A fluorescence decay time study of tryptophan in isolated hemoglobin subunits

Jihad Albani°, Bernard Alpert+, Donald T. Krajcarski and Arthur G. Szabo*

Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A OR6, and +*Laboratoire de Biologie Physico-chimique, Universite de Paris VII, 75251 Paris Cedex 05, France*

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The time-resolved fluorescence behavior of tryptophan residues in isolated human hemoglobin subunits was determined using a sync-pumped dye laser system and time-correlated single photon counting detection. Two decay components having values near 80 ps and 2 ns were found in the fluorescence decay of the α-subunit. The data for the β-chains were best fitted with 3 decay components of 90 ps, 2.5 ns and 6.4 ns. We propose that the decay times correspond to conformations of the proteins in which the disposition of the tryptophan to the heme residue differs.

Tryptophan *Hemoglobin subunits* *Conformational heterogeneity* *Fluorescence decay*

1. INTRODUCTION

The existence of a weak fluorescence from the protein moiety of human hemoglobin has been clearly established [1–5]. This intrinsic emission has been utilised to investigate phenomena such as tryptophan-heme energy transfer [6] and R-T structural transitions in hemoglobin [3–5]. Only two of these reports, however, specifically addressed dynamic aspects of the tryptophan fluorescence which may provide information on segmental motion of the fluorophore environment [6,7].

Human hemoglobin possesses 6 tryptophan residues, one in each *a*-subunit (α_{14}) and two in each β-subunit (β_{15} and β_{37}) [8]. The isolated subunits thus offer a simplified system for studying the dynamic aspects of the protein matrix of hemoglobin. The α-subunit, for example, has only a single tryptophan residue and a single heme group with which it might interact, and there is no possibility of R-T structural changes. The β-subunit, although it possesses two tryptophan residues, will have a different structure than intact hemoglobin. Time-resolved fluorescence experiments on these isolated subunits should provide additional information to assess the interpretation of the fluorescence decay behavior of hemoglobin.

2. MATERIALS AND METHODS

Human hemoglobin A was extracted from red blood cells according to standard procedures [10] and the α- and β-chains were prepared according to the method of Geraci et al. [11]. The homogeneity of the subunits and their capacity for association and reconstitution to reform the hemoglobin tetramer were verified by high-voltage electrophoresis. During isolation of the subunits the SH groups were oxidized by treatment with mercaptoethanol in aerated solution for ½ h for the α-subunit and 1 h for the β-subunit. Deoxygenation of the samples was accomplished by slow equilibration of the oxygenated samples with nitrogen

° Present address: Dept. of Pharmacology, University of Texas Health Science Center at Dallas, Dallas, TX 75235, USA

* To whom correspondence should be addressed

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gas. The carbomonoxy forms were similarly prepared from the deoxy derivatives using carbon monoxide. The purity of the various derivatives was confirmed from their visible absorption spectra which were measured with a Cary 219 spectrophotometer. All samples (2.4×10^{-5} M) were measured in 0.01 M sodium cacodylate buffer (pH 7).

Fluorescence decay experiments were performed at 20°C in scrupulously cleaned suprasil tonometers of 1 cm path lengths. The excitation source ($\lambda_{ex} = 295$ nm) was a Spectra Physics cavity dumped laser system which has been described in detail elsewhere [9]. The emission was monitored at 340 nm (8 nm bandpass) and decay data were collected in 1024 channels of a multichannel analyzer with a resolution of 21.3 ps/channel. A background signal was subtracted from all measurements prior to deconvolution. To minimize photolytic effects in the case of the oxy derivatives, fresh samples were utilized every 10 min until sufficient counts were accumulated in the maximum channel (100 000). Data were analyzed using the DFCM non-linear least-squares iterative convolution method using terphenyl in ethanol as a reference material [9].

3. RESULTS AND DISCUSSION

The fluorescence decay curves for the α- and β-subunits are shown in fig.1. Analysis of the decay data showed that the fluorescence of the various derivatives of the α-chains are best fitted with two exponential decay components while those for the β-subunit are best fitted with 3 exponential decay kinetics (table 1). The fluorescence of the α-subunit had decay times near 80 ps and 2 ns. These values are similar to those observed for the two shorter components in the intact hemoglobin. The fractional contribution of each component depends upon the state of ligation in the subunit case. We assign these two components to two average conformations of the protein in which the single tyrptophan residue is oriented differently with respect to the heme residue.

The results for the β-chain showed that 3 kinetic components were required to fit the data satisfactorily , with decay times near 90 ps, 2.5 ns and 6.4 ns. The fractional fluorescence of the 90 ps component was significantly greater than that of

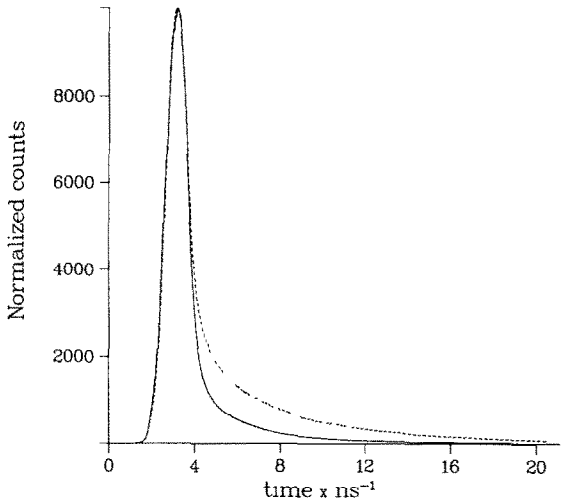

Fig.1. Normalized fluorescence decay profiles for (——) α (oxy) hemoglobin subunit; (---) β (oxy) hemoglobin subunit; resolution 21.3 ps/channel, $\lambda_{ex} = 295$ nm, $\lambda_{em} = 340$ nm.

the short lifetime component in the native hemoglobin derivatives. The fractional fluorescences for the carbomonoxy derivative of the β-subunit are significantly different from those of the other β-derivatives. We are only able to speculate that this may indicate that this derivative has a significantly different structure compared to the other derivatives. We are unable to make a choice at this time as to whether the 3 components correspond to 3 different average conformations of the β-subunit or can be assigned specifically to particular tryptophan residues, of which there are two in the β-subunit.

Upon oxidation of the cysteine residues (α_{104}, β_{93} and β_{112}) a weak band appears near 625 nm in the absorption spectra and there is a significant change in the fluorescence decay curves with an increase in the long time emission. Further the decay kinetics become more complex regardless of the state of ligation.

Earlier, from steady-state fluorescence measurements, it had been reported [3] that tryptophan β_{37} was responsible for the fluorescence of hemoglobin. However, steady-state experiments performed by some of us [2] and the results reported herein clearly demonstrate that the tryptophan located at residue 14 in the α-subunit and residue 15 in the β-subunit also contribute to the hemoglobin fluorescence.

Table 1

Fluorescence decay parameters of human hemoglobin subunits[a]

Derivative	τ_1(ps)	τ_2(ns)	τ_3(ns)	$F(1)$[b]	$F(2)$	$F(3)$
βO_2	95	2.65	6.5	50	21	29
βCO	90	2.55	6.45	41	27	32
β(deoxy)	90	2.3	6.3	50	21	29
αO_2	80	2.2		80	20	
αCO	85	2.3		65	35	
α(deoxy)	65	1.9		77	23	
HbO_2[c]	90	1.9	5.4	24	40	37
Hb deoxy[c]	70	1.8	4.9	30	41	29
HbCO[c]	70	1.8	4.9	30	45	25

[a] The standard errors for each parameter were: τ_1, ± 5 ps; τ_2, ± 0.1 ns; τ_3, ± 0.1 ns; $F(1)$, $F(2)$, $F(3)$, ± 2

[b] $F(1)$, $F(2)$, $F(3)$ are the fractional fluorescence contributions of each component (see [9])

[c] Data from [7]

Our results indicate that the trypotophan residues of the different derivatives of native hemoglobin and the subunits interact differently with the heme residue. Furthermore, they show that there are equilibria between different conformational forms of the subunits and hence in the intact hemoglobin itself. This conformational heterogeneity is affected by the ligation state of the heme residue. The results also indicate that a single tryptophan residue may give rise to two fluorescent components, so that in cases where there is more than one tryptophan in a protein it may not be possible to assign a specific decay component to a specific tryptophan residue [12]. We have shown that time-resolved studies of the intrinsic protein emission of these heme-proteins can provide new insights into their structure and function.

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