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Time resolved emissions in the picosecond range of single tryptophan recombinant myoglobins reveal the presence of long range heme protein interactions

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Abstract

We have analyzed the time resolved fluorescence emission in the subnanosecond range of recombinant wild-type SW myoglobin and its single TRP mutants W7F and W14F. These recombinants carry a methionine at the N1 terminal end. The emission of Trp-7 in the met form of W14F showed residual lifetime components much shorter than those estimated after excitation energy transfer to the heme. We propose that in this recombinant the N1 methionine is close to Trp-7, thereby producing an extra quenching due to either collisions or electron transfer with its sulfur. When the measurements were repeated on its CO-form, the extra quenching of Trp-7 was much decreased, indicating a heme linked conformational change involving the amino terminal end of the protein. This hypothesis is supported by ligand linked conformational changes in myoglobin, reported by Ansari et al. and by Giardina et al. At neutral pH the lifetimes of W7F were consistent with estimations based on the atomic coordinates of SW myoglobin. Those of the wild-type were exactly the combination of the lifetimes of the two mutants. This suggest that the mutations did not affect the overall structure of the protein. However, in the ferric form, substitution of Trp-14 in W14F resulted in low stability at acid pH, as evident from lifetimes modifications at pH 4.8, while no modifications were produced by titrations of W7F to pH 4.5. This suggests a role of Trp-14 in the structural stability of myoglobin.
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Keywords: SW myoglobin; Recombinant; Single tryptophan; Lifetimes

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Abbreviation: CO, carbonmoxide; RWT, recombinat wild-type SW myoglobin, N1-met, 122-Asn; real-RWT, recombinant wild-type SW myoglobin, N1-Val, as in natural myoglobin, 122-Asn; W7F, Trp-7 W ⇒ F mutation of RWT; W14F, Trp-14 W ⇒ F mutation of RWT; **Species I**, myoglobin with heme in normal position; **Species II**, myoglobin with disordered (inverted) heme; **Species III**, myoglobin with reversibly dissociated heme

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1. Introduction

It was initially noted by Weber and Teale [1] that in hemoproteins excitation energy transfer from tryptophans to hemes severely quenches the emission of tryptophyl residues in these systems.

Ultrafast time-resolved fluorescence has shown the presence in these systems of multiple lifetimes in the subnanosecond range, whose origin was referred to the presence of more than one tryptophan in the molecules [2–4]. However, in view of the small Trp–heme separation and the expected massive energy transfer, it was difficult to explain the concomitant presence of lifetimes of several hundred picoseconds and longer than 1 ns. The small amplitude reinforced the hypothesis that they were non-extractive impurities containing tryptophans. However, in many cases those lifetimes were shorter than expected from freely emitting tryptophans.

The observation that excitation energy transfer from tryptophans to hemes in hemoproteins is primarily regulated by a single heme transition moment with well defined orientation in the heme plane broke the impasse [5–7]. In fact the availability of the atomic coordinates of myoglobins and hemoglobins allowed a precise estimation of the expected fluorescence lifetime of tryptophyl residues in these systems. Detailed analyses of radiationless Trp–heme interaction showed that heme inversion, i.e. rotated 180° around the α-γ-*meso*-axis of the porphyrin ring [7] much decreases the rate of excitation energy transfer from tryptophans to hemes, explaining the origin of some long lifetime components of myoglobin [7].

More recently we have conducted a detailed analysis of the emission of horse heart myoglobin [8]. Based on the atomic coordinates of the system we were able to identify in a quantitative mode the emitting species of the system, including species in reversible dissociation equilibrium with heme. This allowed a complete description of dynamic equilibrium of molecular species produced by the heme–protein interactions.

All myoglobins, except that of yellow fin tuna, have two tryptophans in position 7 and 14, respectively. This conservation of tryptophans and of their position across different species, together with the conservation of the classic myoglobin fold, suggest an important role of tryptophans in determining the structure, function and stability of the protein.

The availability of recombinant procedures opens new ways for investigating structural effects of single amino acid substitutions. In order to investigate the role of tryptophanyl residue we used recombinant procedures for eliminating one or the other of the two tryptophans in SW myoglobin. In this report we present comparative measurements of the fluorescence lifetimes of W7F and W14F mutants and of their parent wild type RWT myoglobin (all with the N1-met at the terminal end) to that of the wild type real-RWT, without the extra terminal methionine.

2. Materials and methods

Amino acid sequences were obtained with a Hewlett Packard G1000A sequenator, following the procedure standardized by the manufacturer.

Circular dichroism was measured with an AVIV 60DS instrument. The samples were in 50 mM phosphate buffers at pH 7.0. The total OD of the samples at 222 nm was near OD $\approx$ 0.8.

Recombinant sperm whale myoglobins were obtained following a described procedure [9]. It was later observed that all of these recombinants have Asn in position 122 instead of Asp.

2.1. Treatment of the samples

Before fluorescence measurements the samples were further purified by free phase electrofocusing using a ROTOFOR apparatus (Biorad). If necessary, in order to obtain the carbonmonoxy form of the protein, sodium dithionite was added to a solution of myoglobin previously saturated with carbon monoxide. The solution was then filtered through a Sephadex G25 column before ROTOFOR purification. To change the pH of the samples purified solutions of myoglobin were washed repeatedly by ultrafiltration with the desired buffers during a period of 2–3 h. After this treatment the pH of the samples and their fluorescence response were stable for several hours. We used 0.05 M phosphate buffers at neutral pH,

and 0.05 M acetate buffers below pH 5.0. The absorption spectrum of the samples was independent of pH within the pH range investigated. Protein concentration was measured spectrophotometrically using $\epsilon = 14\,000\ \mathrm{cm}^2\ \mathrm{M}^{-1}$ at 542 nm for its carbonmonoxy derivative [10].

Fluorescence lifetimes were measured using a frequency domain 10 GHz fluorometer equipped with a Hamamtsu 6-μm microchannel plate detector (MCP-PMT) as previously described [11]. The instrument covered a wide frequency range between 7 and 5000 MHz, which allowed detection of lifetimes ranging from several nanoseconds to a few picoseconds. Samples were placed in a 1-cm path 'shielded cuvette', previously described [12], which eliminated stray lights from the emission. The exciting light was tuned at 294 nm. Sample emission was filtered through an ORIEL interference filter centered at 340 nm and a CORNING 7–60 broad band filter. For reference, we used the scatter of the sample solution filtered through an ORIEL interference filter at 289 nm together with neutral density filters. The filters used for the emission and the reference were calibrated, so as to obtain identical optical length at 294 and 340 nm [12].

The governing equations for the time-resolved intensity decay data were assumed to be a sum of discrete exponentials as in

$$I(t) = I_0 \Sigma_i \alpha_i e^{t/\tau_i} \tag{1}$$

where α_i is the amplitude (pre-exponential factor) and τ_i the lifetime of the i-th discrete component. Fractional intensity, amplitude and lifetime parameters were recovered by non-linear least squares procedures using either the Globals Unlimited software [13] or software developed at the Center of Fluorescence Spectroscopy.

2.2. Lifetime computations

For these computations it is necessary to know the parameters that regulate excitation energy transfer between tryptophan and heme in myoglobin. They are the overlap integral, the distance and the angular relationships between donor and acceptor. The overlap integral, J, was computed from the extinction coefficients and normalized emission of heme and heme-free myoglobin, respectively. As reported, the overlap integral is practically independent from the state of ligation of myoglobin [7]. The mutant proteins did not show appreciable modifications of their absorption spectra. The heme–tryptophans distances R_i, the orientation factors κ_i^2 for the Förster radiationless interaction were computed using the atomic coordinates of SW myoglobin from the PDB files, as described [7]. The data bank includes coordinates for oxy-, deoxy- and ferricforms of SW myoglobin. Computations based on all of the available coordinates gave very similar results. On the basis of those data we assumed that the distances of Trp-7 and Trp-14 were 21 and 14 Å, respectively. To calculate the orientation parameter, the transition moment of tryptophan was assumed to be oriented at $-38°$ from the main pseudosymmetry axis of the indole ring [14]. The orientation (angle θ) of the heme transition moment responsible for accepting radiationless excitation energy transfer from tryptophan was assumed to be between 50 and 60° from the α-γ-*meso*-axis of the porphyrin ring, on its proximal side [5–7]. κ^2 values were computed as an average of values calculated at 1° intervals between these two limits (50–60°). This procedure is described in detail by Gryczynski et al. [7].

Residual tryptophans lifetimes (τ_i) in the presence of energy transfer to either normal, or disordered hemes were computed from [15]:

$$\tau_i = \frac{\tau_0}{1 + (R_0 + R_6)^6} \tag{2}$$

where

$$R_0^6 = 8.785 \cdot 10^{-25} K^2 n \phi^{-4} J \tag{3}$$

τ_0 is the lifetime of the tryptophan in the absence of the heme-acceptors, R_i is the distance between the heme and the respective tryptophan, κ^2 is the respective orientation factor, ϕ is the quantum yield of the tryptophan, $\phi = 0.14$ [14], and J is the overlap integral [7]. The ratio $(R_0/R_i)^6$ represents the transfer rate factors between the donor-tryptophan (Trp-7 or Trp-14) and the

heme-acceptor. As previously discussed [8,16], we assumed a value of $\tau_0 = 5000$ ps. This is an arbitrary choice based on the observation that the longest lifetime generated by this proteins and other similar systems, like horse heart myoglobin [8] and hemoglobin [16] is of this magnitude.

3. Results

3.1. Amino acid sequences

We controlled the sequence of the first 16 amino acid residues in RWT, W7F and W14F myoglobins. As expected, the analyses confirmed either the presence or the absence of the tryptophans in the anticipated positions. They also confirmed the presence in RWT, W7F and W14F of the extra initial methionine.

3.2. Circular dichroism

The CD spectra in the far UV region of all recombinant proteins in their ferric forms were superimposable and very similar to what is expected from the natural system.

3.3. Fluorescent species of myoglobin

As shown in a previous publication [7], three emitting species are present in myoglobin systems. They are schematically shown in Fig. 1 and are respectively (1) **species I** with normal heme as shown in the crystal structure; (2) **species II** where heme is inverted, i.e rotated 180° around the α-γ-*meso*-axis of the porphyrin ring; and (3) **species III** in reversible dissociation equilibrium with heme. **Species I** with normal hemes have the shortest lifetimes, up to 150 ps, **species II** with disordered hemes have longer, 'intermediate' lifetimes of a few hundred ps, **species III** with dissociated hemes have the longest lifetimes near 5000 ps.

3.4. Computed lifetimes

The computed lifetimes for Trp-7 and Trp-14 are listed in the lowest rows of Tables 1–3. The

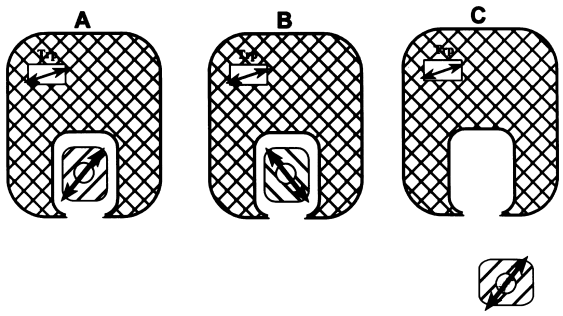

Fig. 1. The three emitting species of myoglobin. Namely, going from the left, **species I** with normally oriented heme, **species II** with inverted heme, **species III** with reversibly dissociated heme. Note the *quasi* orthogonal orientation of heme and tryptophan transition moments produced by inverted heme, which much reduces the excitation energy transfer from tryptophan to heme.

shortest lifetime of Trp-7 (**species I**) is substantially longer than that of Trp-14, because of the larger distance from the heme, which makes the two lifetimes clearly distinguishable. Instead the lifetimes originating from **species II** (disordered heme) and specie III (reversibly dissociated heme) of the two tryptophans are similar and not resolvable in the numerical analysis. Therefore we expected to find three lifetimes components in the time resolved fluorescence decay of the single tryptophan mutants and four components in the wild-type and natural myoglobins with both tryptophans.

Table 1
Lifetimes of Trp-14 in the W7F mutant

Form	pH	τ_1 (ps)	α_1	τ_2 (ps)	α_2	τ_3 (ps)	α_3
met	7.0	34	0.968	1368	0.015	4868	0.017
met	5.0	34	0.967	1368	0.018	4868	0.015
met	4.5	34	0.959	1368	0.022	4868	0.019
CO	7.0	19	0.983	1723	0.013	5102	0.004
CO	4.5	19	0.974	1723	0.021	5102	0.005
Computed lifetimes		36	–	1636	–	5000	–

Notes. The parameters were recovered from simultaneous analyses of the data obtained with the ferric (met) form and CO form, respectively. The lifetime values were the parameters linked across the simultaneous analyses. The standard errors of the measurements were in all case within 3–4% of the estimated values.

Table 2
Lifetimes of Trp-7 in the W14F mutant

Form	pH	τ_1 (ps)	α_1	τ_2 (ps)	α_2	τ_3 (ps)	α_3
met	7.0	34	0.818	244	0.153	3149	0.029
met	5.1	34	0.804	244	0.165	3485	0.031
met	4.8	70	0.845	583	0.082	4862	0.073
CO	7.0	119	0.938	1018	0.029	4375	0.034
Computed lifetimes		114	–	1202	–	5000	–

Notes. Simultaneous analyses were performed only for the data obtained with the ferric (met) form at pH 7.0 and 5.1. The lifetime values were the parameters linked across the simultaneous analyses. The other systems were singularly analyzed. The standard errors of the measurements were in all case within 3–4% of the estimated values.

3.5. Lifetimes of recombinant W7F myoglobin

The lifetimes of this system are those originating from Trp-14 only. The protein was obtained in its ferric form. Part of it was transformed into the ferrous-CO form using sodium dithionite in the presence of carbon monoxide. Some of the frequency-dependent data are shown in Fig. 2. The recovered parameters for the met- and CO-forms between pH 7.0 and 4.5 are presented in Table 1. They indicate a perfect correspondence between experimental and computed lifetimes. Global analyses demonstrated that both in the met and CO forms the lengths of the respective lifetimes were only slightly affected by pH. The effect of pH was limited to their amplitudes, and it was very small. The amplitudes of the intermediate lifetimes for **species II** remained between 1 and 2% in all cases. The fractions of **species III** remained near 2% in the ferric and ferrous form. The decreased amplitude of this component in the CO derivative is consistent with a higher affinity of the ferrous heme for the protein. These lifetime data indicate the presence of a homogenous structure of the protein, fully consistent with the atomic coordinates of natural SW myoglobin.

3.6. Lifetimes of recombinant W14F myoglobin

The lifetimes of this system are those originating from Trp-7 only. Frequency-dependent data are shown in Fig. 3. The recovered parameters for the met-form at pH 7.0, 4.8 and for the CO-form at pH 7.0 are shown in Table 2. In the met form only the data at pH 7.0 and 5.1 could be simultaneously analyzed. The recovered lifetimes of **species I**, **II** and **III** are shorter than those anticipated by the computations. The lifetime pre-

Table 3
Lifetimes of RWT and real-RWT

Form	pH	τ_1 (ps)	α_1	τ_2 (ps)	α_2	τ_3 (ps)	α_3	τ_4 (ps)	α_4
MET-RWT	7.0	33	0.875	165	0.104	1225	0.016	4657	0.006
	4.90	33	0.824	165	0.144	1225	0.025	4657	0.007
	4.55	33	0.826	165	0.106	1225	0.037	4657	0.031
CO-RWT	7.0	37	0.670	149	0.296	1478	0.021	4611	0.009
MET–real-RWT	7.0	40	0.652	158	0.301	2073	0.035	5655	0.010
CO–real-RWT	7.0	47	0.625	166	0.349	2254	0.020	5822	0.005
Computed	Trp-7	–	–	114	–	1202	–	5000	–
lifetimes	Trp-14	36	–	–	–	1626	–	5000	–

Notes. The recovered parameters for the ferric (met) form of RWT were obtained with simultaneous analyses, where the sared, linked, parameters were the lifetimes. The other systems were singularly analyzed. The standard errors of the measurements were in all case within 3–4% of the estimated values.

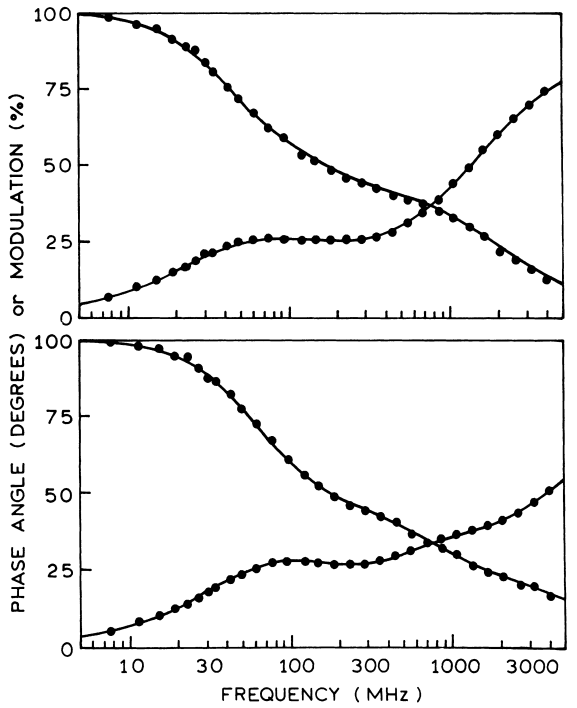

Fig. 2. Frequency dependent phase shift (lower lines) and demodulation (upper lines) of Trp-14 in W7F myoglobin in its met (upper panel) and CO (lower panel) forms. In 50 mM phosphate buffer at pH 7.0 at room temperature. Protein concentration near 1 mg ml^{-1}.

sumably originating from **species I** is of 34 ps rather than the computed 114. The lifetime near 200 ps must be referred to **species II**, and is much shorter than the expected 1200 ps. The lifetime of **species III** near 3000 ps is also shorter than the expected $\sim$5000 ps. The amplitude of the intermediate lifetime, near 200 ps is close to 15%, which may be too large for being referred only to the presence of inverted hemes. The amount of dissociated hemes in **species III** is much higher than that in W7F, near 3%. At pH 4.8 the lifetimes and amplitudes of W14F changed considerably. Simultaneous analyses of the data sets at pH 7.0, 5.1 and 4.8 did not give acceptable results, indicating profound changes in the lifetimes distribution of the system going from pH 5.1 to 4.8. This indicates a significant protein destabilization below pH 5.1. The situation is entirely different for the ferrous CO derivative (also in Table 2). The observed lifetimes are in very good agreement with those expected on the basis of the atomic coordinates of the natural protein. The frequency-dependent data shown in Fig. 3 clearly show the different fluorescent response of the ferric and CO forms of W14F myoglobin. All lifetimes are much longer in the CO form, with τ_1 reaching 119 ps (up from 34 ps), τ_2 at 1018 ps (up from 244 ns) and τ_3 close to 4.4 ns (up from 3.3 ns). Also, the respective fractions are in a much more acceptable range.

3.7. Lifetimes of RWT myoglobin

This is the parent species of the two mutants discussed above. It still contains the N1-met, and emits from both Trp-7 and Trp-14. Frequency-dependent data are shown in Fig. 4. Four lifetimes components are recovered which are almost exactly the combination of the three lifetime com-

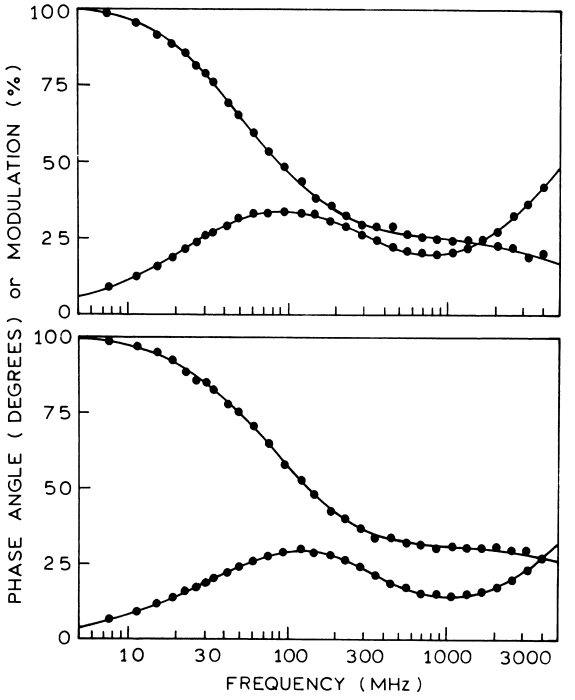

Fig. 3. Frequency dependent phase shift (lower lines) and demodulation (upper lines) of Trp-7 in W14F myoglobin. Ferric (upper panel) and CO (lower panel) forms. In 50 mM phosphate buffer at pH 7.0 at room temperature. Protein concentration near 1 mg ml^{-1}.

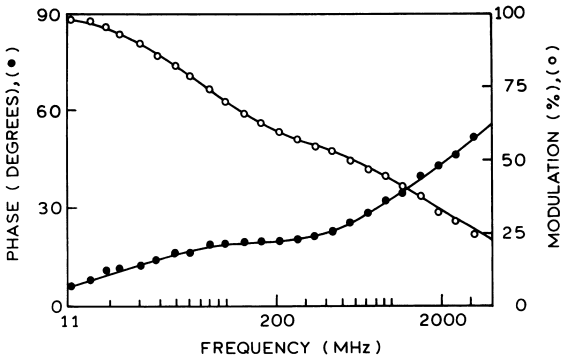

Fig. 4. Frequency dependent phase shift (lower lines) and demodulation (upper lines) of wild type myoglobin (RWT) in its ferric form. In 50 mM phosphate buffer at pH 7.0 at room temperature. Protein concentration near 1 mg ml^{-1}.

ponents detected in the mutant, single tryptophan myoglobins (Table 3). Ferric and CO-ferrous forms have very similar lifetimes. Notably the amount of **species III**, with dissociated hemes, is $< 1\%$, lower than in the two mutants. This amount in the ferric form at pH 4.55 increase to 3%. Notably, the amplitudes, α_2, of the lifetimes near 150 ps change from near 30% in the CO form to approx. 10% in the ferric form, and the amplitudes, α_1 of the near 30 ps lifetimes go from 67% in the CO-form to approx. 85% in the ferric form.

3.8. Lifetimes of real-RWT myoglobin

The amino acid sequence of this recombinant myoglobin does not include the N1-met residue at the beginning of the chain. The recovered lifetimes are shown in Table 3. Both in the ferric and CO-forms they are very similar to those recovered from the CO-form of RWT. In this system, the respective amplitudes of the lifetimes near 30 and near 160 ps are near 65% and 30%, respectively, in both derivatives.

4. Discussion

All of the mutants here investigated carried an aspartyl residue rather than an asparaginyl residue at position 122. This substitution was disregarded. Myoglobin is notorious for non-uniform post-translational amidation of its aspartyl and glutamyl residues.

4.1. Emission of Trp-14 in W7F myoglobin

The lifetimes of this residue were very accurately anticipated by computations based on the atomic coordinates of the natural system. This indicates that the structure of myoglobin is well conserved in the recombinant protein in spite of the extra methionine at the amino terminal end. The amount of inverted and dissociated hemes are not much higher, comparable to those found in horse myoglobin [7]. The changes from ferric to ferrous-CO form did not modify the lifetimes appreciably. The two sets of data, which are reported in Table 1, gave very consistent results in simultaneous analyses. The overall picture is that of a homogeneous, stable system, very similar to the natural protein.

4.2. Emission of Trp-7 in the ferric form of W14F myoglobin

This system is characterized by lower stability than the other two recombinant proteins. This is indicated by the very little resistance of this protein to acid exposure. As shown in Table 2, at neutral pH the lifetimes of Trp-7 do not change between pH 7.0 and 5.1, and can be simultaneously analyzed. Instead the lifetimes at pH 4.8 are consistently longer and cannot be analyzed simultaneously with the data sets obtained at pH 7.0 and 5.1. This indicates an unfolding process, which increases the separation between Trp-7 and heme. It is important to note that the lifetimes recovered by global analysis of the data at pH 7.0 and 5.1 (therefore very consistent internally) do not correspond to those computed on the basis of the atomic coordinates of natural myoglobin. Essentially they are much shorter than anticipated. Notably, also the lifetime of **species III** with dissociated heme is shorter. This indicates that the quantum yield of this residue is in all cases lower than that expected from tryptophan fluorophores. Two explanations can be provided for the lifetimes distribution of this mutant in the met-form: (1) there is a collapse of the myoglobin structure which positions tryptophan 7 in closer proximity

to the heme by at least 5 Å; (2) there is an additional quenching of the tryptophan excited state, additional to that produced by excitation energy transfer to the heme. The first hypothesis is supported by the observation that the system is not very stable at acid pH. However, protein instability would not affect the long lifetimes of the heme missing in **species III**. Also, unfolding processes are more likely to increase the distance between the various segments of the protein, increasing the distance between Trp-7 and heme and producing longer rather than shorter lifetimes for the various myoglobin species. This in fact happens at pH 4.8 where, as mentioned above, the protein starts to unfold producing a significant increase in the length of all fluorescence lifetimes. Very important is the observation that, at neutral pH, the far UV CD spectrum of ferric W14F myoglobin did not indicate losses of secondary structure and was superimposible to that of W7F myoglobin. The hypothesis of an additional quenching is more probable because it involves and shortens all of the lifetime components, even that of **species III** with dissociated heme. The following can be proposed. The lifetime of 34 ps, instead of the expected 114 ps, would result from the extra quenching of **species I**. The lifetime near 250 ps, rather than the expected 1200 ps, is the over-quenched lifetime of **species II**. The one near 3000 ps, instead of 5000 ps, reflects the 'additional' quenching of Trp-7 in **species III** with a dissociated heme. We propose that the very initial portion of the polypeptide chain has a conformation (a loop) which brings the extra initial methionine near Trp-7, resulting in the additional fluorescence quenching due either to collisions or to electron transfer phenomena with the sulfur of the methionine. The major components of the system, **species I** with a lifetime of 34 ps, has an amplitude of only 80%, rather than 95% or more, as in W7F and as in RWT and real-RWT.[1] This suggests that the loop is not stable and fluctuates with the thermal motions of the protein. Therefore the lifetime near 250 ps would include both the over-quenched lifetime of **species II** and some portion of non-over-quenched lifetime of the residual **species I**. This would also explain the large amplitude, approx. 15%, of the lifetime near 250 ps, too large for being referred only to heme inversion. The lifetimes detected at pH 4.8 cannot be interpreted on the basis of the atomic coordinates of natural SW myoglobin. They become longer, consistent with an initial unfolding of the system, which increases the Trp–heme separation.

[1]Both in RWT and real-RWT the total amplitude of the short lifetimes of W7 and W14 is the sum of the respective amplitudes, $\alpha_1 + \alpha_2$.

4.3. Emission of Trp-7 in the CO-form of the W14F myoglobin: heme linked conformational changes

As shown in Table 2, the lifetimes of the CO-form of this mutant are almost exactly those estimated from the atomic coordinates of SW myoglobin. Also, the respective amplitudes of the lifetimes are consistent with data present in the literature on SW myoglobin [2–4] and obtained by us on horse heart myoglobin [8]. The additional quenching of Trp-7, present in the ferric form, is not evident in this form of the W14F mutant, as if the proposed quenching loop would be less efficient in the CO derivative. Whatever is the reason for the additional quenching of Trp-7 in the ferric form, clearly the data indicate a long range heme-linked conformational change of the protein. Contrary to the findings for the ferric form, which started unfolding at pH 4.8, exposure of this mutant in its CO form to acid pH (pH = 4.5) did not affect significantly its lifetimes characteristics. This was probably due to the higher affinity of the protein for ferrous hemes, which stabilizes the folded form of the system.

4.4. Emission of RWT myoglobin

This recombinant myoglobin is the parent of the two mutants, and carries the extra N1 methionyl residue. As judged from their respective length, the lifetime components of RSW are the combination of the components found in the two mutants. All four components match the values anticipated by the computations, however, the respective amplitudes are not what is expected. In

fact if the two tryptophans are equally exposed to incident light the ratio of the amplitudes of the two shortest lifetimes near 30 and 150 ps, originating from Trp-14 and Trp-7 in **species I**, respectively, should be close to 1.0. Instead, the ratio α_1/α_2 of the RWT myoglobin, in Table 3, is close to 7 in the ferric form and to 2 in the CO-form of the system.

This may suggest that there is an additional quenching of Trp-7 produced by the initial N1-met, which is much reduced in the CO form of the system. It is consistent with the presence of long range interactions of the heme with the initial portion of the polypeptide chain.

4.5. Heme linked conformational changes

As shown above myoglobins with the N1 methionine show heme linked conformational changes going from ferric to CO derivatives. The question may be posed whether these conformational changes are present also in real-RWT, i.e. without the extra N1 methionine. We measured the lifetimes of real-RWT in its CO- and ferric-forms. The data presented in Table 3 are very consistent with the data obtained in the past with natural SW myoglobin [2–4]. It should be noted, however, that the ratio α_1/α_2 remains near 2 both in the ferric and CO systems. In other words it appears that also in real-RWT the two tryptophans at position 7 and 14 emit with different intensity, contrary to findings in horse heart myoglobin where that ratio is near 1.0 [8]. Still the atomic coordinates indicate a similar position and exposure to incident light of the two chromophores near the surface of the molecule. Therefore we may speculate that the lower than expected amplitude of the 150 ps lifetime of Trp-7 in real-RWT myoglobin is due to the presence of a quenching loop, or other conformational detail, which, in the absence of the N1 methionine, is less efficient as a quencher. Therefore it gives a weak signal, detectable in the amplitudes, however, not large enough to affect in a clear way the length, of the lifetimes of the system.

It should be stressed that this hypothesis is consistent with recent evidence of ligand linked conformational changes in myoglobin. Ansari et al. [17] report that the heme pocket of myoglobin is sensitive to the presence of ligands in a similar way as for the R to T transition in hemoglobin. Giardina et al. [18] have shown that the oxygen affinity of myoglobin is regulated by the presence of lactic acid, as an allosteric effector. Our data seem to add to these observations, strongly suggesting the presence of conformational interactions between the heme and the initial portion of the polypeptide chain of Sperm Whale Myoglobin.

4.6. Structural relevance of the tryptophans

The perfect correspondence of the computed and experimental lifetimes of Trp-14 in W7F myoglobin suggest little relevance of Trp-7 to the structure of SW myoglobin. The substitution of Trp-14 with phenylalanine is the reason for the unfolding of met-form of W14F already at pH 4.8. This reveals the relevance of Trp-14 to the structure of the system. Its CO form is more resistant to exposure to acid. It may be argued that the hydrophobicity of the substituting phenylalanine in position 14 is capable of sustaining the normal folding of the protein, however, not its stability at acid pH. It may be noticed that the amount of **species III** with dissociated hemes is much higher in both mutants than in the wild types. This may suggest a non-specific effect of the substitutions on the thermal fluctuations of the system, resulting in a decreased affinity of the protein for the heme. It may be anticipated that in the mutants heme transfer to albumin or other acceptors would be faster than in normal myoglobin.

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References

[1] G. Weber, F.J. Teale, Disc. Faraday Soc. 27 (1959) 134–141.
[2] R.M. Hochstrasser, D.K. Negus, Proc. Nat. Acad. Sci. U.S.A. 81 (1984) 4399–4404.
[3] S.M. Janes, G. Holtom, P. Ascenzi, M. Brunori, R.M. Hochstrasser, Biophys. J. 28 (1987) 1508–1512.
[4] E. Bismuto, G. Irace, E. Gratton, Biochemistry 28 (1989) 1508–1511.
[5] Z. Gryczynski, E. Bucci, J. Kusba, Photochem. Photobiol. 58 (1993) 492–496.
[6] Z. Gryczynski, R. Paolesse, K.M. Smith, E. Bucci, J. Phys. Chem. 98 (1994) 8813–8816.
[7] Z. Gryczynski, J. Lubkowski, E. Bucci, Meth. Enzyme 278 (1997) 538–569.
[8] Z. Gryczynski, J. Lubkowski, E. Bucci, J. Biol. Chem. 270 (1995) 19232–19237.
[9] M.S. Hargrove, A.S. Wilkinson, J.S. Olson, Biochemistry 35 (1996) 11300–11309.
[10] E. Antonini, M. Brunori, Hemoglobin and Myoglobin in their Reaction with Ligands, in: A. Neuremberg, E.L. Tatum (Eds.), North Holland, New York, 1997.
[11] G. Lazko, I. Gryczynski, Z. Gryczynski, W. Wiczk, H. Malak, J.R. Lakowicz, Sci. Instrum. 61 (1990) 2331–2337.
[12] Z. Gryczynski, E. Bucci, Biophys. Chem. 48 (1993) 31–38.
[13] J.M. Beechem, E. Gratton, M. Amelot, J.R. Knutson, L. Brand L, in: J.R. Lackowicz (Ed.), Topics in Fluorescence Spectroscopy, vol 1, Plenum Press, New York, 1991, pp. 241–305.
[14] B. Albinsson, M. Kubista, B. Norden, E. Thulstrup, J. Phys. Chem. 93 (1989) 6646–6654.
[15] T. Förster, in: Sinanoglu (Ed.), Modern Quantum Chemistry, part III, Academic press, New York, 1965, pp. 93–137.
[16] Z. Gryczynski, S. Beretta, J. Lubkowski, A. Razynska, I. Gryczynski, E. Bucci, Biophys. Chem. 64 (1996) 81–91.
[17] A. Ansari, C.H. Jones, E.R. Henry, J. Hofrichter, W.A. Eaton, Biochemistry 35 (1994) 5128–5235.
[18] G. Giardina, P. Ascenzi, M.E. Clementi, G. De Sanctis, M. Rizzi, M. Coletta, J. Biol. Chem. 29 (1996) 16999–17001.