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W-F Substitutions in Apomyoglobin Increase the Local Flexibility of the N-terminal Region Causing Amyloid Aggregation: A H/D Exchange Study

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Abstract: Myoglobin is an α-helical globular protein containing two highly conserved tryptophanyl residues at positions 7 and 14 in the N-terminal region. The simultaneous substitution of the two residues impairs the productive folding of the protein making the polypeptide chain highly prone to aggregate forming amyloid fibrils at physiological pH and room temperature. The role played by tryptophanyl residues in driving the productive folding process was investigated by providing structural details at low resolution of compact intermediate of three mutated apomyoglobins, i.e., W7F, W14F and the amyloid forming mutant W7FW14F.

In particular, we followed the hydrogen/deuterium exchange rate of protein segments using proteolysis with pepsin followed by mass spectrometry analysis. The results revealed significant differences in the N-terminal region, consisting in an alteration of the physico-chemical properties of the 7-11 segment for W7F and in an increase of local flexibility of the12-29 segment for W14F. In the double trypthophanyl substituted mutant, these effects are additive and impair the formation of native-like contacts and favour inter-chain interactions leading to protein aggregation and amyloid formation at physiological pH.

Keywords: Amyloid aggregation, apomyoglobin, H/D exchanges, protein folding, protein misfolding, W-F substitution.

INTRODUCTION

Protein misfolding and aggregation are central to the pathogenic mechanisms of several major human neurodegenerative diseases, including Alzheimer's disease, Creutzfeldt-Jakob disease, Huntington's disease, Parkinson's disease, type II diabetes, and Amyotrophic Lateral Sclerosis [1-6]. These diseases involve the formation of amyloid, a specific type of protein aggregate with a number of well defined features. These include long, unbranched fibrils 5-10 nm in diameter, a β-rich circular dichroism spectrum, an X-ray fiber diffraction pattern indicative of cross-β-structure in which β-strands run perpendicular to the fibril axis, and a strong affinity for the Congo red dye [7, 8]. The proteins forming aggregates in pathological conditions do not share any sequence identity or structural homology to each other. Considerable heterogeneity also exists in secondary structure composition or chain length. Moreover, it has been observed that formation of fibrils is not a feature limited to a few proteins or peptides associated with disease, but is a common characteristic of polypeptide chains. In fact, an increasing number of peptides and proteins unrelated to any pathological condition have been found to form similar structures *in vitro*, under selected and appropriate conditions [9-14]. Such

a property arises from the intrinsic tendency of polypeptide chains to self-organize into polymeric assemblies that are stabilized by inter-molecular hydrogen bonds established between the peptide bonds of parallel or anti-parallel polypeptide stretches in β-strand conformation. In this respect, natural proteins can be regarded as amino acid polymers selected by evolution so that their amino acid sequences are optimized to disfavour aggregation whilst favouring folding into compact, yet not rigid, states. This is mainly due to tertiary interactions among the side chains that shield not only the hydrophobic core but also the peptide backbone [15]. Conversely, protein aggregation into amyloid polymers, which are mainly stabilized by secondary interactions, can be considered the result of the emergence, under non-natural conditions, of the intrinsic primordial tendency of the peptide backbone to give secondary intermolecular interactions [9, 15, 16]. Recent high-resolution structural studies have provided insights into this process [17-20]. A number of physicochemical parameters of the polypeptide chain affect similarly both protein folding and aggregation; these include a significant propensity to gain secondary structure, a low net charge and a relatively high content of hydrophobic residues [21]. Mutations neutral to the thermodynamic stability but increasing the mean hydrophobicity and the propensity to undergo local unfolding events or able to reduce the net charge can favour aggregation from the unfolded states in dynamic equilibrium with the folded structure [21]. Thus, protein folding and protein aggregation are considered dis-

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tinct but competing processes, and the environmental conditions dictate which one is favoured for a given polypeptide chain [22].

The target protein of this work is apomyoglobin (apoMb), a well-characterized all α-helical single domain protein whose full length chain folding pathways have been well-studied [23-27]. The apoMb folding proceeds through compact intermediates that have been detected in both kinetic and equilibrium experiments. In most of these intermediates, A, G, and H helices are folded and sterically oriented as in the native AGH subdomain, whereas the remainder of the molecule seems to be unordered [28-33]. Although not involved in any amyloid disease, myoglobin is able to aggregate and form amyloid fibrils under appropriate experimental conditions [34]. Moreover, the replacement of both indole residues located at positions 7 and 14 in the N-terminal region of the protein with phenylalanine residues, i.e. W7F/W14F, renders the protein highly susceptible to aggregation and formation of amyloid fibrils under experimental conditions not drastically different from the natural setting (pH 7.0 and room temperature) [35, 36]. The ability to aggregate has been related to the increased hydrophobicity of the N-terminal segment of apomyoglobin and to the increased aggregation propensity caused by W-F substitutions [36]. The aggregation process starts from a native-like globular state able to bind the prosthetic group with spectroscopic properties similar to those observed for wild-type apoprotein. Nevertheless, the protein rapidly aggregates, forming oligomeric species that slowly convert into proto-fibrils and mature amyloid fibrils [37, 38]. The structure of amyloid fibrils formed by W7F/W14F apoMb has been studied by a battery of biophysical techniques. The rigid core of amyloid fibrils of apoMb includes A, B, E, and part of D and G helices, i.e., not only the N-terminal region but also the molecular regions which form the binding site for the prosthetic group in the native globular state [37, 39]. Studies performed with deleted and circularly permuted mutations on sperm whale myoglobin strongly highlight that the native position of the N-terminus is important for the precise structural architecture of the protein [40]. The apo form of the deleted and permuted mutants show native-like folding and bind heme, but is less stable and exhibits a stronger tendency to aggregate. Moreover, the circularly permuted mutants form cytotoxic fibrils at a rate higher than wild type [41].

We recently reported that the W-F substitutions at position 7 and 14 differently affect the structural organization and dynamics of the AGH subdomain of apomyoglobin. The combined effect of the two substitutions in the double mutant determines further changes that make the polypeptide chain more susceptible to aggregation and amyloid formation [39]. These considerations prompted us to investigate how the single substitution W-F at either position 7 or 14 affects the flexibility of the protein. We followed the hydrogen/deuterium exchange rate of the different protein segments using proteolysis with pepsin followed by mass spectrometry. Differently deuterated peptide fragments reflect the different flexibility of the various protein regions. Mass spectrometry analysis has been recently used to unravel molecular details for other amyloid proteins as well [42, 43]. We focused our attention on the local flexibility properties of

the compact intermediate state predominantly populated at pH 4.0, since it represents the early organized structure from which the native apomyoglobin fold originates [23, 44-46]. The behaviour of single W-F mutants (W7F and W14F) at pH 4.0 was compared with that of the amyloid-forming double mutant (W7FW14F) at pH 4.0, a pH value at which it is soluble without any tendency to aggregate, since aggregation occurs only when the pH is raised from 4.0 to neutrality [35, 36]. The results show that the W - F substitutions at positions 7 or 14 differently affect the flexibility and structural organization of the segment 7-11 in W7F and the segment 12-29 in W14F. The combination of the two effects in the double mutant may certainly contribute to explain the high propensity to aggregate in amyloid fibrils and further corroborates the influence of the N-terminal region in stabilizing the native fold.

MATERIALS AND METHODS

Protein Expression and Purification

Wild type sperm whale myoglobin was obtained expressing the synthetic gene in *Escherichia coli* strain TB-1 [ara, Δ (lac-pro), strA, thi, Φ80dlacZΔM, r-, m+] [47]. The tryptophanyl substitutions were performed as described elsewhere [48]. Wild type, W7F and W14F mutants were essentially purified as described by Springer and Sliger [47]. The culture was grown at 37°C in Luria-Bertrani broth in the presence of ampicillin (200 mg/L), harvested in the late log phase, lysed in lysis buffer (50 mM Tris.HCl, 1.0 mM EDTA, 0.5 mM DTT, 40 units of DNase per mL, 3 units of RNase A per mL, 4 mg of lysozyme per mL, pH 8.0) at 4°C overnight, and sonicated for 30 min on ice. Cell debris was removed by centrifugation, and the supernatant was fractionated by ammonium sulphate precipitation. The 60-95% cut was centrifuged and suspended in 20 mM Tris and 1.0 mM EDTA (pH 8.0) and fractionated on a Sephadex G-50 (Pharmacia) gel filtration column (2.5 x 100 cm) equilibrated in the same buffer. Fractions containing reddish-brown myoglobin were collected and applied to a Whatman DEAE 52 ion exchange column (2.5 x 20 cm) equilibrated and resolved with 20 mM Tris.HCl (pH 8.4). Under these conditions the myoglobin did not stick on the column and was rapidly eluted. Protein purity was controlled by sodium dodecyl sulphate-polyacrylamide gel electrophoresis [49]. The heme was removed from myoglobin by the 2-butanone extraction procedure [50]. The contamination of the apoprotein by myoglobin was assessed spectrophotometrically. In all cases, no significant absorption was observed in the Soret region. W7FW14F myoglobin mutant was purified as N-terminal His-tagged form via affinity chromatography on Ni^{2+}-nitrilotriacetic acid resin [35, 36].

Protein concentration was determined under denaturing conditions measuring absorption at 280 nm for wild type, W7F and W14F mutants and at 275 nm for W7FW14F mutant. The molar extinction coefficients, calculated from the tryptophan and tyrosine content [51, 52] were $\varepsilon_{280} = 13500$, and 9750 M^{-1} cm^{-1} for wild type and single tryptophanyl substituted myoglobins respectively and $\varepsilon_{275} = 3750$ M^{-1} cm^{-1} for W7FW14F mutant. The absorption measurements were carried out on a Jasco V-550 spectrophotometer [53].

H/D Exchange

The H/D exchange reaction was conducted as follows: the protein sample (4 pmol/μL) was allowed to equilibrate for 15 min at 25°C in appropriate buffer (ammonium acetate 10 mM, pH 7.0 or pH 4.0). Deuterium exchange was initiated at 25°C by 5-fold dilution with ammonium acetate 10 mM in D_2O, at the appropriate pD. At various exchange times (15 sec. to 1h), ~ 5 nmol of protein were removed from the labelling solution, and rapidly injected into a 30 x 0.46 mm i.d. reverse-phase perfusion column (POROS 10 R2, Applied Biosystems) coupled to a ZQ single quadrupole instrument (Micromass) (3.8 kV capillary voltage, 40 V cone voltage, 80°C source and desolvation temperature). The protein was eluted at a flow rate of 0.5 mL/min with a 10-60% CH_3CN gradient in 0.1% TFA in 1 min. The HPLC step was quickly performed with cold protiated solvents, keeping the column submerged in an ice-bath, thereby reducing the back-exchange kinetics while removing deuterium from side chains and amino/carboxy termini that exchange much faster than amide linkages [54]. The increase in molecular mass of the protein sample then constituted a direct measurement of deuterium incorporation at peptide amide linkages. Duplicate analyses were performed for each time point. Data were acquired and elaborated using the Biomultiviewer (Applied Biosystems) program and reported as percentage of exchanged protons, considering the number of non-proline residues in the protein.

Pepsin digestion of protein deuterated samples (see above), was performed by 4-fold dilution of each sample (2 nmol) with a 0.1% TFA (pH 2.5) containing pepsin (3.5/1 w/w enzyme/substrate ratio) and incubating for 5 min at 0°C. Peptides were analysed by LC-ESMS on a ZQ instrument coupled to a 2690 Alliance HPLC, Waters using a 30 x 2.1 mm i.d. C-18 reverse-phase column (X-TERRA, Waters). Peptides were eluted at a flow rate of 0.2 mL/min with a 10-60% CH_3CN gradient in 0.1% TFA in 8 min. HPLC solvents and column were kept ice-cold in a bath. Mass spectrometry data were obtained, elaborated, and reported as above described. Non-deuterated samples (2 nmol) were digested with pepsin as above and fractionated using a 250 x 2.1 nm, 300 Å Phenomenex Jupiter C18 column on a HP 1100 HPLC (Agilent Technologies) coupled to a LCQ ion trap (Finnigan Corp.) in order to confirm peptide assignments by MS/MS analysis.

RESULTS AND DISCUSSION

### Hydrogen-Deuterium Exchange Analyses of Intact Proteins

Comparative hydrogen-deuterium exchange experiments were carried out on wild type apomyoglobin and W7F, W14F and W7FW14F mutants. Proteins were diluted in the appropriate D_2O buffers at different interval times and, then, analyzed by fast LC-ESMS. Since protein terminal groups and side chains rapidly back-exchange deuterium during the LC step, the increase in the molecular mass measured by MS is only related to the number of deuterium atoms incorporated in the protein backbone. The single envelope of isotope peaks occurring in the mass spectra of the species indicated an EX2 kinetics of hydrogen-deuterium exchange [54], confirming that the examined protein species are homogeneous and stable.

The experimental molecular mass for the non deuterated species are in full agreement with the mass calculated on the basis of the aminoacidic sequences. The 100% reference sample consisting of fully deuterated proteins was calculated considering the number of non-proline residues. The percentage of deuterium incorporation was calculated at each incubation time in deuterated buffer (from 0 to 60 min) by considering the mass increase and the 100% reference species. Fig. **1a** shows the kinetics of H/D exchange of the intact soluble apoproteins (wild type, W7F, and W14F) at pH 7.0; at this pH the double mutant is insoluble, thus impairing the analysis on W7FW14F. Raw data were corrected for back-exchange and plotted as percentage of exchanged protons as a function of time. Data reported are the average over three independent experiments. Following 60 min of reaction, H/D exchange revealed that wild type protein incorporated 61 ± 1 deuterium atoms. The extent of protons exchanged by wild type apomyoglobin, corresponding to about 40%, is consistent with a structured and homogeneously compact protein, in agreement with data previously reported on apomyoglobins [32, 55]. The two W7F and W14F mutants showed a similar behaviour with a total of 85 ± 2 hydrogen atoms replaced by deuterium, corresponding to about 55% of the total. The 15% increase in deuterium incorporation in the single mutants with respect to wild type apomyoglobin indicates a more flexible conformation, in agreement with fluorescence quenching data previously reported [39].

The time course of H/D exchange for wt, W7F, W14F and W7FW14F intermediates at pH 4.0 is reported in Fig. **1b**. Following 60 min of reaction, when the equilibrium was attained, the wild type protein showed a higher extent of deuterium incorporation as compared to the equilibrium value reached at pH 7.0, with a total of 55% hydrogen atoms exchanged. The increase in deuterium content is consistent with the partial unfolding of the protein at acidic pH to generate the compact intermediate state; however, a significant amount of secondary and tertiary structure should be still retained, since about 45% of the amide protons are shielded to the solvent. A smaller increase of H/D exchange was observed at pH 4.0 for single mutants with respect to native proteins, i.e., 60% vs 55%. This suggests the occurrence of only a moderate unfolding in passing from the native state to the corresponding partially folded intermediate forms. The incorporation of deuterium of the double mutant W7FW14F at pH 4.0 is shown in Fig. **1b**. The exchanging profiles of single and double mutants were almost superimposable, with only a slightly faster incorporation observed for the W7FW14F mutant. These data show that the compact intermediate formed at pH 4.0 by mutant apomyoglobins still consists of a largely structured and compact protein region with a definite degree of conformational flexibility, suggesting the presence of an organized AGH domain analogous to the wild-type protein.

However, this subdomain in W7FW14F mutant seems to be slightly more flexible as compared to W7F and W14F apoMbs.
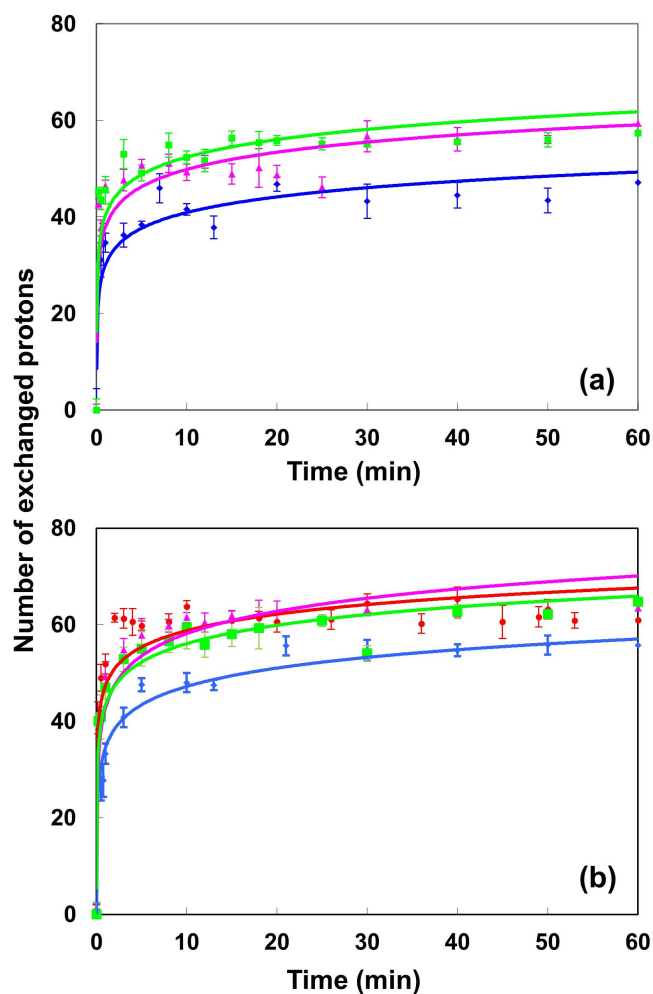
Figure 1. Hydrogen-exchange kinetic profiles of wild type (♦, blue line), W7F (■, green line), W14F (▲, pink line) at pH 7.0 (**a**) and pH 4.0 (**b**), and W7FW14F (●, red line) at pH 4.0 (**b**) apomyoglobins. The number of exchanged protons at each incubation time was determined from the centroid of the isotopic envelopes, averaged and corrected with reference samples.

Flexibility of Protein Regions in the Intermediate State

The differences occurring among the compact intermediates of the apomyoglobin mutants were further investigated by proteolytic digestion with pepsin of the deuterated proteins carried out at acidic pH. Wild-type apomyoglobin, W7F, W14F and W7F/W14F were equilibrated at pH 4.0 and then allowed to exchange for various times before digestion with pepsin under H/D quenching conditions (0°C, pH 2.5). The digested peptide mixtures were directly analysed by fast LC-ESMS, and data were corrected for back-exchange. Peptic fragments were identified by their unique mass values and MS/MS sequence analyses. Several different peptides corresponding to the same protein regions were observed in the digest due to the rather broad specificity of pepsin. The non-redundant series of the shortest peptides covering nearly the entire protein sequence was then considered in the analysis (Fig. **2**).
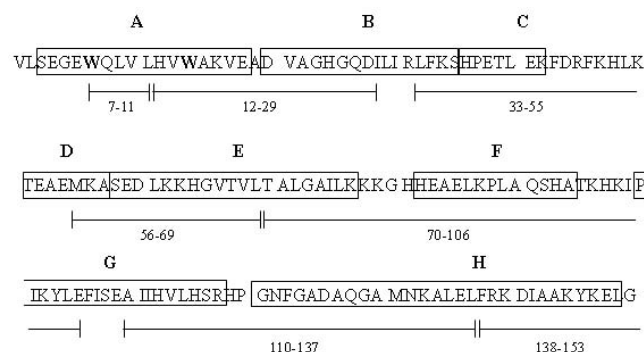
Figure 2. Sperm whale myoglobin sequence. Secondary structure elements are boxed on the sequence and the helices A-H are indicated. Fragments produced by peptic digestion are shown below the sequence.

Fig. **3** shows the percentage of H/D exchange for each selected fragment as a function of D_2O labeling time of the intact proteins. No substantial differences were observed in most of the fragments, i.e. peptides 33-55, 56-69, 70-106, 110-137 and 138-153, encompassing almost the entire protein sequence. However, significant differences in the exchange properties could be detected in the N-terminal region of the intermediates. The double tryptophanyl mutant exhibited a much higher degree of deuterium incorporation than the wild type, particularly in the fragment 12-29, where one of the mutations had occurred. This indicates that the differences in conformational flexibility between the compact intermediate of the double mutant and the wild type are localised within the N-terminal region. The deuteration profile of the 12-29 fragment in the W7F mutant was very similar to that displayed by the wild type protein, whereas the W14F mutant exhibited a higher degree of deuterium incorporation, almost identical to the W7FW14F protein. These data suggest that replacement of W14 with phenylalanine resulted in higher flexibility of the closely surrounding protein region. Moreover, the results reported in Fig. **3** clearly indicate that the physico-chemical properties of the N-terminal end of the protein are heavily affected by the presence of a phenylalanine residue at position 7. In fact, the fragment 7-11 could not be detected either in W7F or in W7FW14F apomyoglobins. A plausible explanation might be the increased tendency of this fragment to aggregate, although an influence of the substitution on pepsin selectivity cannot be ruled out. However, the lack of any new peptides in pepsin digestion compared to wild type that might account for the presence of the segment 7-11 in the longer peptide, support the possibility that aggregation occurred. Chow *et al*. [56] reported that the 1-36 N-terminal fragment of wild type apomyoglobin displays a high level of ß-structure and forms macroscopic aggregates when the pH gets closer to neutrality. The W→F substitution at position 7 certainly contributes to increase the hydrophobicity of the 7-11 fragment, thus favouring aggregation. This result further suggests that the W-F replacement in position 7 could induce local unfolding events which trigger the α - β transition responsible for amyloid aggregation [39]. It is worth mentioning that the exchange behaviour of the 7-11 peptide in the wild type apomyoglobin and W14F
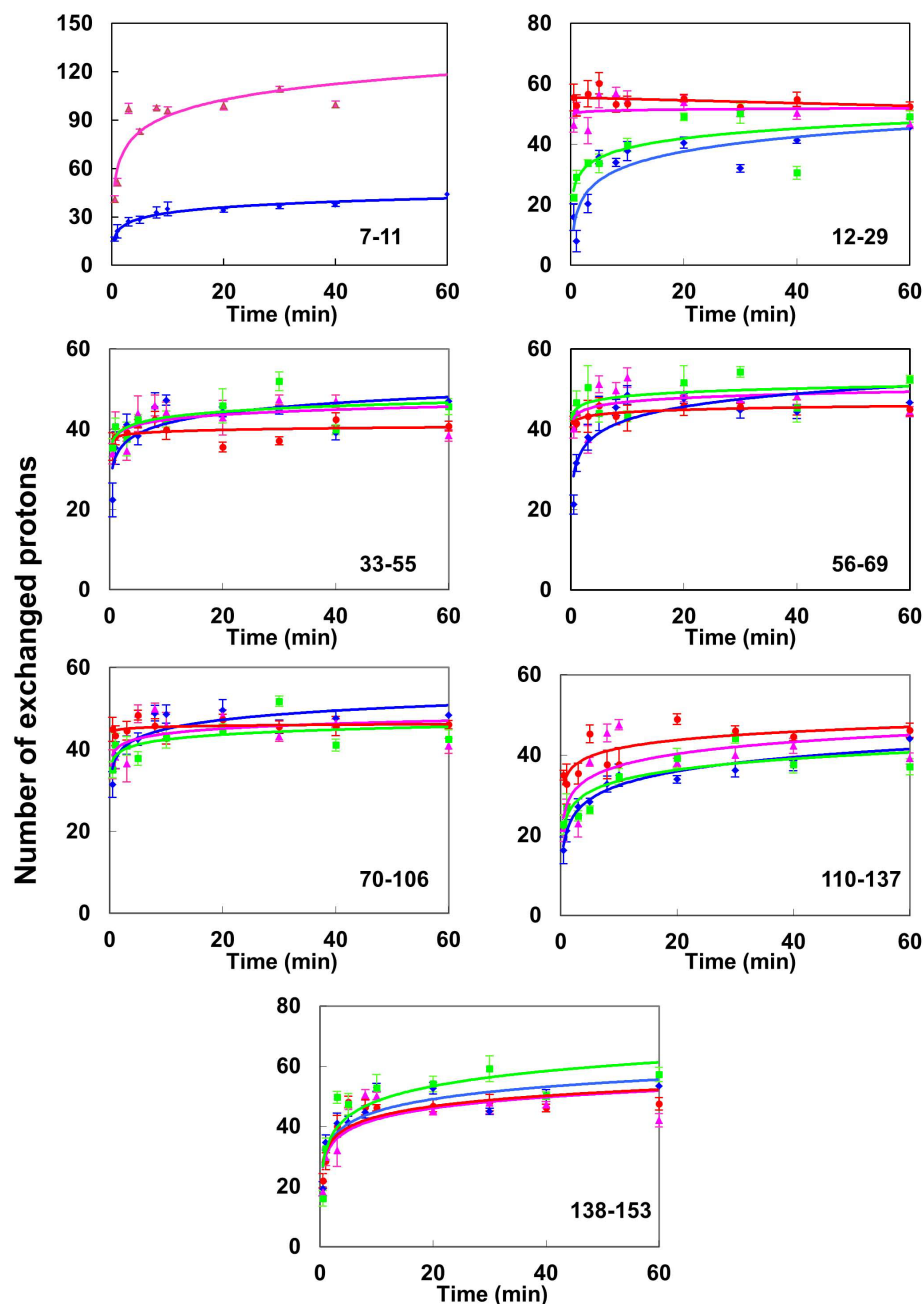
Figure 3. Hydrogen-exchange kinetic profiles of peptides generated by peptic digestion of wild type (♦, blue line), W7F (■, green line), W14F (▲, pink line), W7FW14F (●, red line) apomyoglobins deuterated at pH 4.0. The number of protons exchanged at each time was determined from the centroid of the isotopic envelopes, averaged and corrected with reference samples.

mutant was quite dissimilar, although in this region the two protein species exhibit the same amino acid sequence. This suggests that tryptophanyl substitution in position 14 also affects the flexibility of the adjacent peptide region.

We have previously shown that the simultaneous W-F substitution has a dramatic effect on the structure and flexibility of the apoMb molecule [39]. In particular, the tryptophanyl substitution at position 7 changes the secondary organization of the folded portion of the compact intermediate, which in turn alters the interaction between the AGH subdomain and the remainder of the molecule, perturbing the

docking of AGH core and E helix [39]. The other substitution has a less marked effect on secondary structure but a higher effect on the local conformational flexibility. The data reported in this paper corroborate this picture. Moreover, these data further underline the importance of the N-terminal region in stabilizing the precise structural architecture of myoglobin [40, 41].

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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