Lactoperoxidase folding and catalysis relies on the stabilization of the α-helix rich core domain: A thermal unfolding study

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Abstract

Lactoperoxidase (LPO) belongs to the mammalian peroxidase family and catalyzes the oxidation of halides, pseudo-halides and a number of aromatic substrates at the expense of hydrogen peroxide. Despite the complex physiological role of LPO and its potential involvement in carcinogenic mechanisms, cystic fibrosis and inflammatory processes, little is known on the folding and structural stability of this protein. We have undertaken an investigation of the conformational dynamics and catalytic properties of LPO during thermal unfolding, using complementary biophysical techniques (differential scanning calorimetry, electron spin resonance, optical absorption, fluorescence and circular dichroism spectroscopies) together with biological activity assays. LPO is a particularly stable protein, capable of maintaining catalysis and structural integrity up to a high temperature, undergoing irreversible unfolding at 70 °C. We have observed that the first stages of the thermal denaturation involve a minor conformational change occurring at 40 °C, possibly at the level of the protein β-sheets, which nevertheless does not result in an unfolding transition. Only at higher temperature, the protein hydrophobic core, which is rich in α-helices, unfolds with concomitant disruption of the catalytic heme pocket and activity loss. Evidences concerning the stabilizing role of the disulfide bridges and the covalently bound heme cofactor are shown and discussed in the context of understanding the structural stability determinants in a relatively large protein.

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1. Introduction

Lactoperoxidase (LPO, E.C.1.11.1.7) is a member of the mammalian peroxidase family that includes also myeloperoxidase (MPO) and eosinophil peroxidase (EPO). These are redox heme enzymes that share a high structural and functional homology: they catalyze the oxidation of halides and pseudo-halides at the expense of hydrogen peroxide and generate products with a wide antimicrobial activity [1,2]. LPO is synthesized in the breast secretory epithelial cells [3] and is also found in the salivary and lachrymal glands of mammals and in their secretions, e.g. milk, saliva, tears [4]. It consists of a single polypeptide chain of 612 residues with a molecular mass of 78.5 kDa and a carbohydrate content of about 10% [5]. It contains 14 Cys residues: 12 out of 14 are involved in 6 disulfide bridges, whereas Cys58 and Cys284 display free sulfhydryl groups [6]. The heme group of mammalian peroxidases is protoporphyrin IX: it is covalently bound to the apo-protein through two ester bonds (involving a Glu and an Asp residue) and – in the case of MPO – a sulphonium linkage through a Met residue [7,8]. This feature plays a crucial role in determining the enzyme reactivity [9] and contributes to the structural and functional stability shown by this family of proteins [10].
X-ray structure of bovine LPO with a resolution of 2.3 Å has been recently deposited on the Protein Data Bank (entry 2GJ1). LPO is known to catalyze the oxidation of a wide range of organic substrates, such as phenols [11,12], catecholamines [13] [14–16], arylamines [17]. It has been reported to induce in vivo peroxidation of aromatic amines [18] and of polychlorinated phenyls [19], steroid hormones [20–22] and polycyclic aromatic hydrocarbons [23]. These substrates are regarded as important risk factors for breast cancer [18,19,21,24,25]. The involvement of LPO in breast carcinogenesis has been investigated by several authors: the LPO-catalyzed oxidation of β-estradiol and of its hydroxylated derivatives has been shown to cause redox cycling, oxidative stress, oxidation of glutathione and NADH together with production of superoxide and OH• radicals, responsible for DNA damage [18,20,21]. Activation of aromatic and heterocyclic amines to chemically reactive mutagenic species by LPO has also been suggested [26]. The LPO system has also found application as natural biopreservative in food, feed specialties, cosmetics [1] and in tumor therapy, in association with glucose oxidase [27,28].

One of the most significant aspects of the biological significance of LPO lies in its involvement in the natural host-defense system against bacterial infections [1]. For instance, LPO may replace thyroid peroxidase in the biosynthetic pathway of thyroid hormones [29]. In addition, evidence of the involvement of mammalian peroxidases (e.g. LPO and MPO) in the insurgence of inflammatory diseases have been reported [30,31]. Nitrite, at physiological or pathological levels, has been shown to be a substrate for both MPO and LPO: the formation of reactive nitrogen species (RNS) via peroxidase-catalyzed oxidation of nitrite represents an important contribution to NO•-mediated toxicity. In fact, the LPO antimicrobial system has been proven to be both bacteriostatic and bactericidal to a variety of gram-positive and gram-negative microorganisms [4]. In fact, it has been shown that a functional LPO antibacterial system exists in human airways and mucosa, which is effective against several pathogens including Pseudomonas aeruginosa, Burkholderia cepacia and Haemophilus influenzae [32]. Hydrogen peroxide has been shown to be elevated during airway inflammatory diseases such as asthma and is a major contributor to the inflammatory reactions associated with a variety of airway diseases. The major hydrogen peroxide scavenging activity in airway secretions results from an airway LPO which is identical to that expressed in the mammary gland. Airway LPO is secreted by goblet cells and functions both as a biocide and in controlling the reactive oxygen species in the airway. From its action, an effective protection against infection can be attained [33]. A recent paper has also proven the involvement of LPO in the pathogenesis of cystic fibrosis since the thiocyanate-mediated host defense system is defective in the cystic fibrosis airways epithelium [34].

Despite the complex physiological role of LPO and its potential involvement in carcinogenic mechanisms and inflammatory processes, very little data are available on the structural stability and folding of LPO [35,36]. This may be an important aspect towards a better understanding of the involvement of LPO during inflammatory response, which rely on intrinsically stable and catalytically proficient form of secreted enzyme. In this study we report a detailed characterization of the conformational, structural and catalytic properties of bovine LPO during thermal unfolding.

2. Materials and methods

2.1. Chemicals

Guanidine hydrochloride, potassium mono- and dihydrogen-phosphate, DMF and urea were purchased from Sigma. The MTSL spin-label was purchased from Toronto Chemicals (Toronto, Canada).

2.2. Protein purification

LPO was isolated from bovine milk according to the method described by Ferrari et al. [37]. The purity index of the purified enzyme (Rt = A412/A280) was 0.78 and the molar concentration of LPO was determined spectrophotometrically through the Soret band absorbance A412 (ε412 = 112000 M⁻¹ cm⁻¹).

2.3. Labeling protocol

200 μl of LPO 84 μM in 100 mM phosphate buffer pH=6.8 were mixed with 5 M urea and a 20-fold excess of MTSL. The reaction mixture was kept under stirring for 15–16 h at 4 °C in the dark. The sample underwent several washings with 100 mM phosphate buffer pH=6.8 in order to discard the excess of unreacted spin-label and urea. LPO integrity was checked by optical absorption and ESR spectroscopy as well as by activity measurements. Spin quantitation yielded ~1 MTSL/LPO, showing that a single MTSL was bound.

2.4. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed on a MicroCal VP-DSC MicroCalorimeter controlled by the VP-viewer program and equipped with 0.51 ml cells, following manufacturer’s instructions. Briefly, studies were made using 1 mg/ml of LPO in 0.1 M phosphate buffer and 0.5 M GdmCl, pH 6.3. Heating rates from 0.25 to 1.5 °C min⁻¹ were used from 25 to 90 °C. Calorimetric data were converted to heat capacity by subtracting the buffer baseline and dividing by the scan rate and protein concentration.

2.5. Spectroscopic methods

ESR measurements were performed on an ESP300E Bruker X-band machine equipped with a 4103TM cylindrical cavity and a Stelar VTC-90 temperature control system. ESR spectra were simulated with the EPRSim software 4.9 [38] specific for nitroxide spin-labels. UV-visible spectra were recorded with a Unicam UV300 double-beam spectrophotometer by Thermospectronic, whereas fluorescence spectra were recorded on a Cary Varian Eclipse instrument (slit,ε: 5 nm, slit,em: 5 nm unless otherwise noted) equipped with cell stirring and Peltier temperature control. Far and Near UV CD spectra were recorded typically at 0.2 nm resolution on a Jasco J-815 spectropolarimeter fitted with a cell holder thermostatted with a Peltier device.

2.6. Thermal unfolding studies

Thermal transitions were monitored as a function of increasing temperature, typically at 1 °C min⁻¹ from 25 to 90 °C, unless otherwise noted. Different physical properties were thus followed as a function of temperature, in 100 mM phosphate pH 6.3, 0.5 M GdmCl buffer. For optical spectroscopy measurements, 3.6 μM LPO was scanned in the UV-visible range (250–700 nm) using quartz cuvettes. Trp emission fluorescence measurements were carried out in 5 μM LPO, with excitation at 280 nm or 295 nm and emission monitored from 315 to 450 nm. Typical concentrations for circular dichroism experiments ranged from ~0.1 mg ml⁻¹ (Far-UV) to ~1 mg ml⁻¹ LPO (Near-UV and visible region). A stepwise thermal perturbation protocol was used to monitor the ESR-MTSL
signal, as these measurements cannot be performed at high temperatures. 280 μM LPO were placed in sealed capillary tubes, incubated 3 min at each temperature and cooled down to 298 K prior to recording ESR spectra (instrumental settings: freq. 9.8 GHz, modulation amplitude 1.47 G, microwave power 5 mW). This stepwise approach applied, as a control, to the other spectroscopies yielded identical \( T_m \) values to those obtained by linear temperature increments and measurements at successively higher temperatures. This happens because LPO thermal denaturation is irreversible. A non-linear least-square analysis was used to fit the data to a two-state model which allows the determination of the midpoint \( (T_m) \) of the thermal transition curves; data analysis was carried out in Origin (MicroCal).

2.7. ANS binding

LPO samples (typically 5 μM) were incubated with 200 μM ANS during 15 min prior to measurements. ANS fluorescence emission enhancement of bound ANS at 480 nm was evaluated upon excitation at 370 nm, and always corrected for background emission at 520 nm of a control-free ANS solution in buffer [39].

2.8. Activity measurements

The DMAB-MBTH substrates were used to measure LPO activity. A stock solution of LPO and 0.5 M GdmCl dissolved in 100 mM phosphate buffer pH 6.3 was incubated for 3 min at increasing temperatures in the range 25–95 °C with 5°C steps. After incubation, the following reaction mixture was prepared: 0.85 mM DMAB, 34 μM MBTH, 42 μM hydrogen peroxide, 0.48 nM LPO were mixed and the activity was monitored by following the change at 590 nm. Specific activity was calculated with the following equation:

\[
\text{units/mg} = \frac{100 \cdot \Delta \text{Abs}}{\varepsilon_{590 \text{nm}} \cdot MW_{\text{LPO}} \cdot \text{[LPO]}}
\]

(1)

It was finally normalized to a scale where 100% corresponds to the activity of the native protein.

A similar protocol was used to determine the thermal inactivation constants at 65 °C, 70 °C and 75 °C; specific activity of samples drawn from the mother-solution was measured at regular time intervals throughout the incubation. Inactivation constants (k_{inact}) were calculated by plotting the residual activity expressed as a percentage of the initial activity value and the data were fitted with the following equation:

\[
\text{Residual activity} (\%) = A_0 \cdot e^{-k_{inact} \cdot \text{time(min)}}
\]

(2)

2.9. DTT incubation and thiol titration

LPO was incubated with increasing molar excess of DTT (up to 40-fold). Excess of reductant was removed by extensive washing using 10 kDa cutoff Vivaspin concentrators, and the protein was subsequently incubated with the MTSL spin-label as described above. ESR analysis of the samples, followed by spectral integration, provided the number of free Cys residues coupled to MTSL.

3. Results and discussion

3.1. Effect of temperature on lactoperoxidase

In the pursuit of a detailed characterization of LPO conformational properties, the effect of temperature on the protein was investigated. Preliminary thermal unfolding experiments have shown that upon increasing the temperature from 25 to 90 °C, extensive protein precipitation was observed from 70 °C onwards. In order to overcome this effect, the use of low concentrations of the chemical denaturant guanidinium chloride (GdmCl) to prevent precipitation was investigated. Up to \( \sim 1.5 \) M GdmCl the protein was found to retain its native spectroscopic properties and catalytic activity, as it corresponds to the pre-transition region of the chemical denaturation curve, which has a midpoint denaturant concentration \( (C_m) \) of 2.5 M (not shown). In order to rule out any influence of chloride binding on the thermal unfolding process of LPO, thermal denaturation experiments were also done in the presence of 0.5 M NaCl or (0.5 M NaCl+0.5 M GdmCl); no evidence of a destabilising effect of chloride towards LPO was ever observed and, in the former case, chloride was unable to prevent LPO precipitation. Since the presence of low concentrations of denaturant totally abolished the temperature-induced LPO precipitation and any specific effect of chloride was ruled out, all subsequent experiments were carried out in the presence of 0.5 M GdmCl.

3.2. Lactoperoxidase denaturation is calorimetrically irreversible

The energetics of LPO thermal transition were investigated by differential scanning calorimetry (DSC). A typical calorimetric scan for LPO at pH 6.3 and 0.5 M GdmCl is shown in Fig. 1; a single, mostly symmetric transition is observed, which upon Gaussian deconvolution, corresponds to a transition centered at a \( T_m=72 \) °C. The absence of endergonic transitions in subsequent scans shows that the unfolding transition is irreversible (Fig. 1, trace b). The irreversibility of unfolding and absence of transitions in following scans have not been addressed in previous reports of calorimetric studies on LPO [36]. In our experimental conditions, the irreversibility of the thermal unfolding reaction is not arising from protein precipitation: no precipitate is observed even at the high protein concentrations used in these experiments (1 mg ml⁻¹), nor any exergonic transition typical of protein aggregation is noted. On the contrary, precipitation is observed in experiments carried out in the absence of GdmCl (not shown). Irreversible unfolding reactions are frequent among cofactor-containing proteins, and particularly in metalloproteins [40–43];
disruption of the metal binding pocket or removal of the metal cofactor from the active site is a known cause of unfolding irreversibility. Varying the heating rate from 0.25 up to 1.5 °C min$^{-1}$ had little effect on the apparent $T_m$ obtained, suggesting that the step of irreversible unfolding is not under kinetic control [44].

3.3. Lactoperoxidase denatured states

Protein tertiary structure modifications can be inferred from two main methodologies: near-UV CD signals arising from aromatic residues and intrinsic Trp fluorescence emission. LPO contains several aromatic amino acids, from which 13 are tryptophans. The latter are roughly equally distributed around the protein, but their intrinsic fluorescence is strongly quenched by the heme chromophore and also by cystines. The LPO used in this study, upon excitation at 295 nm, exhibited a broad emission band centred at 337 nm which red-shifts to 346 nm upon thermal denaturation (Fig. 2). These maxima are in agreement with previous reports [35] and denote an increased exposure to solvent upon LPO unfolding. However, in chemically unfolded LPO this effect is significantly enhanced, and a maximum emission is obtained at 356 nm (Fig. 2). This suggests that thermally denatured LPO retains some tertiary structure, whereas the chemically denatured form is closer to a random coil. This is also observed by near-UV CD (data not shown) and far-UV CD (see below). In fact, when a protein is denatured not all of its structure is necessarily lost [45–47]. In particular, it has been reported that horseradish peroxidase, a close relative of LPO, also retains secondary structure at 90 °C [48]. Thermally and chemically denatured states are not necessarily coincident and at high GdmCl concentrations, the denatured conformation is frequently closer to that of a random coil [47].

LPO thermal denaturation was monitored by fluorescence emission of aromatic residues and near-UV CD changes (Fig. 3): a cooperative transition corresponding to a $T_m=70$ °C was obtained, with no significant dependence on the heating rate (from 0.25 to 1.5 °C min$^{-1}$).

3.4. Temperature-induced secondary structure changes

LPO thermal unfolding transition was monitored by far-UV circular dichroism. This technique allows obtaining a detailed scenario of the conformational changes occurring at the level of the secondary structure: the contributions from the protein backbone dominate the far-UV CD spectrum of a protein and reflect its distinct possible conformations. The far-UV CD spectrum of LPO (Fig. 4A) is typical of a helical protein, with prominent negative bands at 208 and 222 nm arising mainly from $\alpha$-helices. These are significantly more intense than those arising from $\beta$-sheet structures, which are characterized by a single negative band ranging from 210 to 225 nm, usually centered at $\sim$216 nm [49]. The far-UV CD signature of LPO is in agreement with the recently available structural data for the protein, which comprises 29 helices and 12 strands. The LPO thermal denaturation was monitored by a temperature/wavelength scan, i.e. recording at 5 °C increments the far-UV CD spectrum upon linearly increasing the temperature at 1 °C min$^{-1}$ (Fig. 4A, only selected spectra are shown for clarity). In agreement with Trp-emission data, it is clear that even at 90 °C LPO retains considerable secondary structure, in comparison to the 7 M GdmCl condition. Monitoring the CD intensity in the far-UV region at 208 and 216 nm as a function of temperature shows a major transition at a $T_m=70$ °C, and a minor change with a $T_m \sim 40$ °C range (Fig. 4B). Clearly the most prominent transition corresponds to protein denaturation, as it agrees with DSC and fluorescence data. Considering that by DSC only a single endothermic peak is obtained at 70 °C, the minor transition observed by far-UV CD corresponds to a conformational...
rearrangement and not to protein denaturation. Since this structural modification, which is insufficient to trigger protein unfolding, is more evident from the CD variation at 216 nm, we hypothesize that it could be accounted by a local destabilization of a region involving mostly β-strands. Inspection of the protein crystal structure (PDB: 2gj1) shows that the β-strands are exclusively located at the protein periphery, in regions which are relatively exterior to the α-helix rich protein core, and whose disruption would not necessarily imply loss of the overall structural protein integrity.

3.5. Structural changes in the peripheral region of LPO monitored through specific spin-labeling

In order to check the feasibility of such hypothesis, we exploited a peculiar structural feature of LPO. In fact, a free cysteine residue (Cys284) lies on the surface of LPO. This residue was selectively conjugated with the methanethiosulfonate (MTSL) spin label and used as a paramagnetic probe to monitor the effect of thermal unfolding on the peripheral regions of the protein. The rationale for this experiment is that higher conformational freedom induced by protein unfolding should translate into an increase of isotropy of the labeled site which, in turn, should be associated with shorter rotational correlation times ($\tau_c$) with respect to the folded form. A possible drawback of this method is that the localization of Cys284 onto the protein surface makes it sensitive to environmental changes such as a temperature-induced viscosity change; this may influence the ESR spectra in a way that mimics non-restrictive rotational motion of the spin-label. In fact, Cys284 is part of a hydrophobic loop next to Phe282, Val283, and Pro285 residues and is highly hindered by its chemical surroundings, as witnessed by the low degree of accessibility of its lateral chain ($\sim 10\%$) in the native protein. Conversely, the value of $\sim 0.3$ ns observed after exposure of LPO to high temperatures, is consistent with high rotational freedom, as expected from our hypothesis. Interestingly, two main steps in the plot of $\tau_c$ vs. T are evident: a main one at $\sim 70 \, ^\circ\text{C}$, corresponding to protein denaturation, and a minor one at $\sim 40 \, ^\circ\text{C}$ (Fig. 5), corresponding...
to the conformational event highlighted by the far-UV CD data. Spin quantitation showed binding of a single MTSL per LPO.

3.6. Probing lactoperoxidase hydrophobic core exposure

The fluorophore 1-anilinonaphalene-8-sulphonate (ANS) is a useful molecule in protein folding studies as its fluorescence becomes significantly enhanced upon its binding to hydrophobic patches within a protein [50]. This makes it particularly useful to characterize molten globule states in which a large hydrophobic area has become solvent exposed as a result of loss of tertiary contacts and maintenance of secondary structure elements. In this study we have used ANS with a distinct but related purpose, aiming to investigate if, and at which point of LPO thermal unfolding, the hydrophobic core exposure started to be relevant. Up to 45 °C, no ANS fluorescence enhancement is observed and only above 60 °C a significant emission increase (up to 2.5 fold) is observed (not shown). This result suggests that no hydrophobic exposure occurs during the conformational change at $T_m \approx 40 ^\circ C$, in agreement with the absence of a DSC transition at 40 °C.

3.7. Heme spectroscopic signature and catalytic activity as reporters of lactoperoxidase core integrity

LPO contains a covalently bound protoheme IX which is located at the protein core within a α-helix motif. This cofactor is coordinated by residues Asp$^{225}$ and Glu$^{375}$ through two ester bonds whereas His$^{468}$ is the proximal ligand. Analysis of the LPO structure shows that the Fe-bound histidine is part of helix XIV which interacts with segments from seven other helices within the core, predominantly with residues from helices XIII and XXIV [51]. Moreover, the catalytic proficiency of peroxidases is strictly dependent from the correct location of specific amino acid residues with respect to the iron centre; crucial residues inside the active pocket of bovine LPO are known to be His$^{226}$ (the distal ligand), Gln$^{222}$ and Arg$^{372}$, all of which are involved in a hydrogen bond network [51]. In the frame of such a complex structural pattern, any modification of the tertiary interactions inside the α-helix core may result in deep perturbations of the scaffold of the heme pocket and compromise catalysis. Heme spectroscopic signatures are particularly useful in characterizing the protein environment and heme moiety. In this respect, both visible absorption and visible CD spectroscopies have been used to monitor conformational changes occurring in the vicinity of the heme pocket during the thermal transition. Exposure of LPO to increasing temperatures results in a modification of its optical spectrum, as shown by the temperature-induced change of the Soret band (Fig. 6A). A similar variation occurs at the visible CD bands typical of heme, namely the δ band at 412 nm [52], whose intensity decreases as temperature increases. In fact, perturbation of the heme moiety starts to be evident above 65 °C, and a sharp transition at a $T_m \approx 70 ^\circ C$ is obtained independently of the spectroscopic method used to monitor the transition (Fig. 6B). The red-shift of the Soret band, the decrease of its molar absorptivity and the modifications of the α and β bands associated with the heme ring transitions (that falls at higher wavelengths and are not shown in the figure) are consistent with the presence of a low-spin iron(III) ion [53] that, in turn, denounces a change of the iron coordination sphere. The trend of the $A_{580 \, nm}/A_{630 \, nm}$ ratio vs. temperature (data not shown) supports this interpretation and highlights the fact that the 65–75 °C range is critical for the unfolding of the heme environment. This is also shown by the activity measurements. Inactivation constants reported in Table 1 clearly show that the enzyme activity is preserved until

<table>
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<tr>
<th>Temperature (°C)</th>
<th>Inactivation constant, $k_{\text{inact}}$ (min$^{-1}$)</th>
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<tr>
<td>65</td>
<td>$4.0(\pm 0.2) \times 10^{-3}$</td>
</tr>
<tr>
<td>70</td>
<td>$1.17(\pm 0.01) \times 10^{-1}$</td>
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See Materials and methods for details.

Fig. 6. Optical spectroscopy and specific activity. (A) Optical spectra of the Soret region of LPO taken during thermal unfolding. Solid line: 25 °C; dotted line: 70 °C; dashed line: 75 °C; short dash-dot line: 80 °C. (B) Variation of the Soret absorption ($A_{412 \, nm}$, open squares), of the specific activity (expressed on a percentage scale, closed squares) and of the Vis-CD heme band (δ band, 412 nm, open triangles). (C) $T_m$ dependence from the DTT/LPO molar ratio in DTT-treated LPO.
65 °C, but it dramatically drops down above that temperature: in fact, $k_{\text{inact}}$ increases by four orders of magnitude in the 65–75 °C range. A good match between the temperature dependence of the specific activity values and the heme spectroscopic signatures is also observed (Fig. 6B): again, it is consistent with structural changes of the heme environment. More specifically, heme covalent attachment involves Glu$^{375}$ from helix II and Asp$^{225}$ from a more peripheral $\beta$-strand, whereas Fe(III) is coordinated by His$^{468}$ from helix XIV, which is involved in inter-helix interactions within the protein core. As a consequence, disruption of the interactions responsible for the correct folding of the $\alpha$-helix core may result in heme distortion, perturbation of the heme coordination sphere and abolish the adequate catalytic pocket required for catalysis.

### 3.8. Disulfide bridges and heme ester bonds contribute to LPO high thermal stability

In order to gain insights into structural factors contributing to LPO stability, the role of disulfide bridges and heme ester bonds was investigated. The nearby six disulfide bridges that clip external regions of the protein are likely to play a role in maintaining protein integrity, even upon destabilization of the $\beta$-strands. On the other hand, the ester bonds involving Asp$^{225}$ and Glu$^{375}$, which keep the heme cofactor in its place, contribute to the topological organization of the $\alpha$-helices in the folded state acting as a key structural element (Fig. 7).

A preliminary investigation of such stabilizing effect of ester bonds was carried out by exposing transiently LPO to low...
acidic conditions (pH 3), and then raising the pH back to neutrality. Although reverse ester bond formation is relatively favored from a kinetic point of view, a lowering of the $T_m$ from 70 to 60 ºC is observed in these conditions. This observation suggests at least partial disruption of ester bonds, which lie inside the very narrow heme pocket, and agrees with the hypothesized stabilizing effect, which will nevertheless need future investigations. In order to probe the stabilizing role of disulfide bonds, LPO was incubated with excess DTT (up to 40-fold, since below this limit no relevant iron reduction is observed), which resulted in breakage of a disulfide bond, as determined from a titration of the free thiols, made by coupling with MTS and quantitation by ESR spectroscopy (data not shown). The melting temperature was found to be inversely proportional to added DTT, and at a DTT/LPO molar ratio of 40, a $\Delta T_m = -6$ ºC is observed (Fig. 6C). Altogether, these preliminary data suggest that both disulfide and ester bonds concur in contributing to the high thermal stability of LPO.

4. Conclusions

The identification of the interactions that stabilize a particular protein structure and modulate its biological activity is essential to characterize the folding of a particular family of proteins and to attain a better understanding of its biological context. Our approach, based on the concerted use of complementary biophysical techniques and biochemical assays, proves successful in providing a broad picture of the protein thermal unfolding mechanism and highlights possible key stabilizing factors.

We have shown that LPO undergoes irreversible thermal denaturation at an apparent midpoint transition temperature of 70 ºC and that the resulting denatured state corresponds to a form with some residual tertiary and secondary structure. The conformational change observed around 40 ºC can be postulated to correspond to a minor structural modification at the level of the protein β-strands, based on the increased mobility of a structural paramagnetic probe lying on the protein surface and on the fact that the CD variation is more notorious at 216 nm. In agreement with the structural modification hypothesis, no DSC transition or exposure of the hydrophobic core is observed up to 50 ºC. In fact, analysis of LPO structure shows that such conformational modification at the peripheral β-strands is unlikely to result in denaturation of the polypeptide.

Protein denaturation occurs only at high temperature, when the catalytic heme pocket is disrupted, although a residual secondary structure is observed in the thermally denatured protein. This may also explain the irreversible character of LPO denaturation, which is most likely associated with a modification of the heme environment that brings about a faulty spatial arrangement of the prosthetic group and of the catalytically active amino acid residues.

In summary, the overall picture that emerges from this study is that of a protein highly resistant to structural destabilisation induced by temperature. In particular, the protein is designed so to maintain the “functional core” active up to high temperature, thanks to an internal domain that is kept tightly packed by a number of covalent interactions and acts like a structural shield for the heme pocket. This feature has a structural and a physiological meaning. The structural relevance comes from the peculiar role played by covalent bonds in keeping the protein in a structured conformation: both the ester bonds involving the heme pocket. This feature has a structural and a physiological meaning. The structural relevance comes from the peculiar role played by covalent bonds in keeping the protein in a structured conformation: both the ester bonds involving the heme group and a number of disulfide bonds, spread over the whole molecule, clip together the helices that form the protein core and maintain LPO active, even in critical conditions. The physiological relevance is related with the increasing evidence that LPO is involved, in direct or indirect ways, in inflammatory processes that imply a local increase of temperature and may implicitly cause protein denaturation. This is the case of airway diseases (the most serious among them being cystic fibrosis) [34], but also carcinogenic processes that often result from the degeneration of inflammatory events [32]. The overall picture coming out from these findings is relevant to identification of the factors accounting for LPO stability in pathological contexts characterized by an altered pH homeostasis or temperature.

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