

## COMMUNICATION

## Probing The Conformational State of Apomyoglobin by Limited Proteolysis

Angelo Fontana\*, Marcello Zambonin, Patrizia Polverino de Laureto  
Vincenzo De Filippis, Angelica Clementi and Elena Scaramella

CRIBI Biotechnology Centre  
University of Padua  
Via Trieste 75  
35121 Padua, Italy

We show here that limited proteolysis can probe the structural and dynamic differences between the holo and apo form of horse myoglobin (Mb). Initial nicking of the polypeptide chain of apoMb (153 amino acid residues, no disulfide bonds) by several proteases (subtilisin, thermolysin, chymotrypsin and trypsin) occurs at the level of chain segment 89-96. In contrast, holoMb is resistant to proteolytic digestion when reacted under identical experimental conditions. Such selective proteolysis implies that the F-helix of native holoMb (residues 82 to 97) is disordered in apoMb, thus enabling binding and adaptation of this chain segment at the active site of the proteolytic enzymes for an efficient peptide bond fission. That essentially only the F-helix in apoMb is largely disrupted was earlier inferred from spectroscopic measurements and molecular dynamics simulations. The results of this study provide direct experimental evidence for this and emphasize therefore that limited proteolysis is a useful and reliable method for probing structure and dynamics of proteins, complementing other experimental techniques such as NMR and X-ray crystallography.

© 1997 Academic Press Limited

**Keywords:** apomyoglobin; limited proteolysis; protein folding; protein dynamics; mass spectrometry

\*Corresponding author

Apomyoglobin (apoMb), myoglobin without the heme, is a small monomeric protein of 153 amino acid residues (Figure 1), devoid of disulfide bonds, that is attracting much interest as a model protein for studies of protein folding (Hughson *et al.*, 1990; Brooks, 1992; Jennings & Wright, 1993; Barrick & Baldwin, 1993; Tirado-Rives & Jorgensen, 1993; Loh *et al.*, 1995) and stability (Griko *et al.*, 1988; Goto & Fink, 1990; Nishii *et al.*, 1994, 1995). The structure of apoMb has not yet been determined by X-ray methods, but the results of a variety of spectroscopic studies have indicated that, in solution and at neutral pH, apoMb has a compact hydrophobic core resembling the tertiary structure of holomyoglobin (holoMb). Circular dichroism measurements have indicated that apoMb has a

reduced helical content with respect to that of holoMb (Breslow & Koehler, 1965; Harrison & Blout, 1965; Nishii *et al.*, 1994, 1995; Kataoka *et al.*, 1995), which consists of eight  $\alpha$ -helices (named A through H), accounting for ~80% helicity (Kuriyan *et al.*, 1986; Evans & Brayer, 1990). Proton NMR spectroscopy provided evidence that native apoMb retains much of the secondary structure observed in the crystal structure of holoMb and, in particular, that helices A, B, E, G and H are still present in apoMb (Cocco & Lecomte, 1990, 1994; Hughson *et al.*, 1990; Cocco *et al.*, 1992; Jennings & Wright, 1993). In a recent study, Lecomte *et al.* (1996) conducted additional NMR studies on the conformational properties of a mutant of sperm whale apoMb and concluded that the majority of helices of holoMb, including helix C and D, is also present in apoMb, with the exception of helix F and the carboxy terminus of helix H. ApoMb, at neutral pH, appears to possess some molten globule-like properties (Lin *et al.*, 1994), since it is less compact (Kataoka *et al.*, 1995), less stable (Griko *et al.*, 1988), unfolds less cooperatively than holoMb (Griko & Privalov, 1994) and binds the hydrophobic and flu-

Abbreviations used: Mb, horse myoglobin; holoMb, holomyoglobin; apoMb, apomyoglobin; Su, subtilisin; Th, thermolysin; Tr, trypsin; Ch, chymotrypsin; MALDI, matrix-assisted laser-desorption ionization; ES, electrospray; MS, mass spectrometry; TFA, trifluoroacetic acid; N, native; U, unfolded; ANS, 8-anilino-1-naphthalenesulfonic acid.

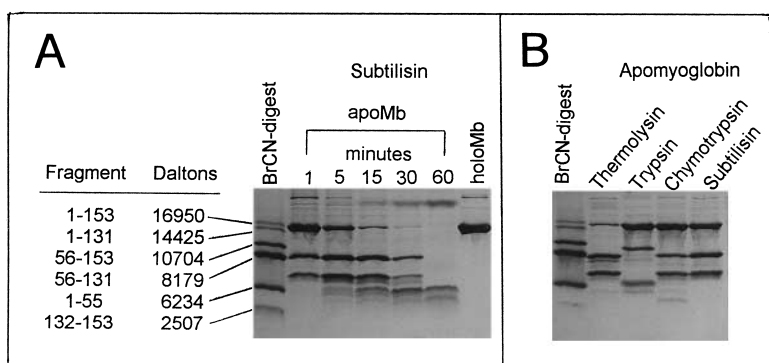
1 Gly-Leu-Ser-Asp-Gly<sup>5</sup>-Glu-Trp-Gln-Gln-Val<sup>10</sup>-Leu-Asn-Val-Trp-Gly<sup>15</sup>  
 16 Lys-Val-Glu-Ala-Asp<sup>20</sup>-Ile-Ala-Gly-His-Gly<sup>25</sup>-Gln-Glu-Val-Leu-Ile<sup>30</sup>  
 31 Arg-Leu-Phe-Thr-Gly<sup>35</sup>-His-Pro-Glu-Thr-Leu-Glu-Lys-Phe-Asp-Lys<sup>45</sup>  
 46 Phe-Lys-His-Leu-Lys-Thr<sup>50</sup>-Glu-Ala-Glu-Met<sup>55</sup>-Lys-Ala-Ser-Glu-Asp<sup>60</sup>  
 61 Leu-Lys-Lys-His-Gly<sup>65</sup>-Thr-Val-Val-Leu-Thr<sup>70</sup>-Ala-Leu-Gly-Gly-Ile<sup>75</sup>  
 76 Leu-Lys-Lys-Lys-Gly<sup>80</sup>-His-His-Glu-Ala-Glu<sup>85</sup>-Leu-Lys-Pro-Leu-Ala<sup>90</sup>  
 91 Gln-Ser-His-Ala-Thr<sup>95</sup>-Lys-His-Lys-Ile-Pro<sup>100</sup>-Ile-Lys-Tyr-Leu-Glu<sup>105</sup>  
 106 Phe-Ile-Ser-Asp-Ala<sup>110</sup>-Ile-Ile-His-Val<sup>115</sup>-Leu-His-Ser-Lys-His-Pro<sup>120</sup>  
 121 Gly-Asn-Phe-Gly-Ala<sup>125</sup>-Asp-Ala-Gln-Gly-Ala<sup>130</sup>-Met-Thr-Lys-Ala-Leu<sup>135</sup>  
 136 Glu-Leu-Phe-Arg-Asn<sup>140</sup>-Asp-Ile-Ala-Ala-Lys<sup>145</sup>-Tyr-Lys-Glu-Leu-Gly<sup>150</sup>  
 151 Phe-Gln-Gly

**Figure 1.** Amino acid sequence of horse myoglobin.

orescent dye 8-anilino-1-naphthalenesulfonic acid (ANS) forming a 1:1 complex (Stryer, 1965).

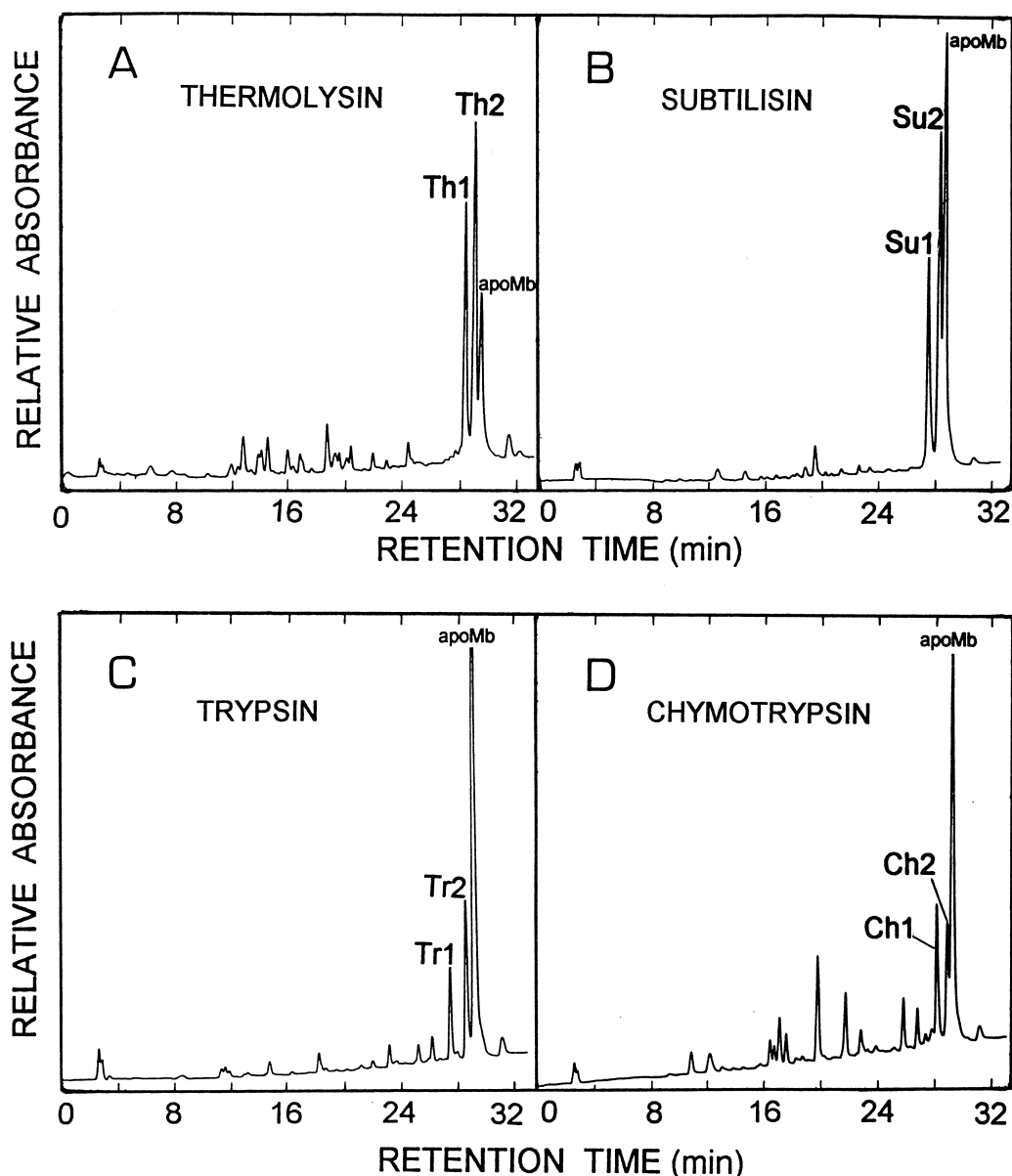
Here we describe the results of limited proteolysis experiments conducted on horse myoglobin utilizing several proteolytic enzymes of varied substrate specificity. The aim of this study was to unravel features of structure and dynamics of apoMb as compared to those of holoMb. The use of proteolytic probes of protein conformation (Mihalyi, 1978; Price & Johnson, 1990; Fontana *et al.*, 1993) is gaining a special momentum, since modern protein chemistry methods, and especially mass spectro-

metric techniques, enable fast and very sensitive analysis of the sites of proteolytic fissions along the polypeptide chain of the protein, even without isolation to homogeneity of the individual protein fragments produced by proteolysis (Brockerhoff *et al.*, 1992; Cohen *et al.*, 1995; Seielstad *et al.*, 1995; Mohsen *et al.*, 1995; Parrado *et al.*, 1996; Zappacosta *et al.*, 1996). We have previously shown that limited proteolysis occurs at flexible loci along the protein chain, as shown by the fact that there is a strict correlation between sites of limited proteolysis and sites of high segmental mobility, the latter monitored by the temperature factor (*B*-factor) determined crystallographically (Fontana *et al.*, 1986). In particular, the results of our own experimental studies (Fontana *et al.*, 1986; Signor *et al.*, 1990; Polverino de Laureto *et al.*, 1994, 1995a,b,c) and those derived from a thorough analysis of previously reported limited proteolysis experiments conducted on a number of globular proteins of known three-dimensional structure allowed us to conclude firmly that limited proteolysis of globular proteins in their native state occurs at flexible loops and never at helical chain segments (Fontana *et al.*, 1993). It is our belief that notions of exposure, accessibility and protrusion (Novotny & Bruccoleri, 1987; Hubbard *et al.*, 1991) are clearly not sufficient to explain the limited proteolysis phenomenon, since it is evident that even in a small globular protein there are many exposed sites (see also Hubbard *et al.*, 1994). Recently, proteolytic enzymes have been used by us to probe



**Figure 2.** Limited proteolysis of holoMb and apoMb monitored by SDS-PAGE. Horse apoMb was prepared by extraction of the heme from holoMb (Sigma, St. Louis, MO; type I) with 2-butanone or acetone (Hapner *et al.*, 1968; Goto & Fink, 1990). The homogeneity of the apoMb preparation was established by reverse-phase HPLC and mass spectrometry. Proteolysis was conducted at 25°C by dissolving holoMb and apoMb (1 mg/ml) in 20 mM Tris-HCl buffer (pH 7.5), containing 1 mM CaCl<sub>2</sub>; the pH of

the proteolytic solution was 8.0 when trypsin was used as a proteolytic enzyme. The E:S ratio was 1:100 (by weight) in each case. Aliquots (15  $\mu$ l) were taken from the reaction mixture and proteolysis was stopped by acidification by adding 0.1% aqueous trifluoroacetic acid (TFA; 30  $\mu$ l). After evaporation *in vacuo* with the Speed-Vac system (Savant, Farmingdale, CT), the samples were dissolved in the SDS-PAGE sample buffer (Schägger & von Jagow, 1987) and subjected to slab gel electrophoresis utilizing the Miniprotean-II (Bio-Rad, Richmond, VA). A sample of a partial BrCN-digest of horse myoglobin at the level of the two Met residues in position 55 and 131 of the chain (producing fragments 1-131, 56-153, 56-131, 1-55 and 132-153, in the order of decreasing molecular mass) was used as molecular mass standard. The gel was stained with Coomassie Brilliant Blue R-250. (A) Proteolysis of myoglobin by subtilisin (Carlsberg): lane 1, BrCN-digest; lanes 2 to 6, apoMb digested with subtilisin for 1, 5, 15, 30 and 60 minutes; lane 7, holoMb digested with subtilisin for one hour. (B) Proteolysis of apoMb with various proteolytic enzymes: lane 1, BrCN-digest; lanes 2 to 5, proteolytic mixtures obtained after one minute (lane 2, thermolysin), 15 minutes (lanes 3 and 4, trypsin and chymotrypsin) and ten minutes (lane 5, subtilisin) reaction. The masses of myoglobin fragments were calculated on the basis of the known amino acid sequence of horse myoglobin, taking into account also the conversion of Met to homoserine upon reaction with BrCN (Fontana & Gross, 1986). The masses of the fragments given in the technical information of Pharmacia-LKB are partly incorrect (Kratzin *et al.*, 1989).



**Figure 3.** Reverse-phase HPLC analysis of the proteolytic mixture of apoMb. The samples analyzed were obtained by proteolysis with thermolysin (A), subtilisin (B), trypsin (C) and chymotrypsin (D) conducted under the experimental conditions described in the legend to Figure 2. A Vydac  $C_4$  column (4.6 mm  $\times$  150 mm; The Separations Group, Hesperia, CA) was employed. Elution was carried out at a flow rate of 0.8 ml/min with a linear gradient of water/acetonitrile containing 0.05% (v/v) TFA from 5 to 60% in 30 minutes. The effluent was monitored by absorbance at 226 nm. The time of proteolysis at 25°C was one minute and ten minutes for thermolysin and subtilisin, respectively, and 15 minutes for both trypsin and chymotrypsin. The peptide/protein material of the labelled chromatographic peaks was recovered by Speed-Vac concentration and further analyzed by mass spectrometry (MS) and N-terminal sequencing. Determination of the molecular masses of the proteolytic fragments separated by HPLC was performed utilizing the matrix-assisted laser-desorption (MALDI) ionization time-of-flight mass spectrometer of Kratos Analytical (model MALDI-I; Manchester, UK) or the electrospray (ES) instrument of Sciex (model API-I; Thornhill, Ontario, Canada). The mass accuracy was in the range of 0.1 and 0.01% for the MALDI- and ES-MS, respectively.

the partly folded states of model proteins (Fontana *et al.*, 1995; Polverino de Laureto *et al.*, 1995a,b). Here we show that apoMb can be proteolytically cleaved at a restricted chain segment encompassed by helix F (residues 82 to 97) of native holoMb. Since the holoprotein is resistant to proteolysis when reacted under identical experimen-

tal conditions, it is concluded that the differences between the apo- and holo-protein appear to be confined largely to the helix F region.

The limited proteolysis approach for probing protein structure requires ideally that the conformational and dynamic state of the protein substrate dictates the proteolysis event and not its

**Table 1.** Analytical data of the proteolytic fragments of apomyoglobin

Protease	Fragment	RT <sup>b</sup> (minutes)	Molecular mass (Da) <sup>a</sup>				Predicted sequence	
			N-Terminal <sup>c</sup> sequence	SDS-PAGE <sup>d</sup> (kDa)	MALDI <sup>e</sup>	Electrospray		
Thermolysin	Th1	28.5	LAQSH	7	7194		7188.22	L89-G153
	Th2	29.3	GLSD	8	9783		9780.06	G1-P88
Subtilisin	Su1	28.5	AQS <sup>f</sup>	7	3525		3517.89	G1-L32
					7094	7074/7091 <sup>g</sup>	7075.06	A90-G153
					6896	6876/6892 <sup>g</sup>	6875.85	S92-G153
					6675	6651/6667 <sup>g</sup>	6651.63	A94-G153
Trypsin	Su2	29.2	GLS	8	9912	9891/9908 <sup>g</sup>	9893.22	G1-L89
	Tr1	27.8	GLSDG	5.5 <sup>h</sup>	3407		3404.73	G1-R31
	Tr2	28.9	GLS/HKI <sup>i</sup>	10/6 <sup>j</sup>	10628		10617.00	G1-K96
Chymotrypsin	Ch1	28.5	AQS	7	6360	7076/7093 <sup>g</sup>	6351.28	H97-G153
	Ch2	29.2	GLS	8	7087	9893/9910 <sup>g</sup>	7075.06	A90-G153
					9933		9893.22	G1-L89

The proteolytic fragments were isolated by HPLC (see the text).

<sup>a</sup> The accuracy of the MALDI and electrospray mass spectrometer employed in this study was about 0.1 and 0.01%, respectively.

<sup>b</sup> Retention time on HPLC

<sup>c</sup> The N-terminal sequence analysis was performed on peptide samples isolated after HPLC utilizing an Applied Biosystems (Foster City, CA) protein sequencer (model 477A) equipped with an on-line analyzer (model 120A) of phenylthiohydantoin-derivatives of amino acids.

<sup>d</sup> Approximate molecular masses in kDa determined by SDS-PAGE analysis using a partial BrCN-digest of myoglobin as standard.

<sup>e</sup> Molecular masses determined by MALDI mass spectrometry were on average higher than expected due to partial oxidation of methionine residues in position 55 and 131 of the polypeptide chain of myoglobin.

<sup>f</sup> Major N-terminal sequence.

<sup>g</sup> Two masses were obtained by electrospray mass spectrometry differing by 16 Da, indicating methionine sulfoxide oxidation.

<sup>h</sup> This fragment has unusual electrophoretic mobility in the SDS-PAGE system.

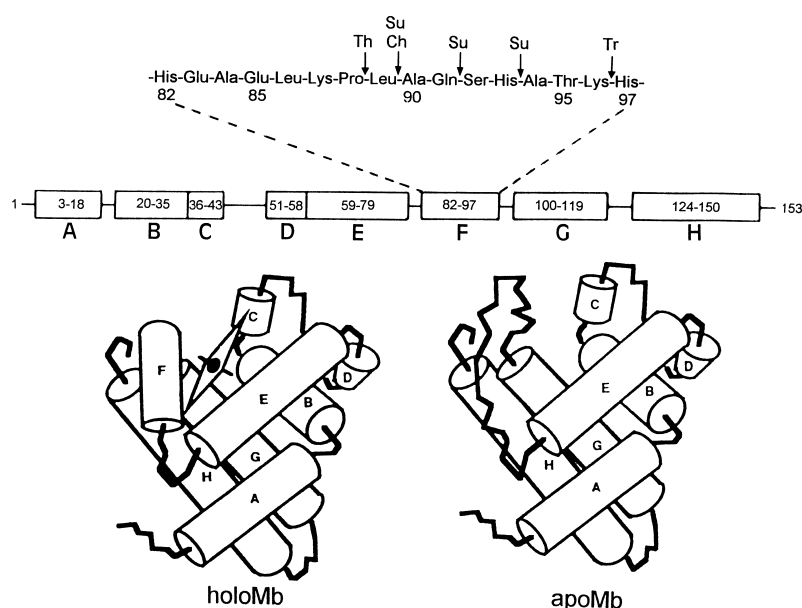
<sup>i</sup> Two N-terminal sequences were observed.

<sup>j</sup> Sample containing two protein fragments.

amino acid sequence. For this purpose, the most useful proteolytic enzymes are those displaying very broad substrate specificity, such as subtilisin and thermolysin. Nevertheless, in this study we also utilized chymotrypsin and trypsin, which cleave mostly at the C-terminus of hydrophobic (Phe, Tyr, Leu) and basic (Lys, Arg) residues, respectively (see Keil, 1982, for a comparative analysis of the substrate specificity of proteolytic enzymes). Thus, along the polypeptide chain of myoglobin (see Figure 1), there are many sites of potential proteolysis by the four proteases employed in this study. Moreover, it must be emphasized that the initial proteolysis events in a native protein are most useful and critical for unravelling structural and dynamic features of a native protein, since the protein fragments or the nicked proteins (if the fragments remain associated) are different conformational and dynamic entities than the native intact protein and are much more easily degraded by proteolysis as a result of their enhanced flexibility (Fontana *et al.*, 1993; Polverino de Laureto *et al.*, 1994, 1995a,b,c).

Proteolysis experiments were conducted by varying the proteolytic enzyme:protein concentration, enzyme:substrate (E:S) ratio, pH, as well as temperature and time of incubation. The pattern of protein degradation was monitored using both SDS-PAGE and reverse phase HPLC. The results of typical experiments of limited proteolysis are shown in Figures 2 and 3. Overall, it is seen that experimental conditions can be devised in order to determine limited proteolysis of apoMb to a few

rather large fragments of comparable size to those of a partial BrCN-digest of the protein at the two Met residues in position 55 and 131 of the chain. The pattern of proteolysis of apoMb by subtilisin, monitored by SDS-PAGE (Figure 2A), shows the presence of two major fragments at rather short reaction times (one to 15 minutes), while after prolonged reaction apoMb is degraded to small polypeptides poorly stained by Coomassie blue (see Figure 2A). The four proteases employed in this study (subtilisin, thermolysin, trypsin and chymotrypsin) produce, from apoMb, a quite clean fragmentation pattern (two major protein bands in the gel, besides that of apoMb, see Figure 2B). Considering that the sum of the molecular masses of the two major fragments, estimated from SDS-PAGE gels, roughly matches that of the intact protein, it can be inferred that the four proteases preferentially cleave apoMb at a single site along its 153-residue chain. On the other hand, holoMb was fully resistant to proteolysis when reacted under experimental conditions identical to those employed for apoMb (Figure 2A). When proteolysis of holoMb was conducted at 25°C for four to six hours or even longer (up to six days), it was found that the protein band gradually disappeared from the SDS-PAGE gel. Intermediate digestion products were not observed in the gel (not shown), implying that the native-denatured conformational transition of the holoprotein dictates the rate of protein degradation and that the unfolded protein only is digested by an all-or-none process to small peptides (not stained by Coomassie blue).



**Figure 4.** Top: Scheme of the secondary structure of holoMb (Kuriyan *et al.*, 1986; Evans & Brayer, 1990). The A through H eight helices along the protein chain are indicated by boxes. The amino acid sequence of helix F (residues 82 to 97) is given at the top of the Figure and the sites of initial proteolytic cleavage by thermolysin (Th), subtilisin (Su), trypsin (Tr) and chymotrypsin (Ch) are indicated by arrows. Notably, helix F encompasses chain segment 82-97 (Evans & Brayer, 1990) and segment 86-94 (Takano, 1977) in horse and sperm whale holoMb, respectively. Bottom: Schematic three-dimensional structure of holoMb (left) and apoMb (right) (adapted from Yang & Honig, 1994). The location of the heme is given in holoMb and the eight helices are indicated by letters (A to H). Note that helix F in the model of apoMb is assumed to be unfolded.

Aliquots of the proteolytic mixtures of apoMb and holoMb were also analyzed by reverse-phase HPLC. The chromatograms shown in Figure 3 indicate that major peaks of protein material are eluted from the column late, with a retention time similar to that of apoMb, while a variety of minor peaks of peptide material are eluted earlier. On the other hand, HPLC analysis of proteolytic digests of holoMb, as obtained by utilizing each of the four proteases employed in this study and up to six hours reaction at 25°C, gave only the peak of the intact protein in the chromatogram (not shown). This is in agreement with the proposal that holoMb is proteolytically degraded  $1 \times 10^{19}$  times more slowly than apoMb (McLendon & Radany, 1978). Since, in this study, we were interested in analyzing the sites of initial peptide bond fissions and thus in the identity of the fragments of higher molecular masses, the protein material of the labelled peaks in the HPLC-chromatograms of Figure 3 were further analyzed. The protein material isolated after HPLC was subjected to SDS-PAGE, N-terminal analysis and mass determination by matrix-assisted-laser-desorption ionization (MALDI) or electrospray (ES) mass spectrometry (MS). From the results of these analyses (Table 1) and their comparison with the known amino acid sequence of apoMb (Figure 1), it was possible to establish the identity of the proteolytic fragments and thus the sites of initial proteolysis of apoMb.

The data of Table 1 deserve few comments. First of all, a general problem encountered in these analyses was that several fragments isolated after HPLC were partly oxidized at the level of Met residue and thus gave two molecular masses differing by ~16 Da in the ES-MS, while MALDI-MS provided

the average masses of the native and oxidized fragment, due to the minor accuracy of the MALDI technique. Notably, the sample of apoMb utilized for the proteolysis experiments was in the reduced state and gave the correct mass by ES-MS (16,950 Da). The protein material of some chromatographic peaks (see Figure 3) was shown to be heterogeneous by both SDS-PAGE, MS and N-terminal sequencing. For example, the peak Th2 (thermolysin digest) contained fragment 1-88 and, as minor component, fragment 1-32; peak Su1 (subtilisin digest) contained fragments 90-153, 92-153 and 94-153; and peak Tr2 (trypsin digest) contained fragments 1-96 and 97-153. Peak Tr1 (trypsin digest) contained fragment 1-31; note that the relative abundance of this fragment in the HPLC chromatogram (Figure 3) is due to the fact that it contains the two strongly UV-absorbing Trp residues of the protein (see Figure 1).

Overall, the results of this study indicate that a variety of proteolytic enzymes initially cleave the apoMb molecule at the chain segment encompassed by helix F in holoMb (residues 82 to 97), as shown in Figure 4. The primary event in the proteolysis of apoMb appears to be the binding and adaptation at the active site of the protease of chain segment 89-96 of the protein, while the actual site of cleavage within this restricted segment (six to eight residues) is dictated by the primary specificity of the protease (Schechter & Berger, 1967; Hubbard *et al.*, 1994). Notably, the tryptic cleavage occurs at Lys96 and not at Lys87, since trypsin does not cleave the X-Pro peptide bond (see Figure 1). If one accepts our view that segmental mobility is the most critical parameter promoting limited proteolysis of globular proteins

(Fontana *et al.*, 1986) and that helices are not cleaved under conditions of limited proteolysis (globular protein in its native state, short reaction times and low E:S ratio; Fontana *et al.*, 1993; Polverino de Laureto *et al.*, 1995c), the results of this study therefore indicate that helix F in apoMb is largely disrupted, as schematically shown in Figure 4. The additional cleavages at residues 31 and 32, residing at the carboxy terminus of helix B, are minor and appear to occur after the initial cut at helix F (see Figure 2). These subsequent cleavages do not relate to intact apoMb, but to fragment or nicked protein species more flexible than the native protein (see also introductory comments above). Alternatively, we can interpret the minor cleavages at position 31 and 32 of the chain as indicating that this site is somewhat flexible in apoMb. Notably, proton NMR data indicate that helix B in sperm whale apoMb is folded up to residue 30 and that "past this residue the constraints are few and fraying is seen" (Lecomte *et al.*, 1996).

In the past, for simplicity, the structure of apoMb has been often assumed to be similar to that of the native, heme-containing holoMb. However, far-UV circular dichroism data indicate partial loss of the helical content of the protein upon removal of the heme (see Hirst & Brooks, 1994, for a recent discussion on the helical content of apoMb calculated from far-UV circular dichroism spectra). Hydrogen exchange experiments using NMR (Cocco & Lecomte, 1990, 1994; Hughson *et al.*, 1990; Cocco *et al.*, 1992; Loh *et al.*, 1995; Jennings & Wright, 1993; Lecomte *et al.*, 1996) fail to detect  $^1\text{H}$ - $^2\text{H}$  protection in the chain segment encompassing helix F, whereas the rest of the hydrophobic helical core of apoMb shows structural and dynamic properties similar to those of native holoMb. Native holoMb and apoMb appear to be indistinguishable in terms of compactness, as given by intrinsic viscosity measurements (Griko *et al.*, 1988). On the other hand, an analysis of the structural characteristics of apoMb, conducted by solution X-ray scattering, indicates that the packing of  $\alpha$ -helices in apoMb is looser than that of holoMb and that the radius of gyration ( $R_g$ ) is larger for apoMb than holoMb (20.1 Å versus 17.5 Å, respectively; Nishii *et al.*, 1994; Kataoka *et al.*, 1995). More recently, a folding pathway of apoMb was proposed on the basis of kinetic NMR measurements (Jennings & Wright, 1993), as  $\text{U} \rightarrow \text{A} \cdot \text{G} \cdot \text{H} \rightarrow \text{A} \cdot \text{B} \cdot \text{C} \cdot \text{CD} \cdot \text{E} \cdot \text{G} \cdot \text{H} \rightarrow \text{N}$ . In this scheme, the native state of apoMb is assumed to be the same as that of holoMb except for helix F (Hughson *et al.*, 1990). Moreover, the results of molecular dynamics simulation of apoMb provided evidence that helix F is substantially more mobile than the core of the protein (Brooks, 1992; Tirado-Rives & Jorgensen, 1993). Therefore, our results of the limited proteolysis experiments provide direct experimental evidence of the enhanced mobility or even unfolding/disruption of helix F in apoMb, previously inferred from spectroscopic and computational analyses.

Our study demonstrates that proteolytic enzymes can be used as reliable probes of protein structure and dynamics, thus complementing other physicochemical methods and approaches (Fontana *et al.*, 1986, 1993, 1995; Signor *et al.*, 1990; Polverino de Laureto *et al.*, 1994, 1995a,b,c).

## Acknowledgements

This work was supported by grants of the Italian National Council of Research (CNR) and the Ministry of University and Scientific Research (MURST). The authors thank Prof. Vincenzo Giancotti for assistance in the ESM measurements and Mrs Anna Mocavero for typing the manuscript.

## References

- Barrick, D. & Baldwin, R. K. (1993). Three-state analysis of sperm whale apomyoglobin folding. *Biochemistry*, **34**, 3790–3796.
- Breslow, E. & Koehler, R. (1965). Combination of protoporphyrin IX with sperm whale myoglobin. *J. Biol. Chem.* **246**, 2266–2268.
- Brockerhoff, S. E., Edmonds, C. G. & Davis, T. N. (1992). Structural analysis of wild-type and mutant yeast calmodulins by limited proteolysis and electrospray ionization mass spectrometry. *Protein Sci.* **1**, 504–516.
- Brooks, C. L. (1992). Characterization of "native" apomyoglobin by molecular dynamics simulation. *J. Mol. Biol.* **227**, 375–380.
- Cocco, M. J. & Lecomte, J. T. J. (1990). Characterization of hydrophobic cores in apomyoglobin: a proton NMR spectroscopy study. *Biochemistry*, **29**, 11067–11072.
- Cocco, M. J. & Lecomte, J. T. J. (1994). The native state of apomyoglobin described by proton NMR spectroscopy: interaction with the paramagnetic probe HyTEMPO and the fluorescent dye ANS. *Protein Sci.* **3**, 267–281.
- Cocco, M. J., Kao, Y. H., Phillips, A. T. & Lecomte, J. T. J. (1992). Structural comparison of apomyoglobin and metaquomyoglobin: pH titration of histidines by NMR spectroscopy. *Biochemistry*, **31**, 6481–6491.
- Cohen, S. L., Ferrè-D'Amarè, A. R., Burley, K. S. & Chait, B. T. (1995). Probing the solution structure of the DNA-binding protein Max by a combination of proteolysis and mass spectrometry. *Protein Sci.* **4**, 1088–1099.
- Eliezer, D. & Knight, P. E. (1996). Is apomyoglobin a molten globule? Structural characterization by NMR. *J. Mol. Biol.* **263**, 531–538.
- Evans, S. V. & Brayer, G. D. (1990). High-resolution study of the three-dimensional structure of horse heart metmyoglobin. *J. Mol. Biol.* **213**, 885–897.
- Fontana, A. & Gross, E. (1986). Fragmentation of polypeptides by chemical methods. *Practical Protein Chemistry: A Handbook* (Darbre, A., ed.), pp. 67–120, J. Wiley and Sons, Chichester.
- Fontana, A., Fassina, G., Vita, C., Dalzoppo, D., Zamai, M. & Zamboni, M. (1986). Correlation between sites of limited proteolysis and segmental mobility in thermolysin. *Biochemistry*, **25**, 1847–1851.

- Fontana, A., Polverino de Laureto, P. & De Filippis, V. (1993). Molecular aspects of proteolysis of globular proteins. *Protein Stability and Stabilization* (van den Tweel, W., Harder, A. & Buitelear, M., eds), pp. 101–110, Elsevier Science Publ., Amsterdam.
- Fontana, A., Zambonin, M., De Filippis, V., Bosco, M. & Polverino de Laureto, P. (1995). Limited proteolysis of cytochrome *c* in trifluoroethanol. *FEBS Letters*, **362**, 266–270.
- Goto, Y. & Fink, A. L. (1990). Phase diagram for acidic conformational states of apomyoglobin. *J. Mol. Biol.* **214**, 803–805.
- Griko, Y. V. & Privalov, P. L. (1994). Thermodynamic puzzle of apomyoglobin unfolding. *J. Mol. Biol.* **235**, 1318–1325.
- Griko, Y. V., Privalov, P. L., Venyaminov, S. Y. & Kutysenko, V. P. (1988). Thermodynamic study of the apomyoglobin structure. *J. Mol. Biol.* **202**, 127–138.
- Hapner, K. D., Bradshaw, R. A., Hartzell, C. R. & Gurd, F. R. N. (1968). Comparison of myoglobin from harbor seal, porpoise and sperm whale. I. Preparation and characterization. *J. Biol. Chem.* **243**, 683–689.
- Harrison, S. C. & Blout, E. R. (1965). Reversible conformational changes of myoglobin and apomyoglobin. *J. Biol. Chem.* **240**, 299–303.
- Hirst, J. D. & Brooks, C. L. (1994). Helicity, circular dichroism and molecular dynamics of proteins. *J. Mol. Biol.* **243**, 173–178.
- Hubbard, S. J., Campbell, S. F. & Thornton, J. M. (1991). Molecular recognition: Conformational analysis of limited proteolytic sites and serine proteinase protein inhibitors. *J. Mol. Biol.* **220**, 507–530.
- Hubbard, S. J., Eisenmenger, F. & Thornton, J. M. (1994). Modeling studies of the change in conformation required for cleavage of limited proteolytic sites. *Protein Sci.* **3**, 757–768.
- Hughson, F. M., Wright, P. E. & Baldwin, R. L. (1990). Structural characterization of a partly folded apomyoglobin intermediate. *Science*, **249**, 1544–1548.
- Jennings, P. A. & Wright, P. E. (1993). Formation of a molten globule intermediate early in the kinetic folding pathway of apomyoglobin. *Science*, **262**, 892–896.
- Kataoka, M., Nishii, I., Fujisawa, T., Ueki, T., Tokunaga, F. & Goto, Y. (1995). Structural characterization of the molten globule and native states of apomyoglobin by solution X-ray scattering. *J. Mol. Biol.* **249**, 215–228.
- Keil, B. (1982). Enzymic cleavage of proteins. *Methods in Protein Sequence Analysis* (Elzinga, M., ed.), vol. 4, pp. 291–304, Humana Press, Clifton, NJ.
- Kratzin, H. D., Wiltfang, J., Karas, M., Neuhoff, V. & Hilschmann, N. (1989). Gas-phase sequencing after electroblotting on polyvinylidene difluoride membranes assigns correct molecular weights to myoglobin molecular weight markers. *Anal. Biochem.* **183**, 1–8.
- Kuriyan, J., Wilz, S., Karplus, M. & Petsko, G. A. (1986). X-ray structure and refinement of carbon-monoxide (Fe-II)-myoglobin at 1.5 Å resolution. *J. Mol. Biol.* **245**, 133–154.
- Lecomte, J. T. J., Kao, Y.-H. & Cocco, M. J. (1996). The native state of apomyoglobin described by proton NMR spectroscopy: The A-B-G-H interface of wild-type sperm whale apomyoglobin. *Proteins: Struct. Funct. Genet.* **25**, 267–285.
- Lin, L., Pinker, R. J., Forde, K., Rose, G. D. & Kallenbach, N. R. (1994). Molten globular characteristics of the native state of apomyoglobin. *Nature Struct. Biol.* **1**, 447–451.
- Loh, S. N., Kay, M. S. & Baldwin, R. L. (1995). Structure and stability of a second molten globule intermediate in the apomyoglobin folding pathway. *Proc. Natl. Acad. Sci. USA*, **92**, 5446–5450.
- McLendon, G. & Radany, E. (1978). Is protein turnover thermodynamically controlled? *J. Biol. Chem.* **253**, 6335–6337.
- Mihalyi, E. (1978). *Application of Proteolytic Enzymes to Protein Structure Studies*, CRC Press, Boca Raton, FL.
- Mohsen, A. A., Aull, J. L., Payne, D. M. & Daron, H. M. (1995). Ligand-induced conformational changes of thymidylate synthase detected by limited proteolysis. *Biochemistry*, **34**, 1669–1677.
- Nishii, I., Kataoka, M., Tokunaga, F. & Goto, Y. (1994). Cold denaturation of the molten globule states of apomyoglobin and a profile for protein folding. *Biochemistry*, **33**, 4903–4909.
- Nishii, I., Kataoka, M. & Goto, Y. (1995). Thermodynamic stability of the molten globule states of apomyoglobin. *J. Mol. Biol.* **250**, 223–238.
- Novotny, J. & Brucoleri, R. E. (1987). Correlation among sites of limited proteolysis, enzyme accessibility and segmental mobility. *FEBS Letters*, **211**, 185–189.
- Parrado, J., Conejero-Lara, F., Smith, R. A. G., Marshall, J. M., Ponting, C. P. & Dobson, C. M. (1996). The domain organization of streptokinase: nuclear magnetic resonance, circular dichroism and functional characterization of proteolytic fragments. *Protein Sci.* **5**, 693–704.
- Polverino de Laureto, P., De Filippis, V., Bertolero, F., Orsini, G. & Fontana, A. (1994). Probing the structure of a human interleukin-6 mutant by limited proteolysis. *Techniques in Protein Chemistry* (Crabb, J. W., ed.), vol. 5, pp. 381–388, Academic Press, New York.
- Polverino de Laureto, P., De Filippis, V., Di Bello, M., Zambonin, M. & Fontana, A. (1995a). Probing the molten globule state of  $\alpha$ -lactalbumin by limited proteolysis. *Biochemistry*, **34**, 12596–12604.
- Polverino de Laureto, P., De Filippis, V., Scaramella, E., Zambonin, M. & Fontana, A. (1995b). Limited proteolysis of lysozyme in trifluoroethanol: Isolation and characterization of a partially active enzyme derivative. *Eur. J. Biochem.* **230**, 779–787.
- Polverino de Laureto, P., Toma, S., Tonon, G. & Fontana, A. (1995c). Probing the structure of human growth hormone by limited proteolysis. *Int. J. Peptide Protein Res.* **45**, 200–208.
- Price, N. C. & Johnson, C. M. (1990). Proteinases as probes of conformation of soluble proteins. *Proteolytic Enzymes: A Practical Approach* (Beynon, R. J. & Bond, J. S., eds), pp. 163–180, IRL Press, Oxford.
- Schägger, H. & von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368–379.
- Schechter, I. & Berger, A. (1967). On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **27**, 157–162.
- Seielstad, D. A., Carlson, K. E., Kushner, P. J., Greene, G. L. & Katzenellenbogen, J. A. (1995). Analysis of the structural core of the human estrogen receptor ligand binding domain by selective proteolysis/mass spectrometric analysis. *Biochemistry*, **34**, 12605–12615.

- Signor, G., Vita, C., Fontana, A., Frigerio, F., Bolognesi, M., Toma, S., Gianna, R., De Gregoris, E. & Grandi, G. (1990). Structural features of neutral protease from *Bacillus subtilis* deduced from model-building and limited proteolysis experiments. *Eur. J. Biochem.* **189**, 221–227.
- Stryer, L. (1965). The interaction of a naphthalene dye with apomyoglobin and apohemoglobin. A fluorescent probe of non-polar binding sites. *J. Mol. Biol.* **13**, 482–495.
- Takano, T. (1977). Structure of myoglobin refined at 2.0 Å resolution. I. Crystallographic refinement of sperm whale met-myoglobin. *J. Mol. Biol.* **110**, 537–568.
- Tirado-Rives, J. & Jorgensen, W. L. (1993). Molecular dynamics simulations of the unfolding of apomyoglobin in water. *Biochemistry*, **32**, 4175–4184.
- Yang, A.-S. & Honig, B. (1994). Structural origins of pH and ionic strength effects on protein stability: Acid denaturation of sperm whale apomyoglobin. *J. Mol. Biol.* **237**, 602–614.
- Zappacosta, F., Pessi, A., Bianchi, E., Venturini, S., Sollazzo, M., Tramontano, A., Marino, G. & Pucci, P. (1996). Probing the tertiary structure of proteins by limited proteolysis and mass spectrometry. The case of Minibody. *Protein Sci.* **5**, 802–813.

*Edited by P. E. Wright*

*(Received 19 September 1996; received in revised form 22 October 1996; accepted 13 November 1996)*

*Note added in proof:* After submission of this paper, a structural characterization by NMR of sperm whale apoMb was published (Eliezer & Wright (1996) *J. Mol. Biol.* **263**, 531–538). Measurements were conducted on a recombinant isotopically labelled (<sup>15</sup>N, <sup>13</sup>C) protein at neutral pH. It was concluded that the structure of apoMb is well-defined and highly similar to that of holoMb with the exception of chain segment 82–102, for which resonances are missing due to conformational fluctuations. Moreover, approximately one turn at both the amino-end of helix G and at the carboxy-end of helix H of holoMb appears to be disrupted in apoMb. The results of this additional NMR study are therefore in full agreement with those of limited proteolysis of horse apoMb here reported.