Interactions of apomyoglobin with membranes: Mechanisms and effects on heme uptake

GRÉGORY VERNIER,^{1,2} ALEXANDRE CHENAL,^{1,3} HEIDI VITRAC,
ROYA BARUMANDZADHE, CAROLINE MONTAGNER, AND VINCENT FORGE

Laboratoire de Biophysique Moléculaire et Cellulaire, Unité Mixte de Recherche 5090, Département Réponse et Dynamique Cellulaires, CEA-Grenoble, 38054 Grenoble cedex 9, France

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Abstract

The last step of the folding reaction of myoglobin is the incorporation of a prosthetic group. In cells, myoglobin is soluble, while heme resides in the mitochondrial membrane. We report here an exhaustive study of the interactions of apomyoglobin with lipid vesicles. We show that apomyoglobin interacts with large unilamellar vesicles under acidic conditions, and that this requires the presence of negatively charged phospholipids. The pH dependence of apomyoglobin interactions with membranes is a two-step process, and involves a partially folded state stabilized at acidic pH. An evident role for the interaction of apomyoglobin with lipid bilayers would be to facilitate the uptake of heme from the outer mitochondrial membrane. However, heme binding to apomyoglobin is observed at neutral pH when the protein remains in solution, and slows down as the pH becomes more favorable to membrane interactions. The effective incorporation of soluble heme into apomyoglobin at neutral pH suggests that the interaction of apomyoglobin with membranes is not necessary for the heme uptake from the lipid bilayer. In vivo, however, the ability of apomyoglobin to interact with membrane may facilitate its localization in the vicinity of the mitochondrial membranes, and so may increase the yield of heme uptake. Moreover, the behavior of apomyoglobin in the presence of membranes shows striking similarities with that of other proteins with a globin fold. This suggests that the globin fold is well adapted for soluble proteins whose functions require interactions with membranes.

Keywords: apomyoglobin; protein/membrane interactions; large unilamellar vesicles; amphitropic proteins; globin fold; heme uptake; protein folding

¹These authors contributed equally.

Present addresses: ²Harvard Medical School, Department of Microbiology and Molecular Genetics, 200 Longwood Avenue, Boston, MA 02115, USA. ³Biochemistry of Macromolecular Interactions Unit, Pasteur Institute, 75724 Paris Cedex 15, France.

Reprint requests to: Vincent Forge, Laboratoire de Biophysique Moléculaire et Cellulaire, Unité Mixte de Recherche 5090, Département Réponse et Dynamique Cellulaires, CEA-Grenoble, 17 Rue des Martyrs, 38054 Grenoble cedex 9, France; e-mail: vincent.forge@cea.fr; fax: 04-38-78-54-87.

Abbreviations: aMb: sperm whale apomyoglobin; dT: diphtheria toxin translocation domain; LUVs: large unilamellar vesicles; ⁻LUVs: anionic LUVs; [±]LUVs: zwitterionic LUVs; EPC: egg phosphatidylcholine; EPA: egg phosphatidic acid; Trp: tryptophan.

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Hemoglobin and myoglobin are heme proteins in charge of the storage and delivery of oxygen (O₂). Hemoglobin carries oxygen in red blood cells, and myoglobin transports it in the cytosol of O₂-consuming myocytes, such as those of cardiac muscles and red skeletal muscle fibers (Wittenberg and Wittenberg 2003). The physiological process of O₂ transport requires the reversible binding of O₂ molecules to the prosthetic ferrous heme group embedded within myoglobin. The insertion of heme within apomyoglobin (aMb) is necessary for the folding into the functional holomyoglobin (hMb) form.

While aMb is produced in the sarcoplasm, the heme is synthesized in the inner mitochondrial membrane (Ferreira 1999; Dailey 2002). Heme partition between

water and lipids is strongly lipophilic, with a partition constant of around 10^5 – 10^6 (Light 3rd and Olson 1990). Hence, considering that aMb and the prosthetic group are synthesized in different cell compartments, and that the protein is hydrophilic while heme is lipophilic, the location and circumstances of heme binding to aMb remain open questions. Previous reports have provided in vitro evidence that the presence of hemoglobin and aMb induces heme efflux from the membrane (Rose and Olson 1983; Cannon et al. 1984; Rose et al. 1985; Szebeni et al. 1985, 1988; Light 3rd and Olson 1990). The interactions of aMb with membranes (Lee and Kim 1992; Basova et al. 2004) suggest that the heme binding within the hydrophobic pocket of aMb requires the interaction of the protein with the mitochondrial outer membrane. However, clear evidence for such mechanisms is still missing. According to one report, the difference in the affinity of heme for the lipid bilayer and the protein allows heme transfer (Rose et al. 1985). The involvement of a chaperone has also been proposed (Jakoby 1978).

Sperm whale myoglobin is a 153-amino-acid protein containing eight α -helices (A to H) (Fig. 1) (Griko et al. 1988). This is the most studied and best known member of the globin family, which is characterized by a structure comprising a bundle of six to 10 α -helices (Holm and Sander 1993a,b). The globin fold is found in numerous proteins driven to interact with membranes for their functions. It can be recognized as an autonomous part of bacterial toxins, within domains specialized in the interactions with membranes. Well-documented examples are pore-forming domains of colicins (Parker et al. 1989; Holm and Sander 1993a,b) and the translocation domain of diphtheria toxin (Choe et al. 1992; Bennett et al. 1994). The globin fold is found also in the proteins of the Bcl-2 family, which are involved in the regulation of the apoptosis (Fesik 2000). Despite a lower stability, the

apo-form of myoglobin possesses a structure close to that of the holo-form (Loh et al. 1995; Eliezer and Wright 1996; Jamin and Baldwin 1998). The heme's largest effect on myoglobin structure is on the stability of the F α -helix, which is much more flexible in the apo-form (Bernad et al. 2004). The folding reaction of aMb in solution has been studied in detail (Griko et al. 1988; Hughson et al. 1990; Barrick and Baldwin 1993; Kataoka et al. 1995; Gulotta et al. 2003; Jamin 2005). A partially folded state is detected during the pH-induced unfolding of aMb (Griko et al. 1988). At pH 6, the protein is folded and contains both secondary and tertiary structures, and it is unfolded at pH 2. The partially folded state, which contains secondary structures but no stable tertiary structure, is stabilized at pH 4. This state possesses the characteristics of the so-called molten globule state (Hughson et al. 1990). In this state, the α -helices A, G, and H are already more stable than the others (Eliezer et al. 1998). The formation of a molten globule state is central in the membrane interactions of proteins with a globin fold. The stabilization of this partially folded state makes the hydrophobic regions of the protein accessible, and diminishes the energetic cost of the topology changes required for the interaction (van der Goot et al. 1991; Johnson and Cornell 1999; Chenal et al. 2002).

In the present work, we aimed to determine whether aMb interacts with membranes to extract heme from the lipid bilayer. We investigated the interaction of aMb with large unilamellar vesicles (LUVs), and measured the influence of aMb membrane binding on the rate of heme uptake. Our results show that all conditions favoring interactions of aMb with membrane destabilize its structure and decelerate the rate of heme binding. This suggests that, in cells, membrane interaction is not necessary for the final step of the myoglobin folding step, i.e., for holomyoglobin formation. The extraction of heme from the membrane is rather controlled by the greater affinity of the heme for myoglobin than for the lipid bilayer.

Figure 1. Ribbon representation of aMb. The α -helices are marked with letters from A to H. The side chains of Trp7 and Trp14 located within the α -helix A are shown in yellow.

Results

Myoglobin contains two tryptophans, which are both located in the N-terminal α -helix (α A) (Fig. 1). The maximum emission wavelength of the intrinsic fluorescence spectrum ($\lambda_{\max}$), which is sensitive to the tryptophan (Trp) environment, has been used to monitor the pH-induced conformational changes and partitioning of aMb between buffer and LUVs. LUVs have been preferred to SUVs as models of membranes because they are more stable, and their curvature is closer to that of the membranes in cells (Lepore et al. 1992; Epand 1998; Heerklotz and Epand 2001; Bigay et al. 2003; Yoshida et al. 2004; Chenal et al. 2005). Figure 2 shows the pH dependence of $\lambda_{\max}$ for the protein in solution (triangles)

Figure 2. Changes in the maximum emission wavelength ($\lambda_{\max}$) of the fluorescence emission spectrum as a function of the pH. aMb (1 μ M) in the absence (triangles) or presence (circles) of $\neg$ LUVs (300 μ M). Both experiments were performed in the presence of 30 mM NaCl.

and in the presence of anionic LUVs ($\neg$ LUVs) (circles). In solution, we obtained the $\lambda_{\max}$ values reported in the literature (Kirby and Steiner 1970; Postnikova et al. 1991; Jamin and Baldwin 1998; Gulotta et al. 2003). At neutral pH, the protein is in the native state, and $\lambda_{\max}$ is around 330 nm, showing that the Trps located in the helix A are within a hydrophobic environment. As the pH decreases, $\lambda_{\max}$ is shifted toward higher values. A first transition is observed between pH 7 and pH 4, leading to a value of $\lambda_{\max}$ equal to 334 nm. During this first transition, the Trps become slightly more exposed to the solvent. The protein is then in the partially folded state at pH 4. This state, which has been extensively studied (Griko et al. 1988; Hughson et al. 1990; Barrick and Baldwin 1993; Kataoka et al. 1995; Eliezer et al. 1998, 2000; Kay and Baldwin 1998; Gulotta et al. 2003), contains native-like secondary structures but lacks tertiary structure. A second transition is observed between pH 4 and pH 2. It is related to the stabilization of the unfolded state of the protein at acidic pH (Griko et al. 1988). Then, $\lambda_{\max}$ is around 344 nm, showing that the Trps are much more accessible to the solvent.

This pH dependence is quite different in the presence of $\neg$ LUVs (Fig. 2, circles). These experiments were carried out with a lipid/aMb ratio (L/P) equal to 300. The pH-dependencies were similar in solution and in the presence of LUVs (between pH 7 and pH 5.5), and diverged as the pH increased further. In the presence of $\neg$ LUVs, the decrease in $\lambda_{\max}$ below pH 5 indicates that the Trps enter a more hydrophobic environment. The lowest value of $\lambda_{\max}$ (327 nm) was observed for pH values around 3.7. An increase was observed for more acidic pH. The latter transition is due to the initiation of the release of protein from the membrane, which is also observed when the binding is monitored by centrifugation (data not shown). This release is correlated with the protonation of the head groups of egg phosphatidic acid (EPA) ($pK_a = 2.9$), leading to the disappearance of the electrical charge on the surface of the membrane. Besides $\lambda_{\max}$ measurements, the ratio between the fluorescence intensities at 325 nm and 365 nm (F_{325}/F_{365}) was used to

monitor the fluorescence changes. Figure 3A (open circles) shows the pH dependence of the F_{325}/F_{365} ratio in the presence of $\neg$ LUVs at low ionic strength, the same conditions as in Figure 2. Both $\lambda_{\max}$ and F_{325}/F_{365} ratio measurements display similar pH-dependence, with a large change between pH 5.2 and pH 3.5 in the presence of $\neg$ LUVs (Fig. 3A, open circles). The changes recorded with aMb in solution (Fig. 3A, open triangles), and in the presence of $\neg$ LUVs (Fig. 3A, open circles), seem to be superimposed for pHs ranging from 7.2 to 5.5.

In order to highlight the contribution of the electrostatic interactions to each step of the interactions between aMb and $\neg$ LUVs, we compared the pH dependence in the presence of 30 and 150 mM NaCl. Increase in ionic strength inhibits the electrostatic interactions and the penetration of Trps in a hydrophobic environment that occurs between pH 4.8 and pH 3 (Fig. 3A). To quantify the effect of the ionic strength, the pH dependence of the

Figure 3. Effects of charge and ionic strength on the interaction of aMb with LUVs as a function of pH. All experiments were carried out with a protein concentration equal to 1 μ M, in the absence of membrane (triangles), in the presence of $\neg$ LUVs (circles), or in the presence of $\pm$ LUVs (EPC only) (squares). Two ionic strengths were used: 30 mM NaCl (open symbols) and 150 mM NaCl (closed symbols). (A) Fluorescence intensity ratio (F_{320}/F_{365}). The data obtained in the presence of LUVs (L/P is 300) were fitted using the Hill equation (continuous lines). (B) Partitioning experiments monitored by centrifugation (L/P = 300). The continuous lines are the best fits to the Hill equation. (C) Observed rate constants (k_{perfo}) of LUV permeabilization by aMb. L/P ratios were equal to 350 and 400 for anionic and zwitterionic LUVs, respectively. The rate constants were normalized to the L/P ratio. To facilitate the comparisons between the various pH dependences, the beginning of the binding of aMb to $\neg$ LUVs monitored by centrifugation is marked by aligned arrows in B and C, and that of the conformation change detected by fluorescence by aligned arrows in A and C.

largest fluorescence change in the presence of lipid vesicles was fitted using the Hill equation (see Materials and Methods). At low ionic strength, the pH of the half transition ($\text{pH}_{1/2}$) and the cooperativity (n_H) were 4.65 and 1.8, respectively. At high ionic strength, the values were 4.4 for $\text{pH}_{1/2}$ and 2.3 for n_H . According to the n_H values, at least two or three protons must bind cooperatively to induce the transition leading to the penetration of Trps in a hydrophobic environment. The shift of $\text{pH}_{1/2}$ in the presence of a high concentration of NaCl highlights the electrostatic contributions to aMb membrane binding. For both ionic strengths, the conformation change of aMb in the presence of LUVs is fully reversible. The fluorescence spectrum of the soluble protein is recovered upon a pH-jump back to pH 7 (data not shown).

The binding of aMb to LUVs was also monitored by centrifugation experiments (Fig. 3B). The Trp fluorescence at 330 nm of the solution before and after centrifugation was used to determine the amount of protein pelleted together with LUVs . At low salt concentration (Fig. 3B, open circles), partition measurements indicated that aMb was completely bound to LUVs below pH 5.5. The binding is quite cooperative and occurs between pH 6.5 and pH 5.5. At pH 7, no binding was detected for lipid/protein molar ratios up to 2500 (data not shown). When the partition experiments as a function of pH were done in the presence of zwitterionic LUVs ($\text{LUVs}^\pm$), only weak binding was detected around pH 3.5 (Fig. 3B, open squares). These results highlight the need for the electrostatic interactions between aMb and LUVs . Curve fitting of the data obtained in the presence of LUVs using the Hill equation gave the half transition pH ($\text{pH}_{1/2}$) as 5.85 and the Hill number (n_H) as 3.7. In the presence of 150 mM NaCl (Fig. 3B, closed circles), the pH dependence of the binding of aMb to LUVs was slightly shifted toward more acidic pH. Curve fitting indicated $\text{pH}_{1/2} = 5.7$ and $n_H = 2.6$ (Fig. 3B). Whatever the ionic strength of the solution, the binding of aMb to LUVs (Fig. 3B) occurs at more alkaline pH than the conformational change detected by fluorescence (Fig. 3A). At low ionic strength, for instance, the binding monitored by centrifugation occurs between pH 6.5 and pH 5.5, while the main fluorescence change is observed between pH 5.2 and pH 3.5. This is more obvious with the values of $\text{pH}_{1/2}$; there is a difference of more than one unit between the values obtained in the two experiments. Therefore, two steps can be distinguished in the interactions of aMb with LUVs : (1) membrane binding, and (2) penetration of the Trps in a hydrophobic environment. The cooperativity of the pH dependence ($n_H > 1$) indicates that the cooperative binding of at least two or three protons to aMb is required for both steps.

To evaluate the extent of destabilization of the lipid bilayer by aMb, we investigated the membrane perme-

abilization induced by aMb. This was monitored by the release of a fluorescent probe, pyranine, trapped inside the lipid vesicles. A quencher of pyranine fluorescence, DPX, was added to the buffer, and its effect was immediate. The fluorescence decay reflects the kinetics of pyranine release from LUVs . In experiments with anionic and zwitterionic LUVs , the L/P ratio was 350 and 400, respectively (Fig. 3C). The time dependence of pyranine release was fitted by a single exponential decay, and the rates given by the fitting procedure (k_{perfo}) were normalized to the L/P molar ratio. At neutral and alkaline pH, when the protein does not interact with the lipid vesicles according to the fluorescence and partition experiments, no release of the pyranine was observed. At low NaCl concentration (Fig. 3C, open circles), membrane permeabilization was detected at pH values lower than 6, and the release rate increased as the pH decreased. At pH 5.5, i.e., the first pH at which membrane permeabilization was observed, the binding of aMb to LUVs was almost complete, while the second transition monitored by fluorescence had just started (Fig. 3, open circles). To facilitate comparisons between pH dependences, the beginning of the binding of aMb to LUVs monitored by centrifugation is marked by aligned arrows in Figure 3B and C, and the start of the conformation change detected by fluorescence by aligned arrows in Figure 3A and C. The start of the conformation change and the end of the first transition are roughly concomitant. Membrane permeabilization seems more linked to the second step of the interaction, i.e., the penetration of the Trp within a hydrophobic environment. This was confirmed in the presence of a high NaCl concentration. The onset of membrane permeabilization was shifted toward more acidic pH and started to be detectable from pH 5.3. At this pH, the binding monitored by centrifugation was complete, while the penetration of the Trps within a hydrophobic environment was ready to begin (Fig. 3, closed circles). Slight permeabilization was detected in the case of the $\text{LUVs}^\pm$ (Fig. 3C, open squares), and was related to the partial binding detected by centrifugation (Fig. 3B, open squares).

The L/P ratio required for membrane permeabilization by aMb ($\text{L/P} \cong 300$) was much lower than that commonly used in the case of bacterial toxins ($\text{L/P} \cong 3000$) (Chenal et al. 2002), indicating that more protein is necessary. The mechanism underlying membrane permeabilization by aMb may be either pore formation or lipid vesicle fragmentation (or micellization). The latter has been described by Lee and Kim (1992), whose electron microscopy experiments showed that micelles accumulate and form large complexes upon the addition of aMb to LUVs . This micellization can also induce the release of pyranine. Vesicle fragmentation decreases light scattering, which can be monitored (Lee and Kim 1992). The

translocation domain (dT) of diphtheria toxin was used for the control experiment (Fig. 4A). In conditions known to induce permeabilization of $\neg$ LUVs by dT (L/P = 3000; 5 mM citrate buffer; pH 5; no NaCl) (Chenal et al. 2002), light scattering remained constant, showing that LUV permeabilization does not occur via a lysis mechanism. In these conditions, the normalized rate was $2 \times 10^3 \text{ sec}^{-1}$ (Chenal et al. 2002), i.e., three orders of magnitude larger than the value observed for aMb (Fig. 3C). In the case of aMb, the protein was not bound to $\neg$ LUVs at neutral pH (Fig. 3B, circles), so no permeabilization occurred and the light scattering was unchanged, while addition of aMb at pH 5 reduced the scattered light intensity (Fig. 4A). The pH dependence of the rate of decay of scattered light was similar to that of the pyranine release (Figs. 3C, 4B). At a given pH, however, the rate was three times slower in the light scattering experiments. This suggests that the lipid bilayer is destabilized upon binding of aMb (leading to the LUV permeabilization), and micellization then occurs.

We used far-UV circular dichroism to probe the secondary structure changes involved in the interaction (Fig. 5). At pH 7, the spectrum of the native state in solution (Fig. 5A, dashed line) has minima around 222 nm and 208 nm, which are typical of a protein with an α -helix content. In the presence of $\neg$ LUVs (Fig. 5A, continuous line), the spectrum was identical to the previous one, as expected, since the protein was not bound to LUVs in these conditions. At pH 5.5, the spectrum of the soluble form (Fig. 5B, dashed line) was still similar to that of the

Figure 4. Light scattering changes. (A) Light scattering was monitored by the ratio between the light intensity measured as a function of time and that before the addition of the protein (I/I_0). Shown are the kinetics with aMb (1 μM ; L/P = 350) in the presence of 30 mM NaCl at pH 7 and pH 5, and with the translocation domain of the diphtheria toxin (100 nM; L/P = 3000) at pH 5. (B) Observed rate constants of light scattering changes (k_{cat}) as a function of pH.

Figure 5. Far-UV CD spectra of aMb in the presence of $\neg$ LUVs at various pHs. (Dashed lines) aMb (2.5 μM) in solution. (Continuous lines) aMb (2.5 μM) in the presence of $\neg$ LUVs (L/P = 300). (A) pH 7; (B) pH 5.5; (C) pH 4.

native state at pH 7. No significant change was observed in the spectrum recorded in the presence of $\neg$ LUVs (Fig. 5B, continuous line), despite a slight increase in intensity. At pH 4, small changes were observed in the spectrum of the protein in solution (Fig. 5C, dashed line). The minimum at 208 nm was slightly more pronounced than that at 222 nm. As reported in the literature, this effect might reflect a small decrease in helical content due to stabilization of a partially folded state of aMb at pH 4 (Griko et al. 1988; Hughson et al. 1990; Barrick and Baldwin 1993; Kataoka et al. 1995; Eliezer et al. 1998, 2000; Kay and Baldwin 1998). However, the helical content of the protein at pH 4 remains high and close to that of the native state. In the presence of $\neg$ LUVs (Fig. 5C, continuous line), the helical content increases slightly upon the binding of the protein to the membrane.

The results described above reveal that the aMb–membrane interaction is pH-dependent. In order to determine whether the membrane interaction is involved in heme uptake, we studied and compared the rate of heme incorporation at several pHs in the presence and absence of membrane. The combination of the heme with aMb, to form the holo-form of myoglobin, induces an enhancement of the Soret band at 407 nm of the heme absorbance spectrum (Fig. 6A). This effect can be used to monitor the extraction of the heme from the lipid bilayer by aMb. The absorbance spectrum of heme trapped in the lipid bilayer (Fig. 6A, 0 s) is close to that in solution (not shown). In our conditions, about 80% of the heme was bound to $\neg$ LUVs at pH 7 and 100% for pHs lower than 6 (Fig. 6B, closed circles). The binding of heme to aMb was

Figure 6. (A) The effect of the binding to aMb on the absorbance spectrum of heme is illustrated with absorbance spectra recorded during the heme uptake at pH 5.6 in the presence of LUVs. The spectrum at “0 s” corresponds to the heme incorporated in LUVs in a preliminary step. The intensity of the Soret band at 407 nm increased upon uptake by myoglobin (spectrum at “49 s”). (B) Amount of heme (closed circles) bound to LUVs as a function of pH (lipid/heme = 40) monitored by centrifugation. Binding of heme (open circles) to aMb (3 μ M) in the presence of $\neg$ LUVs (aMb/heme = 1 and lipid/aMb = 40) monitored by the variation in the absorbance at 407 nm. Binding of heme (open triangles) to aMb (3 μ M) in the absence of $\neg$ LUVs (aMb/heme = 1). The arrows indicate the axis corresponding to the dependences.

observed up to pH 5 and vanished at pH 4, either in the absence or presence of $\neg$ LUVs (Fig. 6B, open symbols). The presence of membrane had no significant effect on the pH dependence of the amount of heme binding. For the experiments in the presence of $\neg$ LUVs, the heme to aMb concentration ratio was 1. Therefore, even at pH 7, when about 20% of heme remains in solution, most of the heme bound to myoglobin was trapped in the lipid bilayer before binding. This is also true for the kinetic experiments described below.

The trapping of heme in the membrane has no effect on the amount of holomyoglobin formed. To investigate a possible effect on the mechanism of heme uptake, we monitored the kinetics of binding in the presence and absence of $\neg$ LUVs (Fig. 7). The pH range of the investigation was 7–4.4 in the presence of $\neg$ LUVs, and 7–5.5 in the absence of membrane. Difficulties in heme handling impair the experiments at lower pH in the absence of $\neg$ LUVs. Examples of heme binding kinetics are shown in Figure 7A, and the pH dependence of the binding rate is illustrated in Figure 7B. Within the pH range explored in solution, heme binding is quite fast (finished within 20 sec) (Fig. 7A, open circles) and weakly sensitive to pH (Fig. 7B, open circles). The kinetics are more sensitive to pH changes in the presence of $\neg$ LUVs (Fig. 7, closed circles). Between pH 7 and pH 6, the heme binding rates

were similar in the presence and absence of membranes (Fig. 7B). Below pH 6, the binding became significantly slower in the presence of $\neg$ LUVs (Fig. 7B). This suggests that the binding of aMb to $\neg$ LUVs, which starts around pH 6 (Fig. 3B, open circles), decelerates the heme uptake. Up to pH 4.5, the reaction further slows down as the interaction of aMb with the lipid bilayer becomes tighter (Fig. 7B, closed circles). The pH dependence of the rate seems to plateau around pH 5, which is the pH intermediate between the two steps of the interaction of aMb with $\neg$ LUVs described earlier (Figs. 3A,B). This suggests that a first deceleration of the heme uptake occurs upon the binding of aMb to $\neg$ LUVs (Figs. 3B,7B), and that the conformation change, which leads to the burying of the Trps in a highly hydrophobic environment (Figs. 2,3A), further slows down the heme uptake (Fig. 7B).

Discussion

We report here an exhaustive study of the interactions of apomyoglobin (aMb) with lipid vesicles. We show that aMb interacts with $\neg$ LUVs under acidic conditions, and that the presence of negatively charged phospholipids is required. Our results differ from those reported by Basova and colleagues (2004). They have shown that aMb interacts with lipid bilayers at neutral pH. The

Figure 7. (A) Kinetics of heme uptake by apomyoglobin. The conditions are the same as in Figure 6. Kinetics (open circles) in the absence of LUVs: The fastest kinetics were recorded at pH 6.5 and the slowest at pH 5.5. Kinetics (closed circles) in the presence of $\neg$ LUVs: From the fastest to the slowest kinetics, the pHs are 6.5, 6.0, 5.8, 5.6, and 4.4. The continuous lines correspond to the best fits of a single exponential decay. (B) pH dependences of the rate of the heme uptake in the absence of LUVs (open circles) and in the presence of $\neg$ LUVs.

discrepancy probably arises from the use by these authors of SUV, sonicated lipid vesicles, which are characterized by a higher curvature, favoring hydrophobic effects within the solvent-membrane interface (Lepore et al. 1992; Heerklotz and Eppand 2001; Chenal et al. 2005). This difference in behavior of aMb in the presence of lipid vesicles of various curvatures highlights that protein membrane interactions are governed by subtle electrostatic and hydrophobic effects.

Two steps can be distinguished in the pH dependence of the interactions. The first step is monitored by partition experiments, and can be related to the binding of the protein to the lipid bilayer (Fig. 3B). The second step is associated with the penetration of the Trps into a more hydrophobic environment (Figs. 2,3A), and to the permeabilization of the lipid vesicles (Fig. 3C). These observations suggest tighter interactions between the protein and the membrane upon this second step. According to the work of Lee and Kim (1992), in conditions similar to those used here, the membrane permeabilization is due to the fragmentation of the lipid vesicles and the formation of large complexes. In these complexes the lipids are still organized in a bilayer (Lee and Kim 1992). In the presence of $\bar{\text{LUVs}}$, the secondary structure of aMb remains native-like over the whole pH range favorable to the binding (Fig. 5). The parallel between the stabilization in solution of the partially folded state at pH 4 and the penetration of the Trps within in a hydrophobic environment suggests that the loss of the tertiary structure of the protein is necessary at that stage of the membrane interaction. The stabilization of such a state, with native-like secondary structure but without tertiary structure, is generally related to the interaction of amphitropic proteins with membranes (van der Goot 1991; Zhan et al. 1994, 1995; Zakharov et al. 1998; Chenal et al. 2002). In the present case, however, we have no direct evidence for the destabilization of the tertiary structure; the weak amplitude of the near-UV CD spectrum of apomyoglobin and the scattering due to the lipid vesicles impede monitoring of the tertiary structure.

Apomyoglobin shares some characteristics of amphitropic proteins with a globin fold, such as bacterial toxin domains (Holm and Sander 1993a,b). Its interactions with lipid bilayers are pH-sensitive and require the presence of anionic phospholipids (Parker and Feil 2005). Moreover, they are linked to the stabilization of a partially folded state, which has the characteristics of a molten globule state. More particularly, the pH dependence of the interactions of aMb with $\bar{\text{LUVs}}$ (Fig. 3) presents striking similarities with that of the translocation domain of diphtheria toxin (Chenal et al. 2002). For both proteins, two steps can be distinguished. The first corresponds to the binding to the lipid bilayer, and the second to the membrane permeabilization. In both cases, the second step is

related to the penetration of Trps within a hydrophobic environment. Concerning the translocation domain of diphtheria toxin, it has been proposed that this movement of the Trps is due to a movement of the amphiphilic N-terminal α -helices within the membrane interface (Chenal et al. 2002). As the pH decreases, the acidic side chains become protonated and lose their negative charges. Then, at acidic pH, the interaction of this amphiphilic α -helix with the lipid bilayer results from electrostatic attractions between basic side chains and anionic headgroups of phospholipids, and from hydrophobic interactions (Chenal et al. 2002). As a consequence, the hydrophobic face of the helix, where one of the two Trps of the domain is located, penetrates deeper into the hydrophobic core of the lipid bilayer. A similar behavior can be proposed for the N-terminal α -helix of aMb, which contains the two Trps of the protein. This helix also contains three glutamates, which can be protonated at acidic pH and lose their charge. Then, the helix can penetrate the hydrophobic core of the membrane, together with the Trps. Hence, a similar behavior is found for two proteins of different functions. This highlights how the pH can regulate the position, within the membrane interface, of amphiphilic α -helices of amphitropic proteins in a way similar to that described for antibacterial peptides (Falnes et al. 1992; Leenhouts et al. 1995; Liu and Deber 1997). This effect of the pH on amphitropic proteins is probably essential for the regulation of their function. Overall, these results also indicate that aMb behaves like other proteins of the globin fold family. Although these proteins have evolved toward divergent functions, they have preserved an ancestral intrinsic propensity to interact with membrane, suggesting that the globin fold is a structural platform well adapted to functions requiring an amphitropic character.

An evident role for the interaction of aMb with lipid bilayers would be to facilitate the uptake of heme from the outer mitochondrial membrane. However, heme binding to aMb is observed at neutral pH when the protein remains in solution, and slows down as the pH becomes more favorable to membrane interactions (Fig. 7). At neutral pH, the heme initially incorporated within the membranes of LUVs is transferred to myoglobin within a few seconds. This suggests that heme has a greater affinity for apomyoglobin than for the lipid bilayer. The heme uptake becomes slower for pHs between 6 and 5.5, when aMb starts to bind to $\bar{\text{LUVs}}$. A possible explanation for this effect is that the affinity of heme for the lipid bilayer becomes greater as the pH decreases. In that case, however, the decrease in the uptake rate should start earlier, because improved heme binding to LUVs is already observed between pH 7 and pH 6 (Fig. 6B), and, obviously, this is not the case (Fig. 7B). The effect of the LUVs on the heme uptake seems more related to the binding of aMb to the membranes. While the protein in

solution is mostly native around pH 6–5.5, the fact that its binding to the membrane slows down the heme uptake raises the question of the structure of the membrane-bound protein. There are two possibilities upon binding of the protein to heme-loaded LUVs: (1) the protein remains in a mostly native-like state, but the heme binding site is not available, or (2) membrane interaction induces unfolding of aMb, and, consequently, the heme binding site is destabilized. The fact that no change is detected by spectroscopy (fluorescence and far-UV CD) suggests that the first possibility is the right one. However, the second possibility cannot be completely excluded. At this stage, we can only conclude that heme binds to aMb in solution.

At least *in vitro*, the interaction of aMb with the membrane is not necessary for heme uptake, and in fact, it decelerates the process. *In vivo*, however, it is possible that, within the crowded cytoplasm of a cell (van den Berg et al. 2000), the interaction with mitochondria membranes increases the probability of heme uptake by aMb. Hence, the amphitropic character would drive aMb in the vicinity of the membrane, favoring its folding reaction. However, the amphitropic character of myoglobin seems necessary to accomplish at least one of its physiological functions, the release by the holo-form of O₂ to the outer mitochondrial membrane.

Materials and methods

Protein expression and purification

A synthetic gene for sperm whale apomyoglobin (aMb) was expressed and purified as described (Weisbuch et al. 2005). Protein concentration was determined by absorbance in 6 M guanidinium chloride using $\epsilon_{280\text{ nm}} = 15,200 \text{ M}^{-1}\cdot\text{cm}^{-1}$ and $\epsilon_{288\text{ nm}} = 10,800 \text{ M}^{-1}\cdot\text{cm}^{-1}$. The recombinant T domain of diphtheria toxin (dT) was expressed and purified as described previously (Chenal et al. 2002).

Preparation of large unilamellar vesicles (LUVs)

Vesicles were prepared at a concentration of 20 mM in 4 mM citrate buffer either with 30 mM NaCl or 150 mM NaCl by reverse phase evaporation. Anionic LUVs (LUVs) were constituted from egg phosphatidic acid (EPA) and egg phosphatidyl choline (EPC) (Avanti Polar Lipids) at 1:9 molar ratio. Zwitterionic LUVs (LUVs) were prepared from EPC only. The monodispersity and size of LUVs (mean hydrodynamic diameter = 150 nm) were checked by a Zetasizer 3000 instrument (10 mV HeNe laser at 632.8 nm) (Malvern Instruments).

Buffers

All experiments were done in 4 mM citrate buffer and either 30 mM NaCl or 150 mM NaCl at 22°C. The protein was kept in pure water and was diluted in a range of buffers of various pHs. The pH of diluted protein was checked after spectroscopic measurements.

Fluorescence spectroscopy

Fluorescence was measured using a FP-6500 spectrofluorimeter (Jasco). The measurements were made in a thermostated cell holder, using a 1-cm pathlength and 3-mL quartz cell. The bandwidth was 3 nm for both excitation and emission beams. The excitation wavelength was fixed at 280 nm. Maximal emission wavelength (λ_{max}) and fluorescence intensity ratio at 325 and 365 nm (F_{325}/F_{365}) were obtained by averaging three scans collected over a 300–450 nm range using 1-nm steps and a scan rate of $100 \text{ nm} \cdot \text{min}^{-1}$. Samples were constantly stirred. Background spectra were collected using the same buffer and lipid concentration. The protein concentration was 1 μM and the lipid/protein molar ratio (L/P) was equal to 300, unless stated otherwise.

Partitioning of aMb to LUVs monitored by centrifugation

The ratio L/P was equal to 300 and the aMb concentration 1 μM . Trp fluorescence was used as the probe of protein concentration. Vesicles were prepared with 0.5% of NBD-phosphatidyl ethanolamine (NBD-PE) (N-360, Molecular Probes). LUVs and proteins were incubated in 5 mL for 2 h at room temperature. Samples were centrifuged in a Beckman L-70 ultracentrifuge using a Ti 70.1 rotor at 4°C for 1.5 h with a speed of 62,000 rpm (265,000g). The efficiency of LUV pelleting was checked by measuring the NBD-PE fluorescence (excitation wavelength: 464 nm, emission: 500–600 nm) before and after centrifugation (supernatant). All experiments were also done without LUVs to check the sedimentation of the soluble aMb. The fraction of partitioned or pelleted aMb (f_{pel}) was determined as follows: $f_{\text{pel}} = (F_0 - F)/F_0$, where F_0 and F are the fluorescence intensities of aMb at 330 nm before and after centrifugation, respectively.

Curve fitting

The pH dependences of the interactions of aMb with LUVs, monitored by either fluorescence (F_{325}/F_{365}) or centrifugation, were fitted using the Hill equation as follows: $P = P_i + (P_f - P_i)/(1 + (K/H)^{n_H})$, where P is the measured parameter (F_{325}/F_{365} or f_{pel}), P_f and P_i are the final and initial values, respectively, H is the proton concentration ($H = 10^{-\text{pH}}$), K is the dissociation constant or $\text{pH}_{1/2}$, and n_H is the Hill coefficient. In the case of the fluorescence measurements, only the data within the pH range of the largest changes were considered for the fit (pH 5.5–3).

Lipid vesicle permeabilization assay

Experiments were performed as described previously (Chenal et al. 2002). The final concentration of aMb was 1 μM . In order to get an optimal signal (magnitude and experiment time) with lipid-saturated conditions, the L/P ratio was equal to either 350 for anionic LUVs or 400 for zwitterionic LUVs. The observed rate constants (k_{perfo}) were normalized to the L/P molar ratio.

Light scattering experiments

The experiments were carried out with anionic LUVs only. The aMb concentration was 1 μM in citrate buffer containing 30 mM

or 150 mM NaCl. Control experiments with diphtheria toxin translocation domain used the same conditions as Chenal et al. (2002), i.e., 5 mM citrate buffer and without NaCl. Both excitation and emission wavelengths were set to 600 nm and the sample was continuously stirred. Kinetics were initiated by addition of the protein to the LUVs solution. The final concentrations were either 1 μ M of aMb with L/P = 350 or 100 nM of diphtheria toxin domain with L/P = 3000. The observed rate constants (k_{scat}) were normalized to the L/P molar ratio.

Circular dichroism spectropolarimetry

CD experiments were performed on a JASCO J-810 spectropolarimeter (Jasco). An average of 30 scans was recorded in the far-UV between 190 and 250 nm at 50 nm $\cdot$ min⁻¹, using 1 nm resolution steps and a bandwidth of 4 nm. The pathlength was 1 mm. CD spectra were recorded for aMb in solution and in the presence of LUVs. The protein concentration was 2.5 μ M in both experiments and the L/P ratio was equal to 300. A low concentration of protein was used in order to minimize LUV-induced light scattering. All spectra were baseline-corrected and then the Savitzky-Golay smoothing algorithm was used with a window width of five values in the Jasco spectra analysis software.

Partitioning of heme to LUVs monitored by centrifugation

The ratio LUV/heme was equal to 40 and the heme concentration 3 μ M. Vesicles were prepared and incubated in 4 mL for 30 min with heme at room temperature. Samples were centrifuged in a Beckman L-70 ultracentrifuge using a Ti 70.1 rotor for 1.5 h at a speed of 62,000 rpm (265,000g). Absorbance spectra (300–500 nm) were recorded before and after centrifugation (supernatant) with a Hewlett-Packard 8453 photodiode array instrument. Control experiments were also done without heme in order to take account of vesicle diffusion (before centrifugation). The fraction of heme bound to vesicles (f_{bh}) was determined as follows: $f_{\text{bh}} = (A_0 - A)/A_0$, where A_0 and A are the absorbencies of heme at 408 nm before and after centrifugation, respectively.

Heme uptake kinetics

The protocol used for these experiments was adapted from that of Olson and colleagues (Rose and Olson 1983; Rose et al. 1985). Heme was solubilized at a concentration of 400 μ M in 10 mM NaOH. LUVs (120 μ M) were loaded with heme at a final concentration of 3 μ M (lipid/heme = 40). The heme uptake kinetics were initiated by adding aMb (3 μ M) (aMb/heme = 1 and lipid/aMb = 40). Absorbance spectra (300–500 nm) were recorded during the kinetics of heme uptake with a Hewlett-Packard 8453 photodiode array instrument. Control experiments of heme uptake by aMb in the absence of vesicles were performed using the same concentrations (3 μ M aMb/3 μ M heme). In order to avoid heme precipitation at acidic pH, heme uptake kinetics in solution were initiated by adding a small volume of heme solution at alkaline pH to a solution of aMb at a pH adjusted in order to get the right pH upon mixing. Absorbance spectra were then recorded as described above. A baseline correction was applied to the spectra in order to take into account the light scattering due to the lipid vesicles. The

aMb/heme ratio used was always equal to 1, and we checked by chromatography that heme was fully bound to myoglobin at pH 7.

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