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Tryptophanyl contributions to apomyoglobin fluorescence resolved by site-directed mutagenesis

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Abstract

The individual emission properties of the two tryptophanyl residues of sperm-whale apomyoglobin have been resolved by examining the fluorescence variations induced by denaturants, i.e., acid and guanidine, on apomyoglobin mutants W7F and W14F. The fluorescence changes have been correlated to the conformational transitions undergone by apomyoglobin on increasing denaturant concentration. The results indicate that the fluorescence decrease, observed for sperm-whale apomyoglobin on going from pH 8.0 to pH 6.0, cannot be ascribed to the formation of a charge transfer complex between a nearby histidine residue and W14 as reported in earlier papers but rather to minor structural changes affecting the microenvironments of both residues. The formation of the acidic partly folded state around pH 4.0 determines an increase of the fluorescence yield and a small red shift (5 nm) of W7 due to removal of sterically interacting K79, which is able to attenuate the emission of this residue in the native state. The fluorescence intensity of the other residue, i.e., W14, is not affected by the acidic transition. Guanidine denaturation experiments revealed an increase of fluorescence yield of W14 upon the intermediate formation, whereas the fluorescence of the other residue remained constant. The results suggest that the unfolding pathway may be different depending on the chemical nature of the denaturant used.

Keywords: Tryptophanyl fluorescence; Apomyoglobin; Site-directed mutagenesis; Protein unfolding

1. Introduction

The 'protein-folding problem' deals with how a linear polypeptide chain can spontaneously acquire the specific tertiary structure necessary for its functioning. The pathway by which this happens is still imperfectly understood. Kinetics studies primarily contribute to the understanding of the molecular events by which the final biologically active conformational state is attained. Equilibrium studies are particularly performed to characterise differences in the structure and stability of the protein states through which the folding pathway occurs. There are a number of biophysical methods that can be used to monitor the folding and unfolding processes of a protein. Among these, fluorescence has many advantages and applications [1]. In particular, intrinsic fluorescence of a protein, dominated by the contribution of tryptophan residues, has been widely employed to detect the conformational changes occurring in globins under pH variations of the medium.

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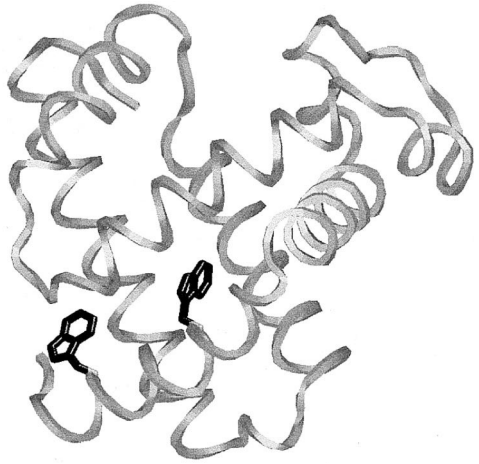

Fig. 1. Schematic picture of apomyoglobin structure indicating the localisation of W7 and W14.

Sperm-whale myoglobins contain two tryptophanyl residues, which fill the invariant positions A-5 (W7) and A-12 (W14) located in the N-terminal region (A-helix) of the protein molecule (Fig. 1) [2,3]. The folding of this protein is known to proceed through a molten globule intermediate, I state, detected in both equilibrium and kinetic experiments [4–9]. 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 [5,8,10–14]. More recently, the occurrence of different intermediates has been reported [15–17].

The structural changes at the N-terminal and in the adjacent region of myoglobin have been extensively studied following the fluorescence of two tryptophanyl residues. Nevertheless, the pH-dependent fluorescence of mammalian apomyoglobins is complicated because the emission properties and the contributions of the two tryptophans to the total emission spectrum are very different. Attempts were made to separate the individual contributions of W7 and W14 by using various quenchers and perturbing reagents [18–22].

In order to resolve the individual fluorescence properties of the two tryptophanyl residues we performed site-directed mutagenesis on sperm-whale myoglobin substituting alternatively the two indolic residues with a phenylalanine. In this paper, we analyse the effects produced by acid and guanidine on the fluorescence emission of wild type and single tryptophan-containing apomyoglobin mutants at low ionic strength. The comparison among the denaturation profiles of the examined proteins allowed us to depict the individual tryptophanyl contribution to the overall fluorescence during the conformational transitions, i.e., from native to I and from I to the acid unfolded state, undergone by apomyoglobin on acid unfolding.

2. Materials and methods

2.1. Mutagenesis of the myoglobin gene

Drs B.A. Springer and S.G. Sligar kindly provided plasmid pMb413 [23] bearing the synthetic sperm-whale myoglobin gene. DNA manipulations were essentially performed as described in Sambrook et al. [24]. The W7 and W14 residues (W7F and W14F substitutions) were mutated with 5′-GTTCT-GTCTGAAGGTGAATTCCAGCT-GGTTCTG-3′ and 5′-GGTTCTGCATGTTTTCGCTAAAGTTG-AAGCTG-3′ oligonucleotides respectively, using the Clontech Transformer site-directed mutagenesis kit. Mutants were screened and confirmed by sequencing double-stranded DNA in the region of the mutation using Sequenase kit purchased from United States Biochemical Corporation. Mutant myoglobins were expressed in *Escherichia coli* strain TB-1 [*ara*, Δ*(lac-pro), strA, thi*, Φ*80dlacZ*Δ*M15, r*$^{-}$, *m*$^{+}$] [23].

2.2. Protein purification

E. coli TB-1 harbouring the pMb413 plasmid was grown at 37°C in LB in the presence of ampicillin (200 mg/l). Protein was essentially purified as described by Springer and Sligar [23]. Briefly, a 10-l culture of cells was harvested in late log phase, lysed overnight and sonicated. Cell debris was removed by centrifugation, and the supernatant was fractionated by ammonium sulfate precipitation. The 60–95% cut was centrifuged and suspended in 20 mM Tris–1 mM EDTA, pH 8, and fractionated on a Sephadex G-50 (Pharmacia) gel filtration column (2.5×100 cm) equilibrated in the same buffer. Reddish-brown myoglobin-containing fractions were collected and applied to a Whatman DEAE 52 ion exchange column (2.5×20 cm) equilibrated and resolved with 20 mM

Tris–HCl pH 8.4. Under these conditions, myoglobin did not stick on the column and was rapidly eluted. Protein purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [25]. Met-myoglobin concentrations were determined using the following extinction coefficient: $\varepsilon_{409} = 157$ mM^{-1} cm^{-1} [26].

2.3. Apomyoglobin

The haem was removed from myoglobin by the 2-butanone extraction procedure of Teale [27]. 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 [28] using molar extinction coefficients calculated from tryptophan and tyrosine content [29]. Concentrations were also checked by absorption methods operating in the peptide absorbing region [30].

2.4. Chemicals and solutions

All common chemicals were reagent grade and were purchased from British Drug Houses. 8-Anilino-1-naphthalene sulfonate (ANS) was a product of Merck Co., and its Mg^{2+} salt was recrystallised twice with the method described by Weber and Young [31]; ANS concentration was determined spectrophotometrically using the following extinction coefficient: 5×10^3 M^{-1} cm^{-1} at 350 nm.

2.5. Spectral measurements

Absorption measurements were carried out on a Perkin-Elmer Lambda Array 3840 spectrophotometer. Steady-state fluorescence measurements were performed on a Perkin-Elmer MPF-66 fluorescence spectrophotometer. In denaturation experiments, the spectral measurements were monitored over time until an apparent equilibrium was reached. The pH of each sample was determined prior to and after measuring fluorescence. Denaturation was found to be highly reversible providing that the time of permanence at acidic condition did not exceed 20 min.

Temperature was kept constant at 20°C using an external bath circulator. For ANS–apomyoglobin complex, the baselines were obtained using the same concentration of free fluorophore in water at each examined pH. In all cases, the fluorescence intensity of the free fluorophore was much smaller than that of the ANS–apomyoglobin complex. Moreover, acid addition to free-in-water ANS did not perturb the emission properties of the fluorophore. Very small variations of the position of emission maximum, i.e., ± 1 nm, were observed at each examined pH.

3. Results and discussion

To obtain information on the individual contribution of tryptophanyl residues to the overall apomyoglobin emission, we designed myoglobin mutants in which one of the two tryptophanyl residues is replaced with a phenylalanine. These recombinant proteins were expressed in *E. coli* as correctly folded globins and purified following the procedure reported by Springer and Sligar [23].

We have already shown that the W7F and W14F replacements have very little effect on the overall globin fold of apomyoglobin as evidenced from near and far ultraviolet circular dichroism (CD) studies and from fluorescence emission spectra [32]. At neutral pH, the emission maximum of W7F is 330.6 nm, whereas that of W14F mutant is slightly red shifted, the maximum being centred at 333.4 nm. No significant variation of fluorescence polarisation at 330 nm was observed for either protein, i.e., the values were 0.146 and 0.157 for W7F and W14F apomyoglobin, respectively. These values are similar to those measured for wild type apomyoglobin, i.e., 331 nm and 0.148. These results indicate that each tryptophanyl replacement does not affect the physical properties of the surroundings of the other residue. This is consistent with the finding that the CD activity of tryptophanyl residues of wild type apoprotein can be obtained by summing the spectra recorded for the two single tryptophanyl-substituted proteins [32]. In order to identify the individual contribution of each tryptophanyl residue to the overall apomyoglobin emission fluorescence, we analysed the acid- and guanidine-induced unfolding of the W7F and W14F apomyoglobin mutants. At low salt concentration,

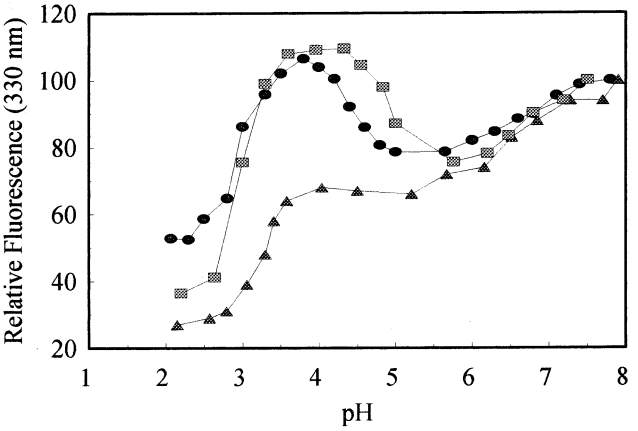

Fig. 2. The acid pH dependence of tryptophanyl relative fluorescence intensity at 330 nm of wild type and mutant apomyoglobins: ●, wild type; ■, W14F; ▲, W7F. Data are from three sets of experiments for each apomyoglobin. Protein concentrations were 1.36×10^{-5} M, 1.6×10^{-5} M and 2.1×10^{-5} M for wild type, W14F and W7F, respectively. Protein solutions contained 0.01 M sodium phosphate, 0.005 M sodium acetate. Excitation was at 295 nm.

the acid-induced unfolding transition of apomyoglobin is a three-state process [33–37]. The first transition, which takes place on going from pH 6.0–5.5 to pH 4.5–4.0, produces a partly folded intermediate. A fully unfolded protein is obtained by lowering the pH from 4.0 to 2.0.

Fig. 2 shows the acid pH dependence of tryptophanyl emission at low salt concentration of W7F and W14F mutants in comparison with the denaturation curve relative to wild type apoprotein. Although very complex, the behaviour of tryptophanyl emission can be rationalised by dividing the denaturation curves into three distinct regions: the first going from pH 8.0 to 5.5 (neutral region), the second from pH 5.5 to 4.0 (acidic region) and the third from pH 4.0 to 2.0 (strongly acidic region). Acidification from pH 8.0 to pH 5.5 causes a 20–25% decrease of the emission intensity without any appreciable variation in the position of the emission maximum. The decrease of fluorescence yield in the neutral region was first attributed to the formation of a charge transfer complex between W14 and a nearby protonated histidine i.e., H119 [18–20,38]. This was further corroborated by the observation that tuna apomyoglobin, which lacks H119, does not show any fluorescence change in the neutral pH range [20]. More recently, the involvement of histidine 119 has been questioned considering that the pK of this residue in the apo form is between pH 5.3 and 5.8 [39,40], i.e., out of the range in which the fluorescence decrease is detected. The results shown in Fig. 2 clearly indicate that the fluorescence decrease between pH 8.0 and 5.5 cannot be exclusively attributed to W14; in fact, in the neutral region, the same fluorescence change was observed for W14F, wild type and W7F apoproteins. An alternative possible explanation for the fluorescence decrease observed as pH lowers from 8.0 to 5.5 is that a minor conformational change takes place and affects the microenvironment of both W7 and W14. Evidence for such a minor change has been previously suggested by Colonna et al. [19].

A large increase of fluorescence intensity with a 5 nm red shift of the emission maximum is observed on lowering the pH from 5.5 to 4.0 for wild type apomyoglobin and the W14F mutant but not for the W7F mutant, the fluorescence intensity of which remains constant in the acidic pH range. The fluorescence enhancement is concomitant with the large conformational change, which brings the apomyoglobin structure in a partly folded state, i.e., I state [5,13,20,33]. In this state, about one half of the native helical structure, i.e., B, C, D, E and F helices, is unfolded whereas the other half, i.e., A, G and H helices, retains a native-like folding [8,10,11,13,35]. Early evidence had suggested that the fluorescence yield of W7 in the folded apomyoglobin is reduced because of the presence of a sterically closed lysine residue, i.e., K79 [20], which acts as a potent quencher of the indole fluorescence. The conformational transition undergone by apomyoglobin on lowering the pH from 5.5 to 4.0 unfolds the region containing the sterically interacting K79 [20,38,40], thus causing an increase of W7 fluorescence yield. The lack of fluorescence increase observed for W7F mutant is fully consistent with the above explanation.

The fluorescence increase, detected in the acid region for wild type apomyoglobin and W14F mutant, is not superimposed. In particular, the mid-point of W14F is increased 0.5 pH unit with respect to that of wild type, thus indicating that this substitution decreases the stability of the native state. This observation is consistent with the results reported by Kay and Baldwin [41]. These authors reported that the ΔG of $N \rightarrow I$ transition, calculated from urea-induced

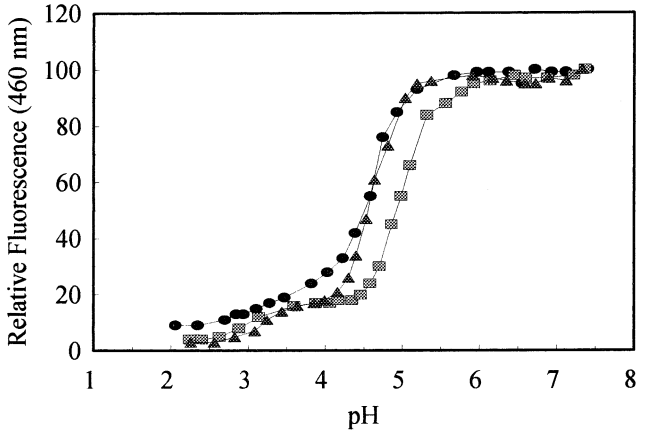

Fig. 3. The acid pH dependence of ANS relative fluorescence intensity at 460 nm of wild type and mutant apomyoglobins: ●, wild type; ■, W14F; ▲, W7F. Data are from three sets of experiments for each apomyoglobin. ANS/apomyoglobin molar ratio = 0.5. Protein concentration was 3.6×10^{-5} M. Excitation was at 350 nm.

far UV CD denaturation curves, is significantly lower than wild type, i.e., $\Delta(\Delta G)$ equal to 0.6 kcal mol^{-1} at 5°C, a value which indicates a stability decrease of about 10%.

Finally, a further pH decrease from pH 4.0 to 2.0 reduces the fluorescence intensity because of a second structural conformational change. This transition brings the fluorescent residues in contact with the solvent as documented by the large red shift of tryptophanyl emission, which becomes similar to that of the free monomeric residue in water, i.e., 350 nm. The small differences detected in the mid-point of the $I\rightarrow U$ transition curves of the examined proteins are probably due to changes in the conformational stability of the I-1 state because of the tryptophanyl replacement [41].

The correlation between the pH-induced variation of intrinsic fluorescence and protein structural changes was further investigated by examining the acid pH dependence of the ANS–apomyoglobin complexes at very low ionic strength (Fig. 3). Apomyoglobin is known to bind ANS in the same site of haem in the molar ratio of 1:1 [42]. When bound to apomyoglobin, ANS exhibits a large increase of fluorescence yield, i.e., 200-fold, and a large blue shift, i.e., from 520 to 460 nm. On lowering the pH from 6.0 to 4.0, the ANS fluorescence intensity decreases; in the same pH range, the emission maximum shifts from 460 to 473 nm. However, the fluorescence emission properties of the ANS–apomyoglobin complex at pH 4.0 are different from those of the free fluorophore in water thus indicating that the fluorophore is not released in the medium but still bound to the protein matrix. The position of the emission maximum, i.e., 473 nm, and the reduced fluorescence yield of the extrinsic fluorophore indicate that the binding site at pH 4.0 is somewhat different from the haem site [10]. Thus, the variations of ANS–apomyoglobin fluorescence provide information on the structural organisation of the molecular regions forming the haem pocket, i.e., the helices E, F, C and D, and are consistent with the appearance around pH 4.0 of the partly folded state I. It is evident from the results displayed in Fig. 3 that the transition curves obtained following the fluorescence of the ANS–apomyoglobin complex fully correlate with those obtained following the fluorescence of the indole residues. In particular, the mid-point of W14F mutant is increased 0.5 pH unit with respect to that of wild type and W7F mutant, thus indicating that the substitution W14F decreases the stability of the native state. The superimposition of the fluorescence curves obtained following two different structural probes located in different protein region, i.e., tryptophanyl residues and ANS, gives strong evidence of the cooperativity of the molecular transition from N to I.

Further acidification to pH 2.0 causes the disappearance of ANS fluorescence, whose residual emis-

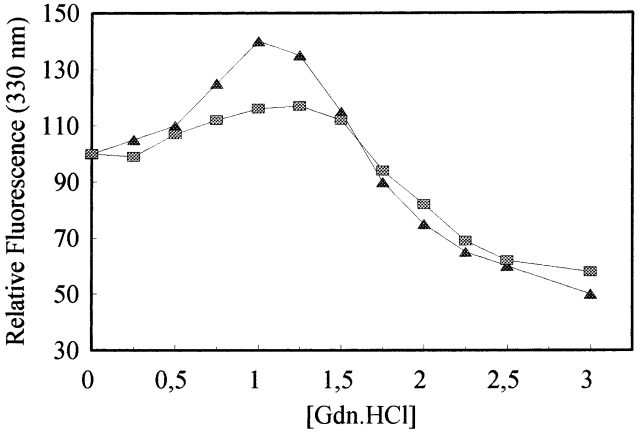

Fig. 4. The effect of increasing Gdn.HCl concentration on tryptophanyl relative fluorescence intensity at 330 nm of W14F (■) and W7F (▲) mutant apomyoglobins. Protein concentration was 5.4×10^{-6} M. Excitation was at 295 nm.

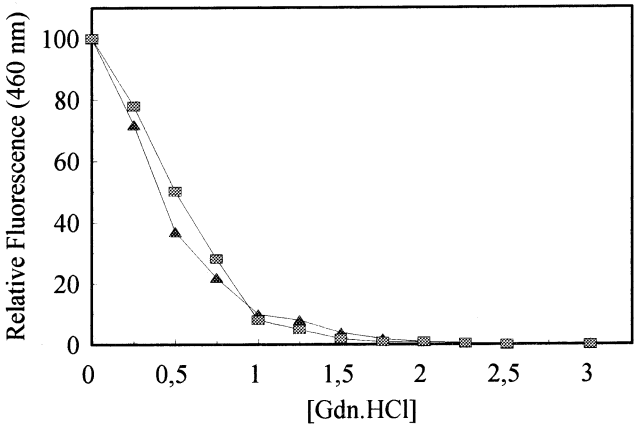

Fig. 5. The effect of increasing Gdn.HCl concentration on ANS relative fluorescence intensity at 460 nm of W14F (■) and W7F (▲) mutant apomyoglobins. ANS/apomyoglobin molar ratio = 0.5. Protein concentration was 3.6×10^{-5} M. Excitation was at 350 nm.

sion becomes similar to that of the free fluorophore in water.

Guanidine unfolding of apomyoglobin at neutral pH is not a two-state process since it involves two subsequent transitions, the first of which produces the appearance of a partly folded form not able to bind the extrinsic fluorophore ANS [43]. Early studies had shown that the guanidine-induced transition curve obtained following the tryptophanyl emission is biphasic. On increasing the guanidine concentration from 0 to 1.0 M, the tryptophanyl fluorescence of mammalian apomyoglobin increases with a concomitant small shift of the emission maximum from 331 to 335 nm. At higher denaturant concentrations, the emission intensity decreases and the emission maximum shifts to 350 nm [20,43]. The increase of tryptophanyl fluorescence emission observed at relatively low guanidine concentrations is concomitant with the loss of ability to bind ANS [33,44,45]. Comparing the guanidine-induced unfolding with that induced by acid, it was generalised that the formation of intermediate partly folded forms of apomyoglobin is always associated with the increase of fluorescence yield of W7 due to the removal of the sterically interacting K79, irrespective of the chemical nature of the denaturant used.

Fig. 4 shows the denaturation curves obtained following the intrinsic fluorescence of the apomyoglobin mutants W7F and W14F on increasing the guanidine concentration at pH 7.0. Fig. 5 shows the denaturation curves obtained following the fluorescence of the extrinsic fluorophore ANS. It is evident that the loss of the ability to bind ANS occurs at denaturant concentrations much lower than those bringing the tryptophanyl residues into full contact with the solvent. Surprisingly, the W14F mutant did not show an increase of tryptophanyl fluorescence in the concentration range of denaturant producing disappearance of ANS fluorescence. An increase was, instead, observed for W7F. These results do not support early suppositions [20,43]. The main conclusion which can be drawn from the results displayed in Figs. 4 and 5 is that the intermediate state formed by apomyoglobin at acidic pH is somewhat different from that appearing at low guanidine concentrations. The differences regard the microenvironments of the indole residues and the ability of the intermediate state to bind the extrinsic fluorophore.

Acknowledgements

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References

[1] M.R. Eftink, M.C.R. Shastry, Fluorescence methods for studying kinetics of protein-folding reactions, Methods Enzymol. 278 (1997) 258–286.
[2] C. Balestrieri, G. Colonna, A. Giovane, G. Irace, L. Servillo, Amino acid composition and physicochemical properties of bluefin tuna (*Thunnus thynnus*) myoglobin, Comp. Biochem. Physiol. 60B (1978) 195–199.
[3] E. Bismuto, G. Colonna, F. Savy, G. Irace, Myoglobin structure and regulation of solvent accessibility of heme pocket, Int. J. Peptide Protein Res. 26 (1985) 195–207.
[4] E. Bismuto, I. Sirangelo, G. Irace, Salt-induced refolding of myoglobin at acidic pH: molecular properties of a partly folded intermediate, Arch. Biochem. Biophys. 298-2 (1992) 624–629.
[5] F.M. Hughson, D. Barrick, R.L. Baldwin, Structural characterization of a partly folded apomyoglobin intermediate, Science 249 (1990) 1544–1548.
[6] Y. Goto, L.J. Calciano, A.L. Fink, Acid-induced folding of proteins, Proc. Natl. Acad. Sci. USA 87 (1990) 573–577.
[7] Y. Goto, A.L. Fink, Phase diagram for acidic conformational states of apomyoglobin, J. Mol. Biol. 214 (1990) 803–805.
[8] P.A. Jennings, P.E. Wright, Formation of a molten globule

intermediate early in the kinetic folding pathway of apomyoglobin, Science 262 (1993) 892–896.
[9] R.V. Pappu, D.L. Weaver, The early folding kinetics of apomyoglobin, Protein Sci. 7 (1998) 480–490.
[10] I. Sirangelo, E. Bismuto, G. Irace, Solvent and thermal denaturation of the acidic compact state of apomyoglobin, FEBS Lett. 338 (1994) 11–15.
[11] M. Kataoka, I. Nishii, T. Fujisawa, T. Ueki, F. Tokunaga, Y. Goto, Structural characterization of the molten globule and native states of apomyoglobin by solution X-ray scattering, J. Mol. Biol. 249 (1995) 215–228.
[12] D. Eliezer, J. Yao, H.J. Dyson, P.E. Wright, Structural and dynamic characterization of partly folded states of apomyoglobin and implications for protein folding, Nature Struct. Biol. 5 (1998) 148–155.
[13] R. Gilmanshin, S. Williams, R.H. Callender, W.H. Woodruff, R.B. Dyer, Fast events in protein folding: relaxation dynamics and structure of the I form of apomyoglobin, Biochemistry 36 (1997) 15006–15012.
[14] R.H. Callender, R.B. Dyer, R. Gilmanshin, W.H. Woodruff, Fast events in protein folding: the time evolution of primary processes, Annu. Rev. Phys. Chem. 49 (1998) 173–202.
[15] S.N. Loh, M.S. Kay, R.L. Baldwin, Structure and stability of a second molten globule intermediate in the apomyoglobin folding pathway, Proc. Natl. Acad. Sci. USA 92 (1995) 5446–5450.
[16] M. Jaming, R.L. Baldwin, Two forms of the pH 4 folding intermediate of apomyoglobin, J. Mol. Biol. 276 (1998) 491–504.
[17] Y. Luo, R.L. Baldwin, Trifluoroethanol stabilizes the pH 4 folding intermediate of sperm whale apomyoglobin, J. Mol. Biol. 279 (1998) 49–57.
[18] E.P. Kirby, R.F. Steiner, The tryptophan microenvironments in apomyoglobin, J. Biol. Chem. 245 (1970) 6300–6306.
[19] G. Colonna, G. Irace, G. Parlato, S.M. Aloj, C. Balestrieri, The effect of evolution on homologous proteins. A comparison between the chromophore microenvironments of Italian water buffalo (*Bos bubalus* L.) and sperm whale apomyoglobin, Biochim. Biophys. Acta 532 (1978) 354–367.
[20] G. Irace, C. Balestrieri, G. Parlato, L. Servillo, G. Colonna, Tryptophanyl fluorescence heterogeneity of apomyoglobins. Correlations with the presence of two distinct structural domains, Biochemistry 20 (1981) 792–799.
[21] Z. Wasylewski, H. Coloczek, A. Washiowska, Fluorescence-quenching-resolved spectroscopy of proteins, Eur. J. Biochem. 172 (1988) 719–724.
[22] I. Sirangelo, E. Bismuto, G. Irace, Resolution of the individual tryptophanyl contributions to the near-ultraviolet dichroic activity of apomyoglobin, Photochem. Photobiol. 59 (1994) 611–614.
[23] A. Springer, S.G. Sligar, High-level expression of sperm whale myoglobin in *E. coli*, Proc. Natl. Acad. Sci. USA 84 (1987) 8961–8965.
[24] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[25] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[26] E. Antonini, M. Brunori, Hemoglobin and Myoglobin in their Reactions with Ligands. American Elsevier, New York, 1971.
[27] F.W.J. Teale, Cleavage of the heme protein by acid methylethylketone, Biochim. Biophys. Acta 35 (1959) 543.
[28] H. Edelhoch, Spectroscopic determination of tryptophan and tyrosine in proteins, Biochemistry 6 (1967) 1948–1954.
[29] D. Wetlaufer, Ultraviolet spectra of proteins and amino-acids, Adv. Protein Chem. 17 (1964) 303–390.
[30] R.K. Scopes, Measurement of protein by spectrophotometry at 205 nm, Anal. Biochem. 59 (1974) 277.
[31] G. Weber, L.B. Young, Fragmentation of bovine serum albumin by pepsin, J. Biol. Chem. 239 (1964) 1415–1423.
[32] I. Sirangelo, E. Bismuto, S. Tavassi, G. Irace, Near-ultraviolet circular dichroic activity of apomyoglobin: resolution of the individual tryptophanyl contributions by site-directed mutagenesis, Eur. Biophys. J. 27 (1998) 27–31.
[33] G. Irace, E. Bismuto, F. Savy, G. Colonna, Unfolding pathway of myoglobin: molecular properties of intermediate forms, Arch. Biochem. Biophys. 244 (1986) 459–469.
[34] E. Bismuto, G. Colonna, G. Irace, Unfolding pathway of myoglobin. Evidence for a multistate process, Biochemistry 22 (1983) 4165–4170.
[35] F.M. Hughson, R.L. Baldwin, Use of site-directed mutagenesis to destabilize native apomyoglobin relative to folding intermediate, Biochemistry 28 (1989) 4415–4422.
[36] D. Barrick, R.L. Baldwin, The molten globule intermediate and the process of protein folding, Protein Sci. 2 (1993) 869–876.
[37] D. Barrick, R.L. Baldwin, Three state analysis of sperm whale apomyoglobin folding, Biochemistry 32 (1993) 3790–3796.
[38] G.B. Postnikova, Y.E. Komarov, E.M. Yumakova, Fluorescence study of the conformational properties of myoglobin structure, Eur. J. Biochem. 198 (1991) 223–232.
[39] M.J. Cocco, Y.H. Kao, A.T. Phillips, T.J.J. Lecomte, Structural comparison of apomyoglobin and metaquomyoglobin: pH titrations of histidines by NMR spectroscopy, Biochemistry 31 (1992) 6481–6491.
[40] I. Sirangelo, E. Bismuto, S. Tavassi, G. Irace, Apomyoglobin folding intermediates characterized by the hydrophobic fluorescent probe 8-anilino-1-naphthalene sulfonate (ANS), Biochim. Biophys. Acta 1385 (1998) 69–77.
[41] M.S. Kay, R.L. Baldwin, Packing interactions in the apomyoglobin folding intermediate, Nature Struct. Biol. 3 (1996) 439–445.
[42] L. Stryer, Interaction of a naphthalene sulfonate dye with

apomyoglobin and apohemoglobin: a fluorescence probe of non polar binding site, J. Mol. Biol. 13 (1987) 482–495.
[43] M.S. Hargrove, D. Barrick, J.S. Olson, The association rate constant for heme binding globin is independent of protein structure, Biochemistry 35 (1996) 11293–11299.
[44] C. Balestrieri, G. Colonna, A. Giovane, G. Irace, L. Servillo, Equilibrium evidence of non-single step transition during guanidine unfolding of apomyoglobin, FEBS Lett. 66 (1976) 60–64.
[45] G. Colonna, C. Balestrieri, E. Bismuto, L. Servillo, G. Irace, Heme and cysteine microenvironments of tuna apomyoglobin. Evidence of two independent unfolding regions, Biochemistry 21 (1982) 212–215.