monomeric form. Relying on the experiments with α^{PCMB}- and β^{PCMB}-chains existing preferably in the form of monomer, we conclude that the oxygenation reaction between O_2 and β^{PCMB}-chains occurs about 1.2 times faster than the oxygenation reaction between O_2 and α^{PCMB}-chains.

The kinetic recordings enable us to measure the quantum yields of photodissociation for α^{PCMB}- as well as for β^{PCMB}-chains. Using the comparative method for determination of the reaction quantum yield (4) and taking γ^{st} to be the standard value, we have calculated the photodissociation quantum yields for the individual α^{PCMB}- and β^{PCMB}-chains at 100-μM concentration. Determination of photodissociation quantum yields for the solutions at 2 μM concentrations is accompanied by

centration range, wherein β^{SH} exists preferably as monomer, are technically difficult to fulfill. As for β^{PCMB}-chains, their monomer forms can be produced by the dilution of the protein solution to the concentration of 1 μM heme [18]. The production of monomer forms for HbA α-chains requires less dilution compared with β^{PCMB}-chains, and at 10 μM concentration they exist preferentially as monomers. Based on these considerations we measured kinetics for the isolated α^{PCMB}- and β^{PCMB}-chains, and the processing of these data revealed the following regularity. The oxygenation kinetics for isolated α^{PCMB}- and β^{PCMB}-chains (Fig. 2) can be approximated by the exponential function:

$$\Delta A = a\cdot\exp(-t/\tau), \qquad (7)$$

where ΔA is the change in sample absorption and a and τ are the amplitude and characteristic time of the exponential. The change in α^{PCMB}-chain concentration within the range from 2 to 100 μM (by heme), as demonstrated by

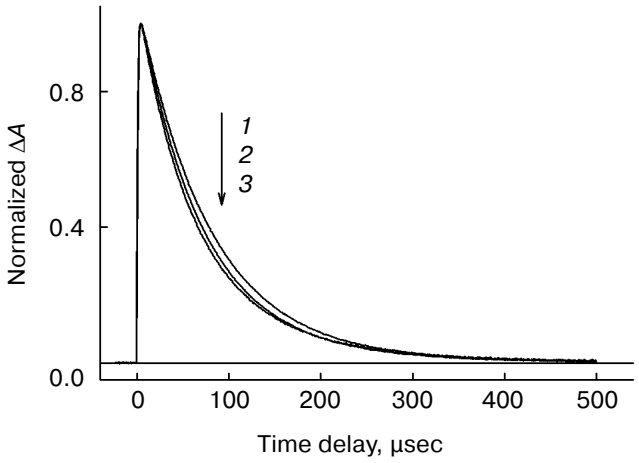

Fig. 2. Normalized kinetics for the recombination of O_2 with triliganded R-state HbA and its individual chains. Wavelengths of excitation and detection are 532 and 430 nm, respectively. [α^{PCMB}], 100 μM (*1*); [β^{PCMB}], 100 μM (*2*); [HbA], 100 μM heme (*3*).

Table 2. Kinetic parameters of oxygen binding to isolated α- and β-chains of HbA

Chain	Buffer	C, μM	k, $\mu M^{-1} \cdot sec^{-1}$	γ	Reference
α^{PCMB}	0.05 M potassium phosphate, pH 7.4	100	44 ± 2	0.056 ± 0.006	our data
α^{PCMB}	0.05 M potassium phosphate, pH 7.4	2	44 ± 2		our data
β^{PCMB}	0.05 M potassium phosphate, pH 7.4	100	49 ± 1	0.065 ± 0.006	our data
β^{PCMB}	0.05 M potassium phosphate, pH 7.4	2	51 ± 1		our data
α^{SH}	0.1 M phosphate, pH 7.0, 20°C	20-50	50 ± 8	0.055 ± 0.023	[27]
α^{SH}	0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4	10-30	41 ± 3	0.053 ± 0.005	[12]
α^{SH}	0.05 or 0.1 M phosphate, pH 7.0	0.2-0.5 1.20	55 ± 4 57 ± 7.1		[29]
α^{PCMB}	0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4	10-30	43 ± 3	0.050 ± 0.005	[12]
α^{PCMB}	0.05 or 0.1 M phosphate, pH 7.0	0.2-0.5 1.20	55 ± 8 43 ± 5.1		[29]
β^{SH}	0.1 M phosphate, pH 7.0	20-50	60 ± 10	0.040 ± 0.010	[27]
β^{SH}	0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4	10-30	71 ± 6	0.045 ± 0.004	[12]
β^{SH}	0.05 or 0.1 M phosphate, pH 7.0	0.2-0.5 1.20	75 ± 15 68 ± 6		[29]
β^{PCMB}	0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4	10-30	53 ± 4	0.11 ± 0.01	[12]
β^{PCMB}	0.05 or 0.1 M phosphate, pH 7.0	0.2-0.5 1.20	74 ± 2 49 ± 3.8		[29]

Note: The experimental data are given as means with 95% confidence intervals.

technical difficulties, so the studies must of necessity be done at higher solution concentrations. Thus the quantum yields for α^{PCMB}- and β^{PCMB}-chains are determined respectively as 0.056 ± 0.006 and 0.065 ± 0.006.

DISCUSSION

Many experimental approximations have been developed to solve the problem of the reaction differences between the R- and T-states of hemoglobin subunits. Among them, the construction of valence and so-called "metallic hybrids" of hemoglobin in which one of two subunit types contains metal (Mn, Cr, Mg, or Ni)-porphyrin group inert to oxygen, whereas the second type contains heme groups reactive to oxygen. This approximation allows unique attribution of the apparent rate constants based on the assumption that only one subunit type in a molecule is capable of interacting with ligands. The hemoglobin hybrids were used previously for unambiguous determination of rate constants for O_2 binding with α- and β-subunits of T- and R-state HbA [21]. Our results demonstrated that β(Fe)-subunits of hemoglobin in R-like state are characterized by the association/dissociation rate constants twofold higher than those for α(Fe)-subunits (see Table 1). Nevertheless, it is always necessary to remember the difference in functional properties of this protein type and the corresponding native tetramers.

The next approximation on the way to the solution of the set task is selective modification of key amino acid residues in the heme pocket region of α- and β-HbA chains. A body of intriguing data has been acquired in this way [9, 22, 23]. The individual rate constants for the α- and β-subunits of HbA tetramer in R-conformation were determined from the patterns of kinetics for both the native and chemically modified protein. It should be particularly noted that for all instances in the studies [9, 22,

23] the liganding kinetics were approximated by the function type:

$$\Delta A_t = \Delta A_0 \cdot 0.5 \cdot [\exp(-k_\beta \cdot t) + \exp(-k_\alpha \cdot t)], \quad (8)$$

where ΔA_t is the absorption change at a given time t, ΔA_0 is the absorption change at zero time, and k_β and k_α are the apparent constants attributed to the oxygenation reactions between molecular oxygen and β- and α-subunits of tri-liganded proteins. The assumption on the amplitude equality of the two processes may result in erroneously determined apparent rate constants. Applying this technique to the β-subunits in R-state HbA, the bimolecular rate constants of O_2 binding to the tri-liganded proteinhave been found to be 2-4 times higher than those for α-subunits (see Table 1). Note that only two parameters of Eq. (8) were varied for the approximation. Nevertheless, the error of the determined parameters (20-50%) was quite large, because each kinetic trace was approximated by the function of two exponents with constants differing sometimes no more than twofold. Note that monoexponential pattern of the association reaction was generally recorded (Table 3) in most studies on native HbA [11, 12, 24-27]. As for a direct detection and recording of spectral signals from the liganding of individual subunits of the intact tetramer, this task of the parameter determination is of even greater complexity due to the necessity of simultaneous search for the rate constants and their amplitudes. Two bimolecular constants of O_2 binding with α- and β-subunits of tri-liganded tetrameric R-state HbA were first measured, and the amplitudes of these reactions were evaluated (Table 1) in study [28].

Thus, in spite of the multiplicity of reported studies devoted to the functional properties of the hemoglobin molecule, no rate constant for the association of individual subunits of the intact tri-liganded R-state HbA and no quantum yield value for the photodissociation of α- and β-subunits in oxygenated HbA tetramer are commonly accepted at present.

The analysis of bimolecular stages of the oxygenation reaction, which was performed both on the individual HbA chains and on subunits in composition of the native tri-liganded HbA tetramer in R-conformation, has revealed the following. The values of association rate constants and quantum yields of photodissociation for the isolated α^{PCMB}-chain ($k_\alpha = 44 \pm 2$ μM^{-1}·sec^{-1}, $\gamma_\alpha = 0.056 \pm 0.006$) exceed the corresponding values for α-subunit composing HbA ($k_\alpha = 27.2 \pm 1.3$ μM^{-1}·sec^{-1}, $\gamma_\alpha = 0.0120 \pm 0.0017$). Lesser differences are observed in kinetic parameters for the isolated β^{PCMB}-chain ($k_\beta = 51 \pm 1$ μM^{-1}·sec^{-1}, $\gamma_\beta = 0.065 \pm 0.006$) and for β-subunit composing HbA ($k_\beta = 62.9 \pm 1.6$ μM^{-1}·sec^{-1}, $\gamma_\beta = 0.044 \pm 0.005$). As discussed earlier [12], the rapid phase of geminal recombination ($\tau \sim 200$ psec) is virtually absent or very short in the individual α^{PCMB}- and β^{PCMB}-chains. Its contribution is significant for native HbA (~30%). This circumstance is apparently the reason for the fact that γ (0.028 ± 0.003) and respectively ρ values are lower for the oxygenated HbA in R-conformation than for the isolated α^{PCMB}- and β^{PCMB}-chains. This fact evidently results in significantly decreased photodissociation quantum yield for α-subunits of oxygenated HbA in R-state in comparison with β-subunits. The last fact enables us to assign the above-mentioned phase of geminal recombination chiefly to the rapid phase of geminal O_2 recombination with α-subunits of the native HbA in R-conformation. Detailed discussion on both geminal and bimolecular reaction stages will be presented in a separate paper.

The available experimental data clearly suggest an important effect of the chain folding process with a formation of symmetric structure of the hemoglobin tetramer on the functional properties of its components. The substantial decrease in the rate constant k and the quantum yield of the photo process γ for α-subunits is observed. β-Subunits of completely oxygenated HbA compared with α-subunits are characterized by less effective geminal recombination and thus higher probability of O_2 dissociation into solution. At the same time, the data presented above suggest that the distal pocket in β-subunits of tri-liganded HbA in R-conformation is more broadly open for O_2 located in the surrounding environment. The ligand binding to α-subunits of the same state protein is more hampered by the amino acid residues

Table 3. Kinetic parameters for oxygen binding to the tri-liganded R-state HbA, when the reaction is considered as monoexponential

Buffer	[HbA], μM	k_{HbA}, μM^{-1}·sec^{-1}	γ_{HbA}	Reference
0.1 M Bis-Tris, 0.1 M KCl, pH 7.0	~50	42 ± 4		[9]
0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4	10-30	47 ± 4	0.028 ± 0.003	[12]
50 mM phosphate, pH 7.0	5		0.052	[26, 27]

Note: The experimental data are given as means with 95% confidence intervals.

located at the distal side of the heme pocket. We emphasize in conclusion that the technique available allows us to conduct simultaneous kinetic monitoring of α- and β-subunits composing the tri-liganded R-state HbA, as well as starting both studies on the influence of various effectors (protons, organic phosphates, chlorine ions) on the interaction of O_2 with α- and β-subunits of native HbA and studies on the oxygenation of other possible HbA tetramer forms.

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