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# The effect of molecular confinement on the conformational dynamics of the native and partly folded state of apomyoglobin

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**Abstract Inclusion in agarose gel significantly affects the conformational dynamics of native and acidic partly folded states of tuna apomyoglobin, a single tryptophan containing protein, as documented by frequency domain fluorometry investigations. The heterogeneity of the tryptophanyl emission decay increases on gel inclusion compared to that observed for free-in-solvent protein at both neutral and acidic pH, thus suggesting that the interconversion rate among conformational substates is somewhat reduced. The observation that this effect is much more pronounced for the partly folded state suggests that confined environments such as those existing in the living cells might favor the sequential folding process avoiding that structured intermediates rapidly convert into less structured ones.** 

*Key words:* Apomyoglobin, partly folded state; Conformational dynamics; Frequency domain fluorometry; Protein, gel entrapment

## 1. Introduction

Biochemical processes involving proteins are conventionally studied under relatively idealized conditions, i.e. low protein and moderate salt concentrations, selected in order to minimize the effects of non-specific interactions and to extract intrinsic quantities and information reflecting the properties of the isolated macromolecule. The interior of a living cell differs from the idealized conditions because of the very high concentrations of macromolecules, proteins and nucleic acid, which occupy a significant fraction of the total cell volume, i.e. 30–40% in the cytoplasm of *Escherichia coli* [1]. The high-volume occupancy might cause significant changes in many parameters and this can have consequences on the structure and stability of individual protein macromolecules. Using a statistical-thermodynamic model, Minton [2] proposed that molecular crowding or uniform confinement favor compact globular conformations. More recently, Eggers and Valentine [3] presented experimental evidence that molecular confinement as achieved in silica gel influences protein structure and enhances thermal protein stability.

Most of the studies on protein folding, so far, have been carried out examining how pure denatured proteins refold when the denaturant is removed. Since the conditions used for refolding in vitro are only distantly related to those found in vivo, most of the conclusions must be evaluated with great care. In fact, the first contacts of a newborn polypeptide chain may be critical for determining its fate. The biological medium, particularly in eukaryotes, contains not only a large concentration of soluble macromolecules but also a network of extended structures such as F-actin, microtubules, intermediate filaments and membrane boundaries. Contacts with these soluble or structural macromolecules may affect the sequential folding pathway of a given protein.

Apomyoglobin folding is known to proceed through a partly folded state, also referred to as molten globule intermediate, which has been detected both in equilibrium and in kinetic experiments [4–8]. In this intermediate, A, G and H helices are folded as in the native state, while the remainder of the molecule seems to be unordered [5,8–10]. The aim of this paper is to explore the effects produced by agarose gel confinement on the conformational dynamics of the native and partly folded state of tuna apomyoglobin, a single tryptophan containing protein, studying the intrinsic tryptophanyl decay by frequency domain fluorometry. The results clearly show that the fluorescence emission of tuna apomyoglobin in whatever conformational state, i.e. native, partly folded or fully unfolded, is strongly affected upon inclusion of the protein in 1% agarose gel. In particular, a strong increase of fluorescence heterogeneity has been observed. The increase of fluorescence heterogeneity has been related to a decrease of interconversion rate among conformational substates produced by the confinement of protein molecules in the gel network.

## 2. Materials and methods

### 2.1. Myoglobin

The main component of tuna myoglobin was prepared according to the method described elsewhere [11]. The protein was used after a further purification step performed by fast liquid chromatography using a Superdex-75 column (10 mm × 25 cm) equilibrated with 0.05 M phosphate pH 7.0. Myoglobin concentrations were determined spectrophotometrically in the Soret region using the following extinction coefficient: 139 000 $M^{-1}$ $cm^{-1}$ at 407 nm [12].

### 2.2. Apomyoglobin

The heme was removed from myoglobin by the 2-butanone extraction procedure of Teale [13]. The contamination of the apoprotein by myoglobin was assessed spectrophotometrically. In all cases no significant absorption was observed in the Soret region.

The concentration of apomyoglobin was determined in 6.0 M guanidine, pH 7.0, by absorbance at 280 nm [14] using a molar extinction coefficient calculated from tryptophan and tyrosine content [15], i.e. 8000 $M^{-1}$ $cm^{-1}$ at 280 nm. Concentrations were also controlled by absorption methods operating in the peptide absorbing region [16].

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*2.3. Fluorescence emission decay measurements*

Frequency domain techniques [17] were used to measure the fluorescence decay in the range 5–200 MHz using a multifrequency phase shift and modulation cross-correlation fluorometer GREG 200 (ISS, Urbana, IL, USA). The emission was observed using an optical filter combination of UV34 and U340 (Oriel Corp., Sarl, Paris, France). The reference was a glycogen solution as scatterer. The temperature was monitored continuously during measurements by attaching a thermocouple to sample cuvette. Readings of the thermocouple were monitored by means of an omega digicator (Omega Engineering, Stanford, CT, USA) with an accuracy of ±0.1°C. The absorbance of the sample did not exceed 0.1 at the excitation wavelength. The protein samples entrapped in 1% agarose (A 0701, Type VII-A Low Gelling Temperature from Sigma, St. Louis, MO, USA) were directly prepared in cuvettes adding the protein to the agarose mixture at 32°C before each measurement. The lifetime analysis was performed by Global Unlimited (University of Illinois, Urbana, IL, USA) according to Beechem et al. [18]. The effect of agarose scattering on multifrequency phase-modulation measurements was controlled using *N*-acetyltryptophanylamide (NATA) as control. Fluorescence lifetime of NATA in 1% agarose was substantially similar to that of the fluorophore free in the solvent.

## 3. Results and discussion

The fluorescence emission of tuna apomyoglobin arises from the contribution of a single tryptophanyl residue, i.e. W14, located along the A helix in the N-terminal region of the molecule [12]. The steady state emission spectrum recorded at 20°C, with excitation at 295 nm, shows a maximum at 325 nm, a value which is blue shifted compared to that of the monomeric tryptophanyl residue, i.e. NATA in aqueous solution, which occurs at 348–350 nm. The inclusion of the protein in 1% agarose did not modify the shape of the spectrum and the position of the emission maximum.

The fluorescence emission decay of tuna apomyoglobin was subjected to a detailed investigation by using the multifrequency phase-modulation technique [17]. The phase shifts and demodulation factors were collected by using modulated excitation at 295 nm with frequency ranging between 10 and 200 MHz. The fluorescence emission was filtered by means of an optical filter combination of UV34 and U340. The observed frequency response is complex as a consequence of the large fluorescence heterogeneity related to the intrinsic protein dynamics [19]. The emission decay was analyzed as the sum of a few discrete exponential components or using distribution models of analysis having different shape [20]. The most appropriate model to best fit the emission data relative to free and agarose-entrapped tuna apomyoglobin appeared to be a bimodal lifetime distribution with both components having a Lorentzian shape as judged on the basis of

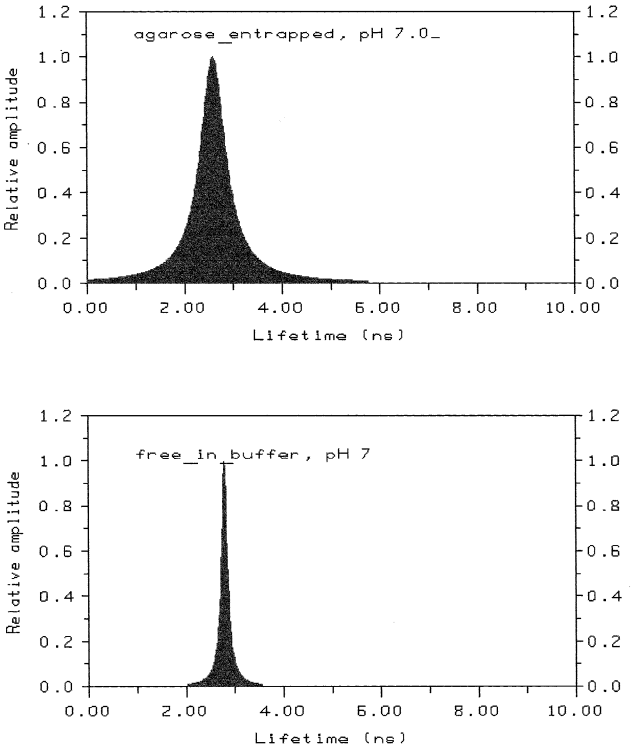


Fig. 1. Tryptophanyl lifetime distribution of agarose-entrapped tuna apomyoglobin at neutral pH and 18.1°C in comparison with the distribution of the free protein at the same pH and temperature. All solutions contained: 0.01 M phosphate, pH 7.0. Excitation was at 295 nm; emission was observed through an optical filter combination of UV34 and U340.

$\chi^2$ values. However, whatever model is considered, the most important conclusion is that the emission decay of this protein can be interpreted in terms of two main lifetimes or distribution centers which exhibit a similar temperature dependence. Because of the marked similarity between the results from discrete and distribution analysis, we will discuss the experimental results in terms of bimodal distribution of lifetimes. It is our opinion that the interpretation of the emission decay in terms of continuous lifetime distributions is much more convincing than that obtained by means of discrete components, not only on a statistical basis, but mainly because of the fluorescence heterogeneity arising from a great variety of conformational substates. The distribution parameters relative to the emission decay of native and partly folded acidic apomyoglobin are shown in Tables 1 and 2.

Table 1
Lorentzian tryptophanyl lifetime distribution analysis of free-in-solvent and 1% agarose-entrapped tuna apomyoglobin at neutral pH

| *T* | Center(1) | Width(1) | Fraction(1) | Center(2) | Width(2) | Fraction(2) | $\chi$-square |
|---|---|---|---|---|---|---|---|
| Free-in-solvent apomyoglobin | | | | | | | |
| 3 | 3.179 | 0.201 | 0.954 | 0.726 | 0.05 | 0.046 | 0.593 |
| 18.1 | 2.785 | 0.154 | 0.917 | 0.837 | 0.05 | 0.083 | 0.546 |
| 28.5 | 2.238 | 0.13 | 0.889 | 0.726 | 0.05 | 0.111 | 0.909 |
| 38 | 1.795 | 0.196 | 0.931 | 0.447 | 0.05 | 0.069 | 2.18 |
| 47.7 | 1.4 | 0.28 | 0.96 | 0.373 | 0.05 | 0.04 | 1.164 |
| 1% agarose-entrapped apomyoglobin | | | | | | | |
| 8.1 | 2.983 | 0.619 | 0.993 | 0.007 | 0.05 | 0.007 | 0.817 |
| 18.1 | 2.584 | 0.642 | 0.992 | 0 | 0.05 | 0.008 | 1.725 |
| 28.5 | 2.157 | 0.664 | 1 | | | 0 | 2.734 |
| 38 | 1.796 | 0.05 | 0.859 | −2.367 | 0.05 | 0.141 | 1.574 |
| 47.7 | 1.456 | 0.05 | 0.759 | −2.37 | 0.05 | 0.241 | 1.571 |

Table 2
Lorentzian tryptophanyl lifetime distribution analysis of free-in-solvent and 1% agarose-entrapped tuna apomyoglobin at pH 3.4

| $T$ (°C) | Center(1) | Width(1) | Fraction(1) | Center(2) | Width(2) | Fraction(2) | $\chi$-square |
|---|---|---|---|---|---|---|---|
| Free-in-solvent apomyoglobin | | | | | | | |
| 8.1 | 3.952 | 0.858 | 0.792 | 0.906 | 1.315 | 0.208 | 1.527 |
| 18.1 | 3.524 | 0.88 | 0.77 | 0.82 | 0.99 | 0.23 | 1.472 |
| 28.6 | 3.064 | 0.915 | 0.741 | 0.737 | 0.485 | 0.259 | 1.334 |
| 38 | 2.453 | 0.914 | 0.751 | 0.53 | 0.05 | 0.249 | 1.686 |
| 48 | 1.912 | 0.941 | 0.745 | 0.483 | 0.05 | 0.255 | 2.905 |
| 1% agarose-entrapped apomyoglobin | | | | | | | |
| 8.1 | 4.418 | 1.995 | 0.378 | 2.022 | 3.837 | 0.622 | 1 |
| 18.1 | 3.438 | 2.187 | 0.722 | 1.116 | 2.954 | 0.278 | 1.23 |
| 28.5 | 2.507 | 2.668 | 0.96 | 0 | 0.479 | 0.04 | 1.305 |
| 38 | 1.829 | 2.707 | 0.98 | 0 | 0.05 | 0.02 | 1.158 |
| 47.7 | 1.489 | 2.683 | 0.999 | 0 | 0.05 | 0.001 | 4.231 |

Fig. 1 shows the tryptophanyl lifetime distribution of agarose-entrapped tuna apomyoglobin at neutral pH and 18.1°C in comparison with the distribution of the free protein. The emission decay of free apomyoglobin at neutral pH is essentially represented by a single narrow Lorentzian distribution. The short lifetime component which contributes negligibly to the total fluorescence probably originates from weakly scattered light or other unpredictable instrumental contributions [21]. Gel inclusion determines a considerable increase of fluorescence heterogeneity as documented by the increase of distribution width, i.e. from 0.201 to 0.619 ns. Since the fluorescence width is related to the number of conformational substates that the indole fluorophore experiences during its permanence in the excited state and to the interconversion rate among them, it is reasonable to hypothesize that gel entrapment reduces the interconversion rate among substates. A plausible explanation is that the agarose gel network because of its high hydrophilicity strongly reduces the mobility of water molecules interacting with the protein surface, thus introducing surface restraints on the outer shell of the molecule. This would produce a reduction of the conformational freedom of the rest of the protein and, hence, a decrease of the interconversion rate among conformational substates.

Fig. 2 shows the temperature dependence of the distribution center and width of the agarose-entrapped tuna apomyoglobin at neutral pH in comparison with that of free protein. No difference is observed following the distribution center, which monotonically decreases because of the increase of thermal motion in the tryptophan environment. By contrast, the temperature dependence of distribution width of gel-entrapped apomyoglobin at neutral pH differs from that of the free protein. In fact, between 30 and 40°C, the width of the fluorescence lifetime distribution of the immobilized protein decreases from 0.664 to 0.05. This result can be explained considering that the width of lifetime distribution depends both on the interconversion rate among conformational substates and the number of conformations accessible to the polypeptide. A temperature increase determines a reduction of lifetime distribution width because of the augmented rate of interconversion [19]. By contrast, thermal induced unfolding increases the distribution width [11]. Since gel entrapment enhances protein stability making the protein less susceptible to denaturation, the reduction of width observed for agarose confined apomyoglobin at higher temperatures can be reasonably ascribed to the increased rate of interconversion among conformational substates. For the protein free in solvent, the increase of distribution width due to thermal unfolding masks the increase of interconversion rate. Thermal denaturation of apomyoglobin was controlled by examining the fluorescence emission maximum. At the highest examined temperature, the fluorescence emission spectrum showed the appearance of a shoulder at 350 nm indicative of the co-existence in solution of denatured apomyoglobin.

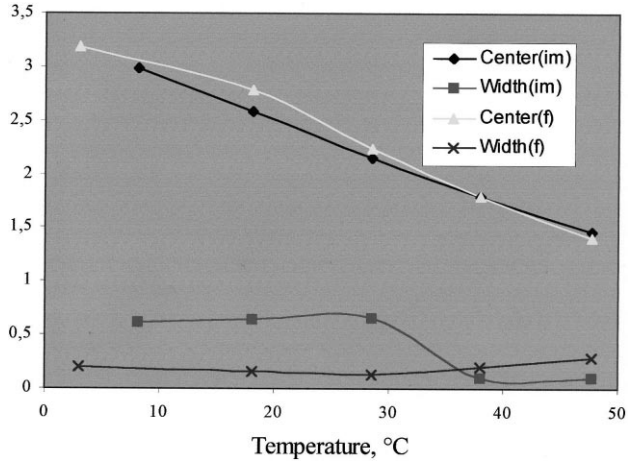


Fig. 2. Temperature dependence of tryptophanyl fluorescence distribution parameters of free (f) and gel-entrapped (im) tuna apomyoglobin at neutral pH. The other experimental conditions are reported in Fig. 1.

At pH 3.4 in the presence of moderate salt concentration, the most populated conformational state of tuna apomyoglobin is the partly folded state which is also referred as molten globule state. The steady state emission spectrum recorded in these conditions, with excitation at 295 nm, shows a maximum at 338 nm, which is red shifted compared to that of native apomyoglobin because of the increased accessibility of solvent to indole ring. The inclusion of the protein in 1% agarose at acidic pH did not modify the shape of the spectrum and the position of the emission maximum. Fig. 3 shows the tryptophanyl lifetime distribution of agarose-entrapped tuna apomyoglobin at pH 3.4 and 18.1°C in comparison with the distribution of the free protein in similar experimental conditions. The tryptophanyl fluorescence decay of free-in-solvent apomyoglobin at acidic pH is represented by two well separated distribution components centered at 3.5 and 0.8 ns. In a previous work, we showed that the two peaks actually represent physically distinct species each with characteristic internal

dynamics. In particular, the long-lived component arises from partly folded apomyoglobin molecules whereas the short-lived component is produced by fully unfolded molecules [22,23]. The strong heterogeneity documented by the rather large width of both distributed component detected in the lifetime distribution, i.e. 0.88 and 0.99 for the long- and short-lived component, respectively, is consistent with the idea that the fluorescence decay of tuna apomyoglobin at acidic pH mainly arises from partly and fully denatured molecules, in which the conformational space accessible to the indole residue is not longer constrained by tertiary interactions. The inclusion of tuna apomyoglobin in 1% agarose at acidic pH determines a further broadening of both fluorescence distribution components, which cannot be distinguished at the highest temperatures. The enhanced fluorescence heterogeneity corroborates the hypothesis that gel entrapment reduces the interconversion rate among conformational substates.

Fig. 4 shows the temperature dependence of the distribution parameters, i.e. center and width, of tuna apomyoglobin at pH 3.4 in 1% agarose in comparison with the data relative to free apomyoglobin. At the lowest temperature, the tryptophanyl fluorescence lifetime of agarose-entrapped acidic apomyoglobin is higher than that of the protein free in solvent. A plausible explanation is that the hydrophilic gel network strongly reduces the mobility of water molecules interacting with the exposed fluorophore, thus contributing to increase the fluorescence lifetime. Moreover, gel-entrapped apomyoglobin exhibits a further increase of distribution width between 20 and 30°C and a steeper thermal dependence of the distribution center compared to that of free-in-solvent protein.

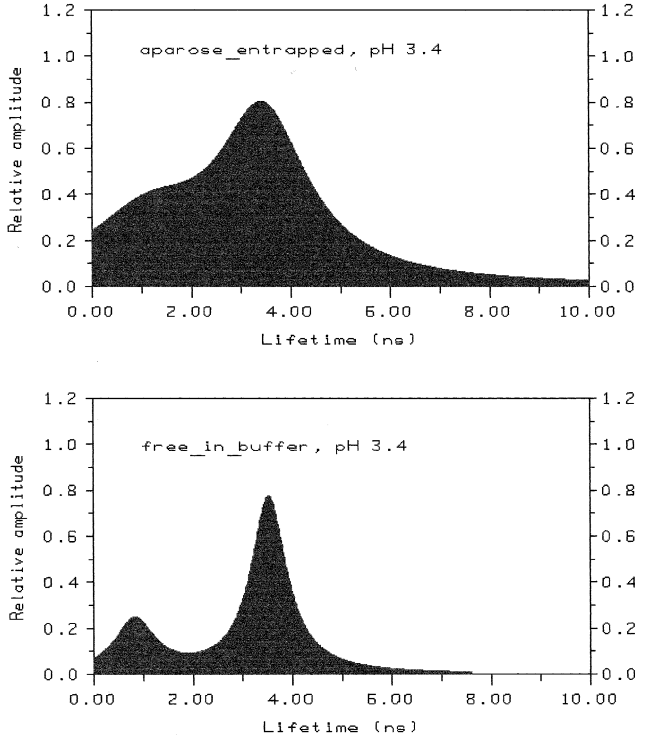


Fig. 3. Tryptophanyl lifetime distribution of agarose-entrapped tuna apomyoglobin at pH 3.4 and 18.1°C in comparison with the distribution of the free protein in similar experimental conditions. All solutions contained: 0.01 M phosphate, pH 3.4. The other experimental conditions are reported in Fig. 1.

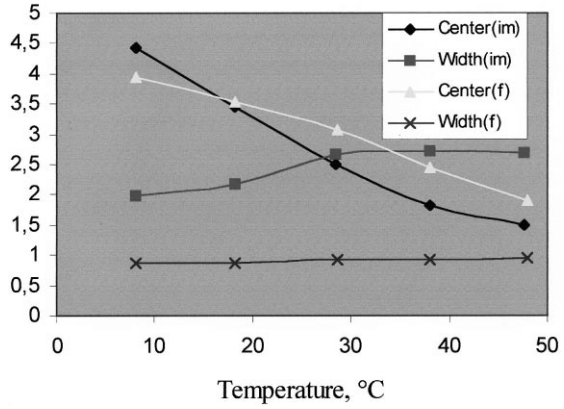


Fig. 4. Temperature dependence of tryptophanyl fluorescence distribution parameters of free (f) and gel-entrapped (im) tuna apomyoglobin at acidic pH. The other experimental conditions are reported in Fig. 1.

The increased susceptibility to temperature induced quenching observed for apomyoglobin at pH 3.4 when entrapped in agarose gel is probably due to the gel network that hinders the aggregation process that is commonly occurring in free solution. The coalescence of the tryptophanyl containing region of an apomyoglobin molecule with hydrophobic segments of other partly folded molecules could mask the indole residue from the contact with solvent molecules and intramolecular groups acting as fluorescence quenchers.

The results reported in this paper indicate that the confinement in agarose gel strongly affects the conformational dynamics of apomyoglobin, reducing the rate of interconversion among conformational substates. Flash photolysis experiments performed on carbon-monoxy myoglobin embedded in trehalose–water matrix had previously shown that protein dynamics is strongly inhibited [24–26]. More recently, Cordone and co-workers [27,28] reported that the specific motions arising from thermal fluctuations of myoglobin molecule among conformational substates are hindered for the protein in trehalose–water matrix. The observation that the dynamics of the acidic partly folded state is significantly affected suggests that confined environments, such as those existing in the living cells, might favor the sequential folding process, avoiding that structured intermediates rapidly convert into less structured ones. The confinement also reduces the intermolecular aggregation of proteins which is commonly observed under extreme experimental conditions, such as those employed for studying partly folded states having conformational properties similar to those of the intermediate species appearing during spontaneous folding. In conclusion, the use of confined environment and macromolecular crowding should become routine for studying biological macromolecules under more physiologically relevant conditions.

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