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Effects of urea and acetic acid on the heme axial ligation structure of ferric myoglobin at very acidic pH

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Abstract

The heme iron coordination of ferric myoglobin (Mb) in the presence of 9.0 M urea and 8.0 M acetic acid at acidic pH values has been probed by electronic absorption, magnetic circular dichroism and resonance Raman spectroscopic techniques. Unlike Mb at pH 2.0, where heme is not released from the protein despite the acid denaturation and the loss of the axial ligand, upon increasing the concentration of either urea or acetic acid, a spin state change is observed, and a novel, non-native six-coordinated high spin species prevails, where heme is released from the protein.

Keywords

Myoglobin; unfolding process; urea; resonance Raman spectroscopy; magnetic circular dichroism spectroscopy; electronic absorption spectroscopy

1. Introduction

Myoglobin (Mb) is a protoheme-containing globin that has become a widely used model molecule in protein folding studies, since it is suitable for a large number of spectroscopic techniques, including optical and magnetic resonance spectroscopies. It was the first protein to have its 3D structure solved by X-ray crystallography nearly half a century ago [1] and for which the dynamic X-ray structures become available [2–4]. A variety of external triggers, including pressure [5–7], temperature change [6,7], pH change [8–12], chemical denaturants like urea and guanidium hydrochloride [12–23], strong anionic surfactants like sodium dodecyl sulfate [24,25], have been used to study the denaturation of Mb. Many investigations have regarded the role of the heme in the stabilization of the holoprotein with respect to the apo-form and the effect of structural modifications of the chromophore, which are relatively easy to obtain [26,27]. Unfolding studies have revealed that heme binding clearly stabilizes the globin tertiary structure with respect to both the intermediate and unfolded states [14,28,29].

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Moreover, loss of the heme chromophore has been recognized as a key event in guanidium hydrochloride-induced unfolding [13–15] and heme loss, a consequence of the breakage of the iron-proximal histidine bond, is probably the main contribution to the denaturation of Mb [30]. Mb unfolding typically was found not to be reversible due to aggregation of the heme group following its release from the unfolded protein [31,32]. The re-folding reaction has also been extensively studied. Heme association, the first step of the folding reaction, was found to be driven by nonspecific binding to the empty but partially folded heme pocket for apoMb [29,33–35]. Once the heme is within the pocket, large-scale folding occurs in the globin to obtain the native conformation, including binding of the proximal histidine to the iron atom. A second pathway of the folding reaction was found to involve nonspecific binding of the heme outside the heme pocket, from where it must dissociate to enter the pocket so that the final folding steps can occur.

The investigation of the different unfolded conformations of a protein at equilibrium under denaturing conditions is important for many reasons. First, unfolded forms have been proved to represent the analogs of transient folding intermediates [36]. Secondly, partially or totally unfolded species are supposed to initiate amyloid formation, [37–39] which is responsible for many pathological conditions, including Alzheimer's disease, Parkinson's disease, Huntington's disease, prion encephalopathies, and sickle-cell anemia disease [40–43]. Interestingly, native Mb forms fibrils when incubated with an amyloidogenic peptide in aqueous solutions [44,45]. The fact that Mb, a globular protein, can relatively easily form fibrils, was rather unexpected, since partially folded states of Mb were found to be significantly helical [46].

Urea is a chemical agent which is often used to induce protein denaturation and thus to assess protein stability [12,17–23]. Despite its widespread use, the molecular mechanism underlying urea-induced protein denaturation has not yet been fully understood. Mainly two opposing mechanisms are controversially discussed. The first is the so-called indirect mechanism in which urea acts indirectly, altering water structure and dynamics, thereby reducing the hydrophobic effect and facilitating the exposure of the hydrophobic core residues, leading to protein unfolding [47]. The second is the direct mechanism, which proposes that the polypeptide is solvated by both urea and water. Urea participates in the unfolding process by binding to protein residues and peptide backbone. Recently a model based on ^{1}H-NMR spectroscopic analysis and molecular orbitals calculations has been proposed for the denaturation of RNase A at low concentration urea [48]. A direct interaction between urea and protonated histidine at the active site is proposed as the initial step for protein inactivation followed by hydrogen bond formation with polar residues, while the breaking of hydrophobic collapse is the final steps for protein denaturation. In addition, many models propose that urea favors unfolding by stabilizing the denatured state [49–51]. Recently it has also been shown that addition of high concentrated urea solutions to ferrous Mb at neutral pH either slow down or speed up the self diffusion of water, in contrast with the generally reported water structure-breaking mechanism of urea and the associated rise in the overall water mobility. Moreover, urea competes with water in the binding to the protein [18]. Therefore, it cannot be excluded that a possible effect of urea on Mb is the binding of the carbonyl group of urea to the heme to form axial ligation in the denatured state.

Despite the ongoing efforts in the area of Mb folding and unfolding, the relevance of the axial ligands of heme iron to this process in the presence of urea, is still missing to date. As far as apoMb is concerned, recent investigations have revealed that it forms a soluble monomer species under strongly denaturing conditions (highly concentrated urea solutions and low pH values), whereas aggregates are predominant under milder unfolding conditions (highly concentrated urea solutions pH 6.0) [52].

With this in mind, UV-visible (UV-Vis) absorption, magnetic circular dichroism (MCD) and resonance Raman (RR) spectroscopic techniques have been employed in the present study to investigate the axial ligation of denatured met-(holo)Mb at acidic pH values in the presence of urea or acetic acid. MCD has been demonstrated to be a powerful spectroscopic technique to elucidate Mb heme coordination structure for (a) native Mb and its ferric and ferrous ligand complexes including the oxyferrous form [53–55], (b) chlorin-reconstituted Mb as model for His-ligated iron chlorin proteins [56], (c) cavity mutants of Mb and mixed ligand complexes as models for heme proteins [57–59]. On the other hand, RR is a well established technique to study the heme environment and coordination. The frequencies of the core size marker bands in RR spectra furnish important information on the oxidation, spin and coordination states of the iron atom [60,61].

2. Materials and methods

2.1 Materials

Horse Mb, bovine hemin, urea, HCl and NaOH were purchased from Sigma Chemical Co. Glacial acetic acid was purchased from Merck. Mb concentration was determined using published molar absorptivities [62]. HCl and NaOH were used throughout to readjust the pH.

2.2 Sample preparation

Mb samples were prepared by dissolving the lyophilized protein directly in the following appropriate solutions: 10 mM phosphate buffer, pH 2.0 and pH 7.0; 0.9 M acetic acid, pH 2.5; 9.0 M urea, pH 2.0; 8.0 M acetic acid, pH 1.8. Hemin in 9.0 M urea, at pH 2.0 was prepared by mixing concentrated hemin solution in NaOH 0.1 M to 9.0 M urea, pH 2.0 and the pH re-adjusted with HCl. In urea at pH 2.0, the concentration of both hemin and Mb was kept lower than 5 μM. Upon increasing chromophore concentration, a shoulder at 370 nm appears in the UV-Vis spectrum, indicating that a 5cHS heme grows, probably due to aggregation. In the absence of urea, no dependence of the species in solution upon Mb concentration was observed using a 10–30 μM protein concentration range.

Free heme in Mb solutions at pH 2.0 in 9.0 M urea was separated using YM-10 Centricons (Amicon bioseparations) spun down at 5,000 rpm (small Sorvall rotor) for 30 min at room temperature. The colored filtrate (free-heme) was collected. However, since the retentate solution started precipitating and elution of free-heme (in urea) stops when about half the volume is filtered (see Results and Discussion), spinning was done in six 5 min intervals. The last two fractions were colorless, i.e. no free heme. The partially precipitated retentate was diluted using pH 2.0, 9.0 M urea and spun down again to obtain more colored filtrate.

Refolded Mb was obtained by 10-fold dilution of Mb either in 9.0 M urea at pH 2.0 or in 8.0 M acetic acid at pH 1.8, into pH 7.5 phosphate buffer 100 mM. NaOH was used to readjust the pH to 7.0. Final concentration of refolded Mb was 0.5 μM and 2 μM in urea and acetic acid solutions, respectively, 2 order of magnitude different since heme aggregation occurs in urea at higher extent [12].

2.3 Spectroscopy

UV-visible absorption spectra were recorded using a Cary 400 or a Cary 5 spectrophotometer at room temperature. MCD measurements were carried out at 4°C except for urea-denatured samples which were examined at 20°C. The spectra of the samples in urea (9.0 M) were recorded at 20°C in order to avoid crystallization of urea. UV-Vis MCD data were recorded with a JASCO J600 spectropolarimeter equipped with a JASCO MCD-1B electromagnet operated at field strength of 1.41 T. All spectral measurements were recorded immediately

after sample preparation using quartz cuvettes with 0.1, 0.5 or 1.0 cm path lengths. Data acquisition and processing were done as previously reported with JASCO software [63].

The UV-Vis spectra of refolded Mb were obtained upon concentration of the solution by about 10 times.

RR spectra were obtained at room temperature with excitation at 406.7 nm using a Kr^+ laser (Coherent, Innova 300C) using a triple spectrometer (consisting of two Acton Research SpectraPro 2300i and a SpectraPro 2500i in the final stage with a 3600 grooves/mm grating) working in the subtractive mode, equipped with a liquid nitrogen-cooled CCD detector (Roper Scientific Princeton Instruments). All RR measurements were repeated several times under the same conditions to ensure reproducibility. To improve the signal-to-noise ratio, a number of spectra were accumulated and summed only if no spectral differences were noted. The RR spectra were calibrated with indene as standards to an accuracy of 1 cm^{-1} for intense isolated bands. In the figures the relative intensities of the high-frequency RR bands are normalized on the ν_4 band. RR spectra were collected at room temperature. Prior to the spectra analysis contributions from the unfolding agents and the featureless background were subtracted.

Electronic absorption spectra were recorded before and after MCD and RR measurements to check the sample integrity.

3. Results and Discussion

Figure 1 compares the UV-Vis, RR and MCD spectra of Mb at pH 2.0 with and without 9.0 M urea with the spectra of Mb at neutral pH. It is well established that at pH <4, the spectra of metMb undergoes dramatic changes [64,65]. Most noticeably, upon lowering the pH to 2.0, the Soret absorption band dramatically decreases in intensity, broadens and blue-shifts from 409 to 370 nm, while the bands in the visible region red-shift to 514 and 545 nm and the CT1 band to 650 nm [65] (Figure 1A). These spectral variations are indicative of the conversion of the aqua six-coordinate high-spin heme (6cHS) native form of the protein [denoted as 6cHS (1)] to a five-coordinated high-spin (5cHS) species. In fact, at acid pH the length of the Fe-proximal histidine bond increases [65], and breaks in ferric Mb below pH 4, whereas a water molecule binds to the iron atom [64–66]. Accordingly, the corresponding RR spectrum in the high frequency region (Figure 1B) changes from the core size marker bands typical of a 6cHS heme (ν_3 at 1483 cm^{-1} and ν_2 at 1563 cm^{-1}) [67] to that of a 5cHS heme with a water molecule coordinated to the heme iron atom [64].

Upon lowering the pH from 7.0 to 2.0 significant differences occur also in the spectral features of the MCD spectra (Figure 1C). At pH 7.0 the MCD spectrum displays a derivative-shaped feature with the cross-over point at 409 nm, characteristic of a 6cHS species, and two troughs in the visible region. At acidic pH the MCD spectrum looses the characteristic derivative-shaped feature of the native protein and displays two prominent troughs at 359 and 562 nm. At low pH, it is logical to assume that protonation of the histidine proximal ligand causes the heme to be released from Mb; however, heme solubilization in the aqueous phase is not favorable because it is highly hydrophobic, especially at low pH where the propionic groups of porphyrin become protonated. In order to understand whether the heme is released from the protein, Mb at pH 2.0 was spun down using a Centricon (YM-10). No absorbance was observed in the filtrate, indicating the absence of free heme in solution. Thus, heme remains bound to the protein at pH 2.0 despite the acid denaturation and the loss of axial ligation. Futher, the acidic form of ferric HRP has been previously established by resonance Raman spectroscopy to be a five-coordinate water-ligated high spin complex and the MCD spectrum of this complex has been previously published [68,69]. The spectral similarities in band shape and peak positions of Mb at pH 2.0 and of ferric horseradish peroxidase (HRP) at pH 3.1 (Figure 2)

argue that both species have very similar, if not identical, coordination structures. Therefore, we conclude that Mb loses its histidine ligation, due to protonation of the histidine residue at low pH, and the final form of ferric Mb at pH 2.0 is five-coordinate high-spin heme whose histidine ligand is replaced by a water ligand. This MCD finding is consistent with the report by Sage et al. on low pH Mb studied using resonance Raman spectroscopy [64].

Addition of 9.0 M urea to Mb at pH 2.0 causes dramatic spectral variations. In the MCD spectrum the two deeper troughs at 359 and 562 nm, observed in the absence of urea disappear and the spectrum shows a derivative-shaped feature with cross-over point at 401 nm and two troughs in the visible region at 550 and 637 nm (Figure 1C). Such spectral variations are indicative of a change in the coordination and spin state of the heme iron. Accordingly, the RR spectrum (Figure 1B) clearly shows the formation of a six-coordinate heme (ν_3 at 1484 cm^{-1}), although a large amounts of a 5cHS form (ν_3 at 1493 cm^{-1}, ν_{10} at 1629 cm^{-1}) is still detected. Moreover, unlike Mb at pH 7.0, which is characterized by two coincident ν(C=C) stretching modes at 1620 cm^{-1} [67], on the basis of depolarization ratio measurements and a curve fitting analysis (data not shown), two polarized bands are observed at 1620 and 1624 cm^{-1} which are, therefore, assigned to two vinyl stretching modes. Therefore, in the unfolded protein, a rearrangement of one vinyl group, resulting from interactions with the protein matrix, occurs.

The comparison of the MCD and UV-Vis spectra of the native (pH 7.0) and the unfolded (9.0 M urea at pH 2.0) protein allows us to exclude the regeneration of the native coordination [6cHS(1)]. In fact, both the MCD and UV-Vis spectra of Mb in the denaturants conditions are blue-shifted as compared to the native protein. In particular, the MCD spectrum (Figure 1C) of Mb at acid pH in the presence of urea, is blue-shifted by 8 nm. In the UV-Vis spectrum, the Soret band blue-shifts from 409 to 400 nm, the Q-bands from 504 and 535 nm to 501 and 530 nm, respectively, and the CT1 band from 634 to 628 nm (Figure 1A). The CT1 band, in particular, gives important informations about the strength of the axial ligands of the high spin heme proteins. This transition is associated with the promotion from the highest filled porphyrin π orbitals to the $e_g(d_{xz},d_{yz})$ orbitals of the iron atom. The energy of the $e_g(d_{xz},d_{yz})$ orbitals increases with the strength of the ligand field. Therefore the maximum of the CT1 band shifts to the blue upon decreasing the strength of the axial ligands. Several spectra of 6cHS forms of Mb are reported in the literature where His remains as the proximal ligand and H_2O is replaced by a weaker ligand. For axample, the CT1 band shifts to the blue on going from the native protein (634 nm) to formate (624 nm) and fluoride (606 nm), as a result of the decreasing ligand field on going from water to formate and fluoride [69–72]. Therefore, since the CT1 band shifts to the blue from 634 (native Mb) to 628 nm (Mb urea pH 2.0), it can be concluded that, in the novel 6cHS(2) species, at least one of the axial ligands (His and H_2O) is replaced by a weaker ligand (H_2O or alternatively an urea carboxyl group, -COOH, or another endogenous ligand of the polypeptide chain). The novel 6cHS(2) species is also observed when Mb is unfolded in urea 9.0 M at neutral pH (data not shown). In this latter case, however, the formation of this species depends on Mb concentration, probably as a consequence of protein aggregation. The 6cHS(2) species prevails when Mb is lower than 5μM, whereas 5cHS and 6cLS forms grow in at higher concentration.

In order to obtain further insights into the nature of the axial ligands of Mb in 9.0 M urea pH 2.0, spectra of hemin in the same unfolding conditions have been obtained. Figure 3 compares the spectra of Mb and hemin in 9.0 M urea at pH 2.0. The spectra are almost identical, being a mixture of 5c- and 6cHS hemes. Therefore, not only the 6cHS(2) species observed for acid Mb in the presence of urea is not a native form but this experiment rules out the presence of a polypeptide residue coordinated to the heme iron. In addition, it suggests that in these unfolding conditions the heme is released from the holoprotein. Accordingly, free-heme has been separated from the solution of the unfolded protein through the procedure described in the

Materials and Methods section. Gradual precipitation of Mb in 9.0 M urea in a Centricon during the free heme removal process by centrifugation indicates that the removal of protein-free heme and increase in protein concentration leads to the formation of large aggregates. Dilution of the partially precipitated retentate using 9.0 M urea at pH 2.0 releases more free-heme; because of this reason the percentage of free-heme present in Mb in 9.0 M urea at pH 2.0 could not be determined quantitatively. The release of free-heme in the urea medium may be dependent on several factors including protein concentration. UV-Vis and MCD spectra of the eluted fraction (free heme) are different from both the spectra of hemin and Mb, probably as a consequence of aggregation (Figure 4).

Therefore, we conclude that, when Mb is unfolded at acidic pH, the proximal histidine residue is not bound to the heme iron; however, heme is not released from the holoprotein, since no free-heme is observed upon centrifugation. On the contrary, when Mb is unfolded in urea at acidic pH, heme is released from the protein.

The UV-Vis spectrum of the refolded Mb is identical to that of the native protein (Figure 5). In fact, the absorbance of the Soret band of the native species 6cHS(1) was almost completely restored upon refolding, indicating that i) the unfolding process is completely reversible, and ii) the refolding of Mb leads essentially to the native monomer. On the contrary, the refolding process of the apoprotein under these conditions was found to proceed via two parallel routes: one leading to native monomer, and the other leading to a misfolded and heavily aggregated state bearing some native-like secondary structure [52]. Therefore the heme chromofore stabilizes the native protein.

Further experiments have been performed to confirm or rule out the potential carbonyl ligation to Mb in 9.0 M urea at pH 2.0. In particular, urea has been replaced by CH_3COOH. Figure 6 compares the UV-Vis, MCD and RR spectra of Mb in CH_3COOH 0.9 M at pH 2.5 and in CH_3COOH 8.0 M at pH 1.8 with those of Mb in urea at pH 2.0. The spectra in CH_3COOH 0.9 M at pH 2.5 are very similar to those of Mb at pH 2.0 in the absence of urea. The UV-Vis is characterized by Soret band at 368 nm, Q bands at about 509 and 541, and a CT1 band at 642 nm (Figure 6A). Also the MCD spectra of Mb in the two acidic solutions are very similar, except for a deeper trough at 400-450 nm for Mb in 0.9 M acetic acid at pH 2.5 than for Mb alone at pH 2.0 (Figure 6C). The RR core size marker bands (Figure 6B) at 1493 cm^{-1} (ν_3) and 1629 cm^{-1} (ν_{10}), and the small intensity ratio, $I_{\nu4}/I_{\nu3} = 2$ are indicative of the presence of a 5cHS heme with a weak axial oxygen anionic (such as the case of Fe-H_2O) [65,73].

Upon increasing CH_3COOH concentration from 0.9 to 8.0 M (and consequently lowering the pH from 2.5 to 1.8), the UV-Vis, MCD and RR spectra become very similar to those of Mb at pH 2.0 in urea 9.0 M (Figure 1). The band at 368 nm almost disappears as the intensity of the peak at 398 nm increases and the features in the visible region blue-shift. In particular, the charge transfer band shifts from 642 to 629 nm (Figure 6A). Accordingly, the RR spectrum is characteristic of a mixture of 5c- and 6cHS (Figure 6B). Moreover, the absorbance features translate in the MCD spectrum as a derivative-shaped feature centered at 399 nm in the Soret region (indicating the presence predominantly of a six coordinated species) and two troughs at 547 nm and 638 nm in the visible region (Figure 6C). Therefore, upon increasing CH_3COOH concentration from 0.9 to 8.0 M the 5cHS (Fe-H_2O) is partially converted into the 6cHS(2) species. A rearrangement of the vinyls, as observed in urea 9.0 M pH 2, also occurs.

The six-coordinated species could be either acetic acid/water or bis-acetic acid, but at this very high concentration of acetic acid, the acetic acid/water ligation may be ruled out. If heme is retained in the protein, it may be unlikely to have bis-acetic acid ligation. In fact, free heme was observed in the Centricon filtrate. Therefore, we suggest that in the presence of 8.0 M acetic acid (pH 1.8), the heme iron, likely released from the protein, is coordinated by two

acetic acid molecules (bis-carbonyl). Knowing that Mb in 9.0 M urea at pH 2.0 is a mixture of two or more complexes, several comparisons were made in order to establish the identity of the complexes present. The spectrum of Mb in 9.0 M urea at pH 2.0 shows striking resemblance to the calculated spectrum of the mixture of 50% Mb in 8.0 M acetic acid at pH 1.8 and 50% Mb at pH 2.0 (Figure 7). This leads us to conclude that the Mb at pH 2.0 in 9.0 M urea is a mixture of six-coordinate bis-urea (in other words bis-carbonyl) and five-coordinate mono-water, and to the extent that free heme is seen with both 9.0 M urea and 8.0 M acetic acid, we conclude that the bis-carbonyl ligated heme is released from the protein.

The refolding of Mb (unfolded in 8.0 M acetic acid, pH 1.8) has been proved by UV-Vis spectroscopy. Upon refolding aggregation occurs and some precipitate is observed. Therefore, while the UV-Vis spectrum of the filtrated refolded protein is identical to that of the native protein, the intensity of the band at 280 nm is about three times more intense in the spectrum of the refolded protein as a consequence of the large amount of heme which aggregates and precipitates upon refolding.

In summary, in concentrated urea solutions, both at neutral and at acidic pH, the results suggest that the carbonyl group of urea contributes to the stability of the denatured state by binding directly to the iron atom of unfolded Mb. Since at pH 2.0 the protonation of urea is negligible (ca. 2%) and comparable to that occurring at pH 7.0 [73,74], its carbonyl group is capable to coordinate to the iron atom both at neutral and at acidic pH. At neutral pH, the 6cHS(2), bis-carbonyl species, is not the prevailing form as aggregation occurs giving rise to both 5cHS and 6cLS species, but it becomes the only species in extreme unfolding conditions (high urea concentration and pH 2.0). This was rather unexpected, since the solubilization of the hydrophobic heme chromophore is unfavoured especially at low pH where the propionic groups of the porphyrin are protonated. Similarly, at neutral pH apoMb forms aggregates at high urea concentration, whereas under strongly denaturing environment the large aggregates are absent [52]. At low pH, polypeptides may acquire a net positive charge, leading to an overall increase in intermolecular electrostatic repulsion, which reduces the tendency toward intermolecular complex formation. The solubilization of the heme group therefore is accomplished by the parallel solubilization of the the polypeptide chain.

In unfolded Mb, both at neutral and at acidic pH, the iron-histidine bond cleavage, and the consequent non-native coordination of the carbonyl group, is not driven by the protonation of the proximal histidine ligand, but is mediated by both the global unfolding of the protein and by the solvatation of the heme in the more hydrophobic environment created by urea.

The re-folding of the monomeric unfolded state (concentrated urea, low pH) was found to be reversible for holo-Mb, whereas the parallel formation of severely self-associated species was observed for apo-Mb, thus reaffirming that heme binding is a key event in the stabilization of the native form of the protein.

Protein aggregation is a problem in protein production and biotechnology. It is well known, infact that, upon in vivo production of recombinant proteins, co- or posttranslational aggregation can lead to the formation of heavily insoluble inclusion bodies, which are notoriously hard to handle. Inclusion body solubilization requires high concentrations of denaturing agent such as urea and guanidine hydrochloride [75]. Upon removing the denaturing agent aggregation and precipitation occur, thus reducing the overall yields of properly folded products [76].

In the present work we found that the novel 6cHS(2) species of Mb, formed at pH 2.0 in 9M urea, refolds completely to the native conformation. Therefore, this extreme unfolding conditions, i.e. low pH (where polypeptides acquire a net positive charge and reduce the tendency toward globin aggregation) and urea (which provides a more hydrophobic

environment that reduces heme aggregation), might be useful both to solubilize inclusion body and to reduce protein aggregation and precipitation.

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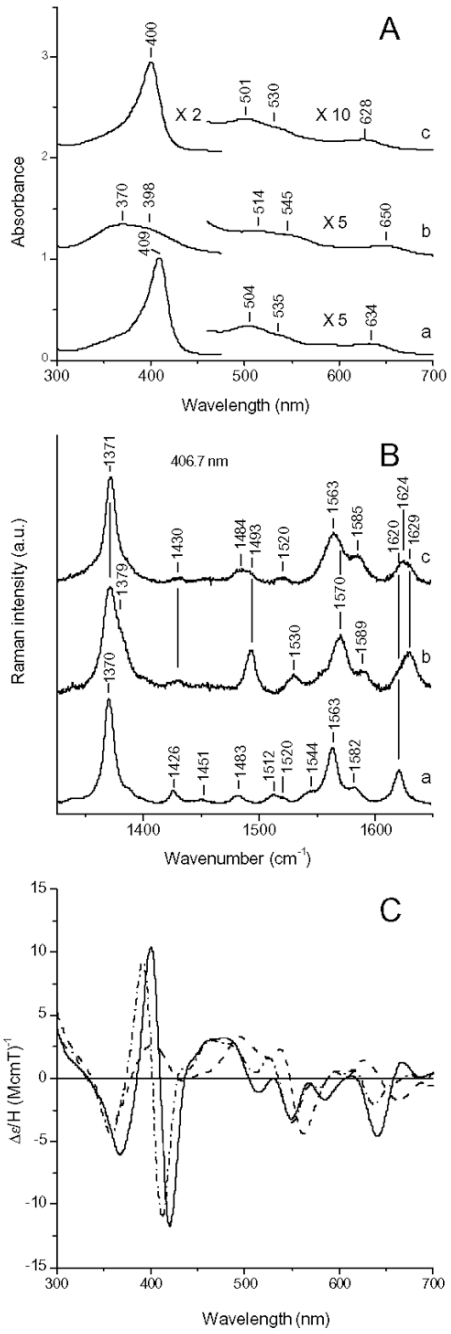

Figure 1.

UV-Vis (A), RR (B) and MCD (C) spectra of Mb at pH 7.0 and at pH 2.0 in the absence and presence of urea 9.0 M. (A): Mb 6 μM at pH 7.0 (a), Mb 6 μM at pH 2.0 (b), Mb 3 μM at pH 2.0 with urea (c). (a,b) The 460–700 nm region in the absorption spectra has been expanded by a factor of five; (c) The 300–475 nm region in the absorption spectrum has been expanded by a factor of two; the 460–700 nm region in the absorption spectra has been expanded by a factor of ten. The spectra have been shifted along the ordinate axis (spectrum b by 1 absorbance unit and spectrum c by 2 absorbance unit) to allow better visualization. (B): Mb at pH 7.0 (a), at pH 2.0 (b), at pH 2.0 with urea (c). Experimental conditions: 406.7 nm excitation wavelength, 3 mW laser power at the sample, 1 cm^{-1} spectral resolution and 60 min (a), 140 min (b), 120

min (c) integration time, respectively. (C) Mb at pH 7.0 (—), at pH 2.0 (------), and at pH 2.0 with urea (- - - -).

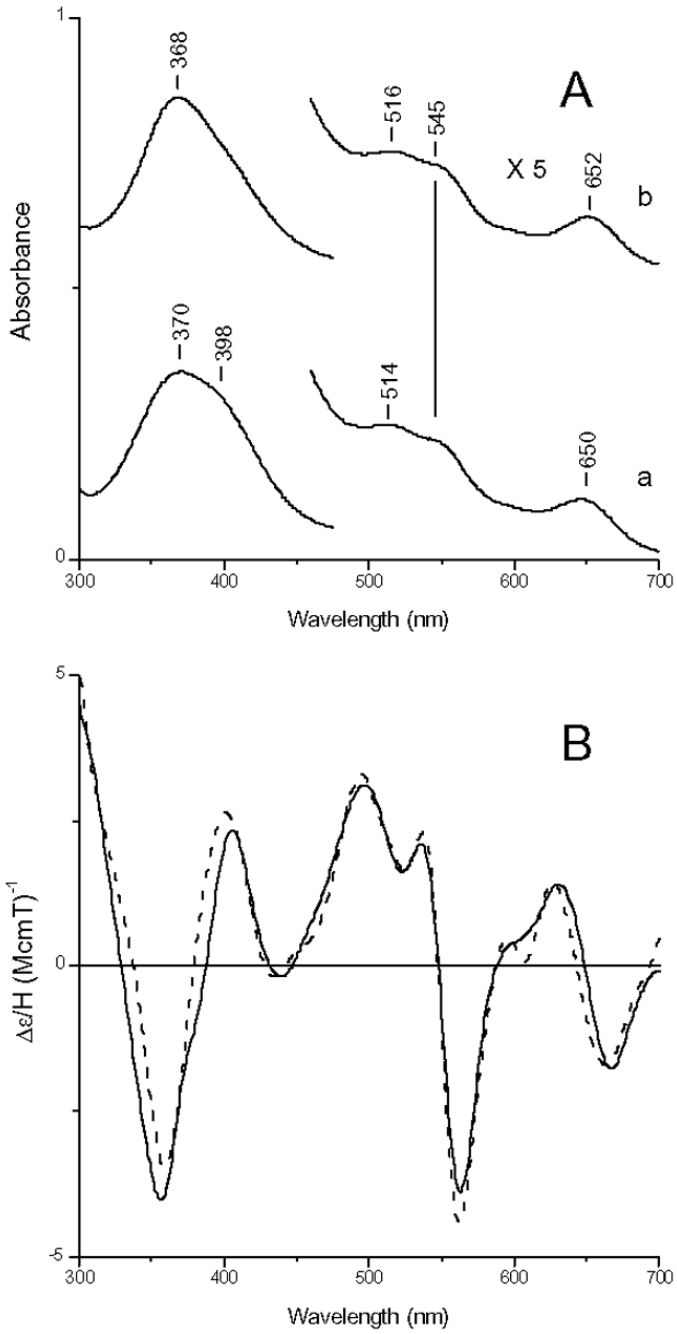

Figure 2.

UV-Vis (A) and MCD (B) spectra of Mb at pH 2.0 and HRP at pH 3.1 (replotted from reference 69). A): Mb (a) and HRP (b). The 460–700 nm region in the absorption spectra has been expanded by a factor of five; spectrum b has been shifted along the ordinate axis by 0.5 absorbance unit to allow better visualization. B): Mb (------) and HRP (—).

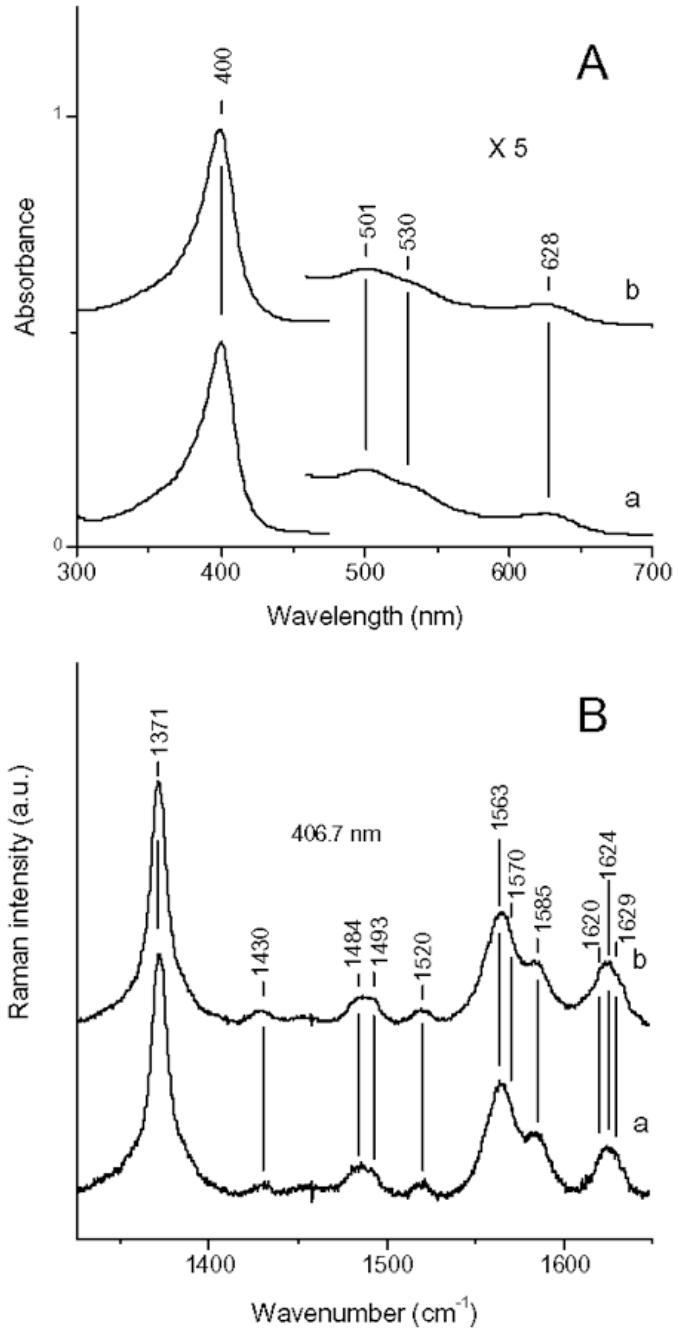

Figure 3.

UV-Vis (A) and RR (B) spectra of Mb (a), and of hemin (b) at pH 2.0 with urea 9.0 M. (A): the 460-700 nm region in the absorption spectra has been expanded by a factor of five; spectrum b has been shifted along the ordinate axis by 0.5 absorbance unit to allow better visualization. (B): experimental conditions: 406.7 nm excitation wavelength, 3 mW laser power at the sample, 1 cm^{-1} spectral resolution and 120 min (a) and 80 min (b) integration time, respectively.

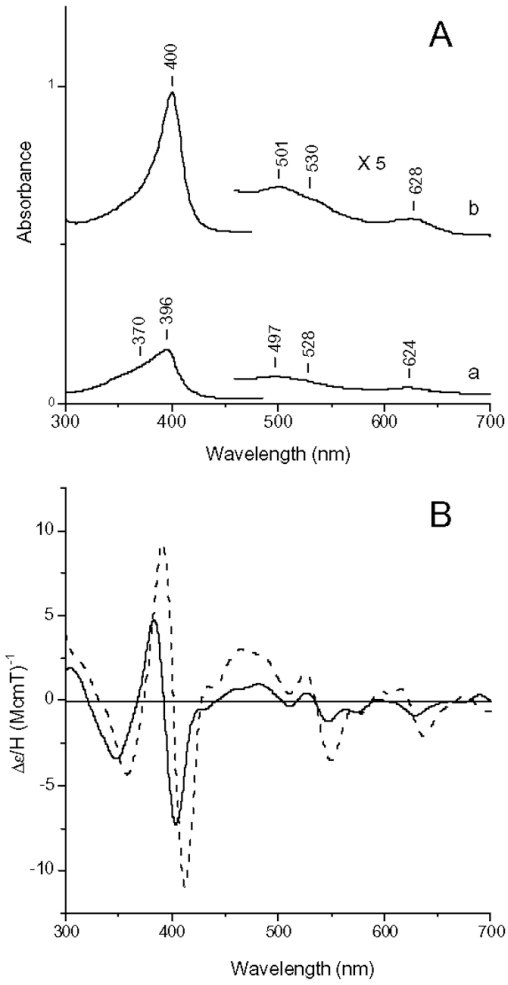

Figure 4.

UV-Vis (A) and MCD (B) spectra of free-heme separated from the solution of the unfolded protein (see Materials and methods) and of Mb in urea 9.0 M at pH 2.0. (A): free-heme (a) and Mb (b). The 460–700 nm region in the absorption spectra has been expanded by a factor of five; spectrum b has been shifted along the ordinate axis by 0.5 absorbance unit to allow better visualization. (B) free-heme (—) and Mb (------).

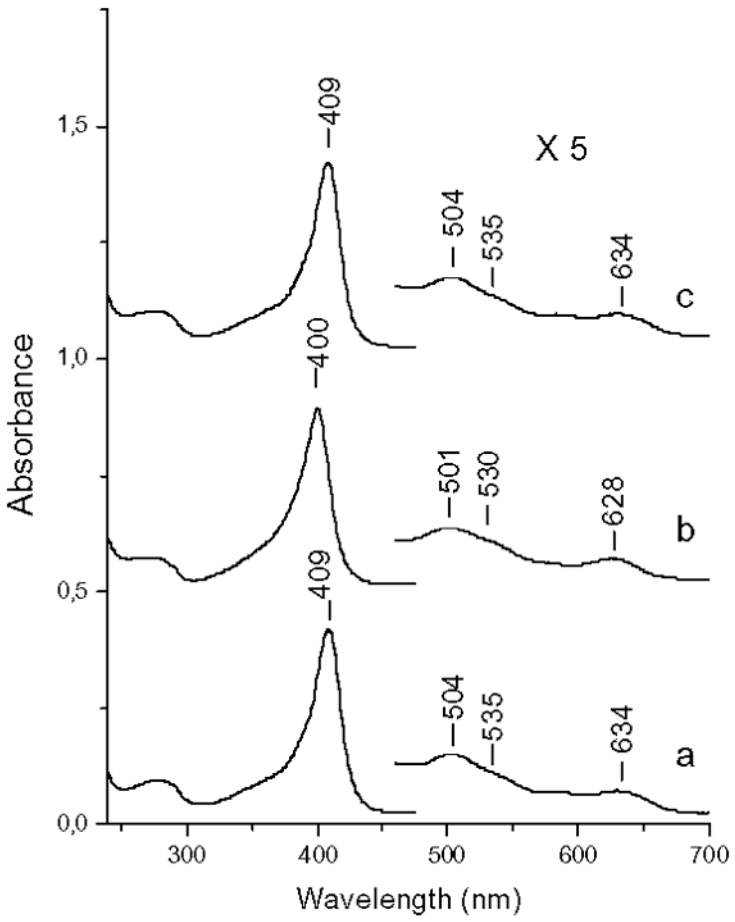

Figure 5.
UV-Vis spectra of ferric protein. (a) native Mb at pH 7.0, 20 μM; (b) unfolded Mb with urea at pH 2.0, 2 μM; (c) refolded Mb, 2 μM, at pH 7.0. The 460–700 nm region has been expanded by a factor of five; the spectra have been shifted along the ordinate axis to allow better visualization, spectrum b by 0,5 absorbance unit and spectrum c by 1 absorbance unit.

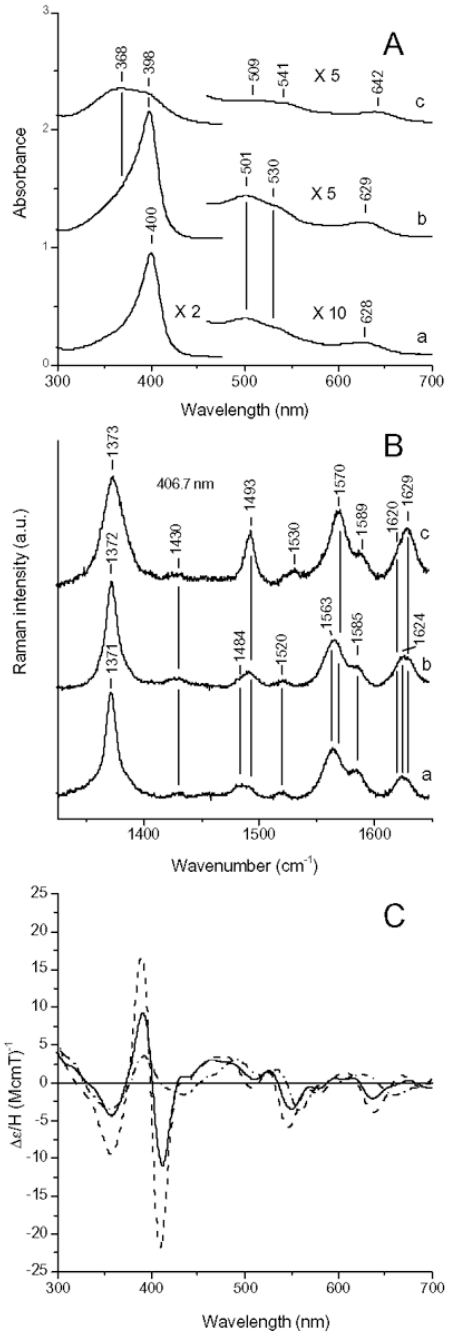

Figure 6.

UV-Vis (A), RR (B) and MCD (C) spectra of Mb in 9.0 M urea at pH 2.0, CH_3COOH 8.0 M at pH 1.8, CH_3COOH 0.9 M at pH 2.5. (A): Mb 2 μM in urea (a), Mb 6 μM in CH_3COOH, pH 1.8 (b), Mb 6 μM in CH_3COOH, pH 2.5 (c). (a) The 300–475 nm region in the absorption spectrum has been expanded by a factor of two; the 460-700 nm region in the absorption spectra has been expanded by a factor of ten; (b,c) The 460–700 nm region in the absorption spectra has been expanded by a factor of five. The spectra have been shifted along the ordinate axis, (spectrum b by 1 absorbance unit and spectrum c by 2 absorbance unit) to allow better visualization. (B): Mb in urea (a), in CH_3COOH, pH 1.8 (b), and CH_3COOH, pH 2.5 (c). Experimental conditions: 406.7 nm excitation wavelength, 3 mW laser power at the sample, 1

cm^{-1} spectral resolution and 120 min (a) and 120 min (a), 160 min (b), 140 min (c) integration time, respectively. (C): Mb in urea (—), in CH_3COOH, pH 1.8 (------), and CH_3COOH, pH 2.5 (- - - -).

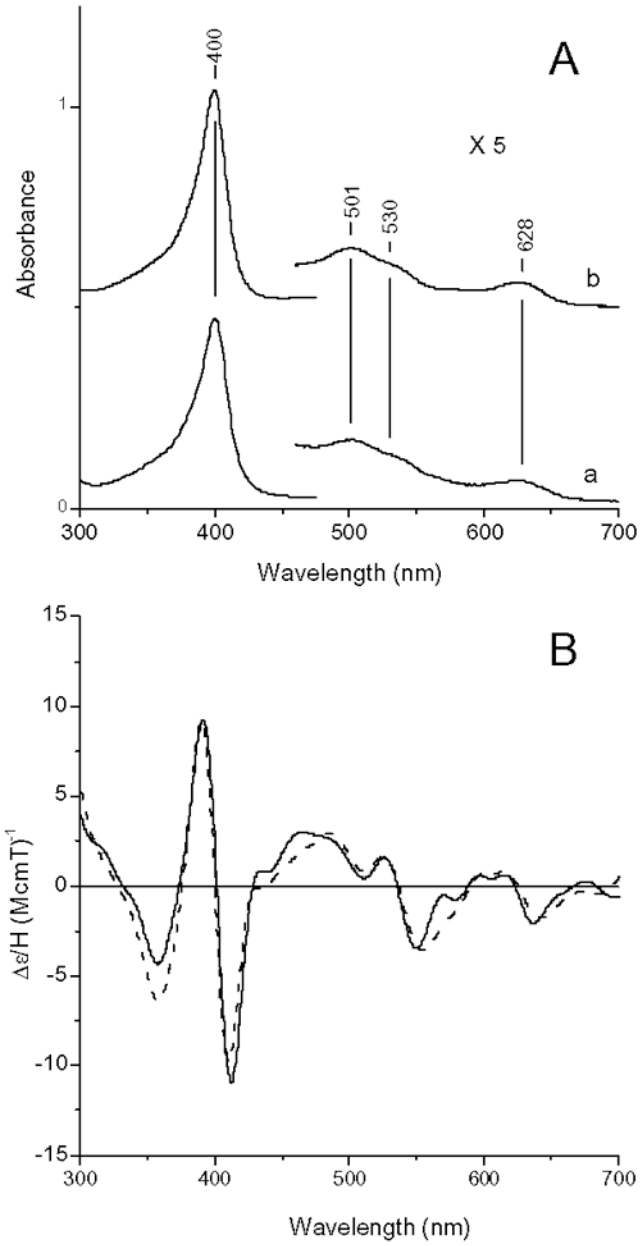

Figure 7.

UV-Vis (A) spectra of ferric Mb in 9 M urea at pH 2.0 (a) and a calculated spectrum for a 50/50 mixture of Mb in 8 M CH_3COOH at pH 1.8 (which is assigned in the paper as bis(CH_3COOH) complex of free heme) and Mb in 10 mM potassium phosphate buffer at pH 2.0 (b); the 460–700 nm region has been expanded by a factor of five; spectrum b has been shifted along the ordinate axis by 0.5 absorbance unit to allow better visualization. (B) experimental MCD of ferric Mb in 9 M urea at pH 2.0 (—) and a calculated MCD spectrum of a 50/50 mixture of Mb in 8 M CH_3COOH at pH 1.8 and Mb in 10 mM potassium phosphate buffer at pH 2.0 (------).