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Acid-Induced Unfolding of Flounder Hemoglobin: Evidence for a Molten Globular State with Enhanced Pro-oxidative Activity

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The acid-induced unfolding of flounder oxyhemoglobin was investigated and the effect on pro-oxidative activity assessed. Hemoglobin exhibited multistep unfolding transitions as pH was lowered, with the major transition between pH 3.5 and 4 5. The protein was maximally acid-unfolded (but not fully unfolded) at ~pH 2.5, and further titration with HCl led to a partially refolded protein due to a stabilizing effect of Cl^- anions. At low pH, the protein retained a sizable amount of secondary structure and had increased ANS binding, suggesting a molten globular form at low pH. Dramatic changes in the heme environment occurred concurrently with the changes in protein conformation. These changes resulted in an enhancement in the pro-oxidative activity of the protein. The results show that an increase in flounder hemoglobin pro-oxidation was correlated with the extent of its unfolding, and they provide useful insight into what may occur with hemoglobin in processes where highly acidic conditions are employed.

KEYWORDS: Hemoglobin; flounder; lipid oxidation; molten globule; acid unfolding; pro-oxidant; conformational changes

INTRODUCTION

Aquatic foods are very susceptible to peroxidative processes due to high levels of pro-oxidants and susceptible polyunsaturated lipids (*1*). Lipid oxidation of fish muscle can lead to highly undesirably off-odors, flavors, and discoloration which negatively affect its quality, appearance, and value. Recent evidence has shown that hemoglobin can play a major role in the oxidation of fish muscle (*2*, *3*). Fish hemoglobin has received little attention in the past, in part due to the misconception that most blood is removed on bleeding fish after harvest. For example, it has been documented for salmon that there was no difference between hemoglobin content in muscle from bled vs unbled fish (*4*). In addition, the longer the delay between harvesting and bleeding, the less blood is removed on bleeding (*5*, *6*). For this reason, a sizable amount of hemoglobin is present in the muscle of postmortem fish and can thus readily participate in oxidative reactions. This has triggered interest in understanding the role hemoglobin plays in the oxidation of fish muscle. Pro-oxidative activity of trout hemoglobin was found to be greatly enhanced as pH was lowered (*2*, *3*). Richards and Hultin (*2*) observed increased deoxygenation as pH was lowered from 7.6 to 6.0, and speculated that deoxygenation may be responsible for an increase in pro-oxidative activity (*2*). A similar connection between pH (6 and 7) and pro-oxidative activity was found by Kristinsson and Undeland (unpublished data) for hemoglobins from four different fish species (Atlantic pollock, winter flounder, mackerel, and menhaden). Recent evidence shows a dramatic increase in pro-oxidative activity of trout hemoglobin at very acidic conditions (pH 2−3) (*3*). This increase was found to be closely linked to significant conformational changes in its structure and heme crevice (*3*).

There is much interest in the author's laboratory to understand how very acidic conditions affect the structure of fish hemoglobins and influence their ability to participate in lipid oxidation. Substantial evidence has accumulated in the past two decades from many laboratories, showing that some proteins may be only partially unfolded at acid pH and may, in fact, refold upon titration with more acid or salts (*7*−*10*). These states have been collectively referred to as "molten globules", as they share similar characteristics, such as retaining sizable amount of native-like secondary structure, losing most of their tertiary interactions, retaining a relatively compact structure, and displaying substantially exposed hydrophobic residues (*12*−*14*). The molten globular state has received much attention, as it is thought to bear a resemblance to intermediates that form in protein folding. Molten globules are also hypothesized to be involved in important processes in vivo, such as enabling a protein to interact with and penetrate into membranes and play a role in protein turnover (*13*). A handful of heme proteins (mostly myoglobin) have been studied in the molten globular form to provide insight into how proteins may be folding. However, no study has addressed the functional role of the molten globular state of heme proteins. It is important to address this, as heme proteins are potent catalysts of lipid peroxidation, which leads to the propagation of dangerous free radicals and membrane and protein damage (*15*). Hemoglobin may encounter

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very acidic environments in vivo that can affect its structure, most notably those surrounding the highly negatively charged phospholipids membranes, which have a high concentration of hydrogen ions in their immediate surroundings. The same situation may emerge in food processing, e.g., when muscle is ground, thus releasing hemoglobin and enabling it to contact membrane surfaces. Previous workers have, in fact, demonstrated that proteins can partially unfold when they are in contact with a charged membrane surface or negatively charged model surfaces (*16*).

There are certain food processes that utilize highly acidic pHs which in turn may affect hemoglobin. Of particular importance to the author's laboratory is a new process that utilizes very acidic pH conditions (pH 2−3) to recover fish muscle proteins from minced fish to produce functional low-fat protein isolates (*17*, *18*). These highly acidic conditions can have a detrimental effect on the conformation of hemoglobin, increasing its pro-oxidative activity. This, in turn, can lead to peroxidative problems with the final protein isolate, which may contain a level of membrane phospholipids high enough to result in significant lipid oxidation. Very little is known about how highly acidic conditions will affect the pro-oxidative function of hemoglobin. From studies with trout hemoglobin, it is evident that hemoglobin's tertiary structure and heme crevice are greatly affected at low pH, while changes in secondary structure were more resistant to acid pH (*3*). The pro-oxidative activity of trout hemoglobins was higher at pH 2−3 than at pH 7 (*3*). Hemoglobins from different fish species can differ markedly in their properties. For this reason, it is important to individually examine hemoglobins from different species to understand how acidic pH conditions affect their function and conformation. Understanding the response of different fish hemoglobins to low pH can allow control of lipid oxidation in the low-pH fish protein isolation process.

This paper reports on the acid-induced conformational changes in flounder hemoglobin, a protein heretofore not studied at low pH, and on accompanying functional changes of the protein as a catalyst of lipid peroxidation. Flounder is a species with a high percentage of polyunsaturated membrane fatty acids and therefore would be highly susceptible to peroxidative processes stemming from hemoglobin. Evidence is presented which shows that flounder hemoglobin partially unfolds at acid pH and yields different stable molten globules at different low pHs. Evidence is also presented that the molten globular form of flounder hemoglobin has greatly enhanced pro-oxidative activities, dictated by the extent of unfolding. This work contributes to our knowledge about how acidic conditions influence fish hemoglobins and will aid in our search to understand how hemoglobin conformation is linked to pro-oxidative activity.

MATERIALS AND METHODS

Materials. Hemoglobin was prepared from blood drawn from anesthetized flounder as previously described (*19*) and stabilized in 50% glycerol prior to frozen storage at −20 °C. The purity of the preparation was verified by SDS−PAGE (*20*) and densitometry, which demonstrated that samples were >98% hemoglobin. Samples were clarified by centrifugation prior to conformational and oxidation studies. Hemoglobin levels were quantified spectrophotometrically as described previously (*21*) with bovine hemoglobin as the standard. Chemicals were obtained from Sigma (Sigma Chemical Co., St. Lois, MO) unless otherwise specified and were of analytical grade: linoleic acid, bovine hemoglobin, 8-anilino-1-naphthalenesulfonic acid (ANS), salts, buffers, and acids. A stock solution of ANS was prepared and its concentration determined using the extinction coefficient of 5000 M^{-1} cm^{-1} at 350 nm (*22*). Electrophoresis-grade guanidine hydrochloride was obtained from Fisher Scientific (Fair Lawn, NJ), and Tween-20 was obtained from Bio-Rad Laboratories (Hercules,CA). All experiments were performed in ultrafiltrated double-distilled water.

Acid Unfolding of Hemoglobin. Acid unfolding of flounder hemoglobin was performed by incubating the protein at pH 1.5−7.6 (5 °C) for 1 h (all samples reached their maximum unfolding values within seconds or minutes). Buffers were H_2O−HCl for pH 1.5−3.5, and 20 mM sodium phosphate for pH 4−7.6. Hemoglobin concentration was 0.8 μM in UV−visible spectroscopy studies, 0.4 μM in tryptophan fluorescence and ANS binding/fluorescence studies, and 8.33 μM for near-UV circular dichroism. Full unfolding was achieved by incubation in 6 M Gu-HCl.

Conformational Studies. Changes in hemoglobin heme peak environment, ligand binding, and oxidation state were studied by scanning in the visible range (350 nm < λ < 700 nm) using a HP 8453 diode array UV−visible spectrophotometer (Agilent Technologies, Palo Alto, CA). Exposure and solvation of tryptophan residues was monitored by the tryptophan fluorescence emission intensity and trytophan fluorescence peak wavelength of hemoglobin. Measurements for tryptophan fluorescence used 280 nm as the excitation wavelength, and emission was followed from 300 to 400 nm, at 0.5-nm intervals, using a Perkin-Elmer LS 45 luminescence spectrometer (Perkin-Elmer Instruments, Norwalk, CT). Hemoglobin's ability to bind to the hydrophobic dye ANS was studied as an indicator of exposure of hydrophobic clusters. ANS was added (40 μM) to hemoglobin at pH 2, pH 7, and at 6 M Gu-HCl, and fluorescence was recorded after 15 min (when it had stabilized) using 380 nm as the excitation wavelength and following emission from 400 to 550 nm, at 0.5-nm intervals. Changes in secondary structure were monitored by scanning hemoglobin solutions in a quartz cell (2 mm width) from 260 to 190 nm using a Jasco J-715 spectropolarimeter (Jasco Inc., Easton, MD). Changes in tertiary structure were monitored from 360 to 260 nm using a 1-cm cell width. Resolution was set at 0.2 nm, bandwidth 1 nm, sensitivity 20 mdeg, response 1 s, and scanning speed 20 mdeg/min. Five spectra were acquired for each sample, and the results were averaged into one spectrum. The fraction of unfolded hemoglobin was assessed according to a two-state unfolding mechanism:

$$f_{app} = \frac{f_U - f}{f_U - f_N}$$

where f_{app} is the fraction of the protein which had unfolded, f represents the observed maximum tryptophan fluorescence intensity under the given conditions, and f_N and f_U are the corresponding values in the native and unfolded states, respectively (*23*).

Conformational Stability of Native and Acid-Unfolded Hemoglobin. Acid-unfolded (pH 2) and native hemoglobin (pH 7) were compared for their conformational stability. This was accomplished by adding hemoglobin (0.4 μM) to solutions between 0 and 6 M Gu-HCl (at 0.5 M intervals) and recording tryptophan fluorescence after a 1 h unfolding time at 5 °C, as described above.

Heme and Iron Loss from Hemoglobin. To detect if heme was lost at pH 1.5−7 from the globin, samples were subjected to ultrafiltration through 3000 and 10 000 Da molecular weight cutoff filters. The filtrate and reconstituted retentate were analyzed for the presence of heme by scanning in the visible range (600−350 nm). Potential iron loss with time under the same solution conditions was determined spectrophotometrically using the ferrozine method (*24*). Total hemoglobin-bound iron was measured according to a modification of the method described by Panter (*25*). Hemoglobin was assayed at 13 μM in both experiments, and all samples were kept at 5 °C.

Pro-oxidative Activity of Hemoglobin. Linoleic acid (20 mg) and Tween 20 (20 mg) were mixed by stirring with a plastic rod and diluted in distilled deionized water to give 2 mL. This solution was emulsified by forcing the solution through a pipet (1 mL) tip five times. To study the pro-oxidative activity of flounder hemoglobin, 150 μL of the stock solution was mixed with 850 μL of sample buffer (25 mM potassium phosphate for pH 7; H_2O−HCl for pH 2−3) and 10 μL of hemoglobin added to give a hemoglobin concentration of 0.9 μM. Controls were run without hemoglobin. All assays were performed at 5 °C. Develop-

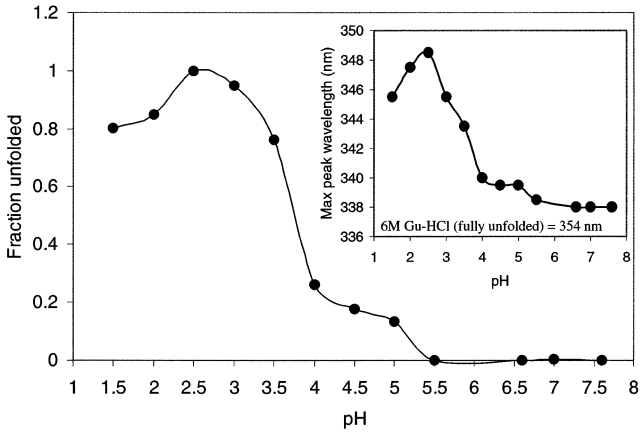

Figure 1. Conformational changes in flounder hemoglobin with pH as assessed by intrinsic tryptophan fluorescence intensity. The main figure shows the fraction unfolded hemoglobin (normalized) as assessed by changes in the intensity of tryptophan fluorescence with pH. The inset shows the shift in peak wavelength with changing pH. Protein concentration was 0.4 μM, and fluorescence was recorded after 30 min at 5 °C. Excitation wavelength was 280 nm, and emission spectra were recorded from 300 to 450 nm.

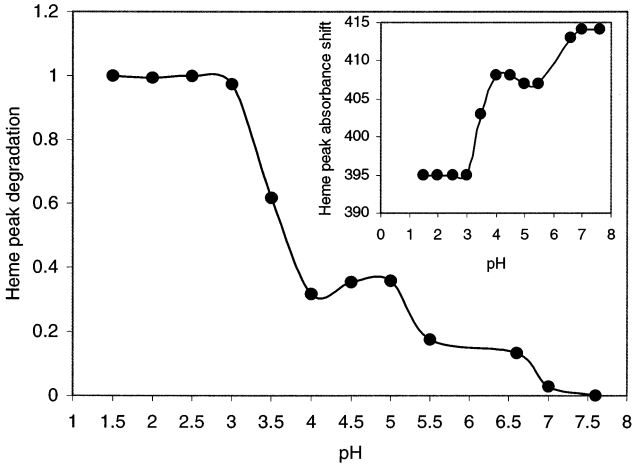

Figure 2. Conformational changes in flounder hemoglobin with pH as assessed by UV–visible spectroscopy. The main figure shows the degradation in the heme peak from the protein's Söret spectrum (350–450 nm) as a function of pH. The data were normalized such that 1 represents maximum degradation, while 0 refers to a native heme peak. The inset shows the wavelength shift in the heme peak maximum absorbance. Protein concentration was 0.8 μM, and spectra were recorded after 1 h at 5 °C.

ment of lipid oxidation was followed by measuring the rate of conjugated diene formation during a 10-min reaction period by recording absorbance changes at 234 nm using a HP 8453 diode array UV−visible spectrophotometer.

RESULTS AND DISCUSSION

Hemoglobin Global Conformational Changes Induced by Low pH. To obtain comprehensive information on the conformational changes in hemoglobin from pH 1.5 to 7, several different structural probes were employed. The unfolding transition at acid pH was monitored from the intrinsic tryptophan fluorescence of the protein, which is a sensitive probe for protein unfolding, and from UV−visible spectroscopy, which is a probe for changes in the heme crevice and oxygen binding state of hemoglobin (*3*). Hemoglobin contains a number of buried tryptophan residues which are confined to very hydrophobic areas in relatively close proximity to the heme group (*26*, *27*). Under native conditions, these residues are quenched from surrounding groups and have limited exposure to the solvent, and hence low fluorescence and a fluorescence maximum around 338 nm (**Figure 1** inset). However, as the pH was lowered, the tryptophan fluorescence dramatically increased, and the fluorescence wavelength shifted up (**Figure 1**). This is most likely the result of exposure of the buried tryptophans to the surrounding solvent as the protein unfolds. It is interesting to note from the tryptophan fluorescence data that flounder hemoglobin exhibited two unfolding transition as the pH was lowered (**Figure 1**). The first transition, between pH 5 and 5.5, was relatively small; however, the second transition, between pH 3.0 and 4, was large. A single unfolding transition had been observed previously with trout hemoglobin around pH 3.5 (*3*). This cooperative unfolding transition was ascribed in large part to the protonation of buried histidine residues as pH was lowered, which destabilizes the native state and partially unfolds the protein at acid pH (*28*, *29*). Recent evidence with myoglobin, which has a similar transition at acid pH, points to protonation of only one partially buried histidine residue (*30*), which would explain a sudden cooperative unfolding within a limited pH range (∼0.5 unit). The cooperative unfolding transition seen with flounder hemoglobin between pH 3.5 and 4 supports that the unfolding is driven by protonation of only one or very few histidine residues. Histidine residues would typically become protonated at a pH higher than 3.5−4; however, factors such as proximity of other charged nearby residues and screening effects in the protein could dramatically shift the pK_a of partially buried histidine residues (*30*, *31*), which explains why some become protonated at the very low pH where flounder hemoglobin started to unfold. The first partial unfolding transition seen for the tryptophan residues below pH 5.5 could therefore be ascribed to protonation of more solvent-exposed histidine residues, which do not have a greatly suppressed pK_a like the one(s) responsible for the second acid-unfolding transition.

The results from the UV−visible data partly agree with the two unfolding transitions seen in the tryptophan fluorescence data, with the exception that a third conformational transition is seen starting below pH 7 (**Figure 2**). This transition could be linked to the autoxidation of the heme in hemoglobin, which yields a degraded heme peak as pH is lowered below pH 7 and is not due to significant global unfolding. The heme group in hemoglobin is the key to its function as a pro-oxidant, and the environment surrounding the heme largely dictates its function. The visible spectrum of hemoglobin therefore provides important information on the heme−protein interactions, oxidation, and ligand binding state of the protein. In its native state, the heme group is a five-coordinate high-spin complex (*32*), and it is stabilized by a bond between the proximal histidine and the iron in the heme as well as hydrophobic residues in the heme pocket, and to some extent by hydrogen bonding via bound ligands to a distal histidine residue (*33*). At pH 7, flounder hemoglobin was in its oxygenated state, with a Söret band absorption maximum at ∼414 nm (heme peak) (**Figure 2** inset and **Figure 3**). However, as the pH was lowered, the Söret bands were substantially blue shifted. This change in the heme peak spectra indicates major conformational changes in the heme pocket, where the heme group likely loses its contact with the proximal histidine, converting the heme to a four-coordinate low-spin species at and below pH 3 (*3*, *28*, *34*). The data strongly suggest that flounder hemoglobin forms three stable, partially unfolded species with different levels of heme group exposure (Figure 2 inset): one between pH 4 and 5.5, where the heme is

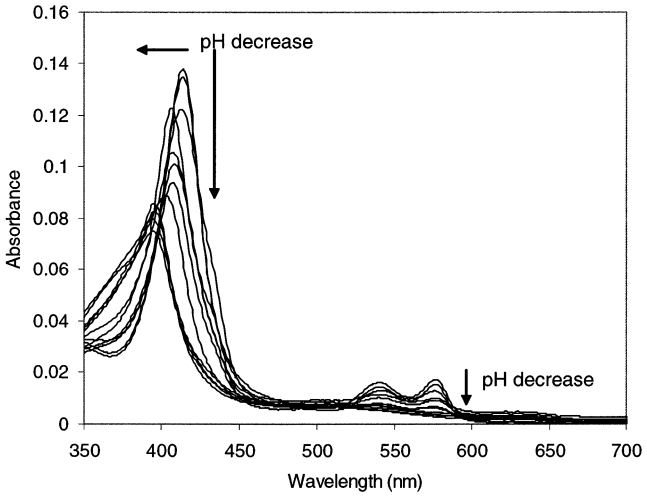

Figure 3. UV–visible spectra of flounder hemoglobin from pH 1.5 to 7.6. Arrows indicate the drop in absorbance (heme peak at 395–414 and oxygen peaks at 541 and 576 nm) and shift in heme peaks in the direction of pH decrease. Protein concentration was 0.8 μM, and spectra were recorded after 1 h at 5 °C.

Table 1. Loss of Iron from Hemoglobin at Low pH Compared to Neutral pH[a]

sample	μg iron/mL in solution	% of hemoglobin iron in solution
hemoglobin at pH 2.5	0.035 ± 0.012	4.8
hemoglobin at pH 7	0.02 ± 0.009	2.7
hemoglobin treated to release all bound iron	0.734 ± 0.08	100

[a] Hemoglobin was assayed at 13 μM, and all samples were kept at 5 °C.

likely still bound to the proximal histidine but slightly displaced from the heme crevice; another at pH 3.5, which could represent a mixture of displaced heme unbound or bound to the proximal histidine; and a third species, from pH 1.5 to 3, which has lost all contact with the proximal histidine and has a substantially displaced heme. These data are consistent with those reported by Tang et al. (*35*) and Sage et al. (*28*), who found several different myoglobin heme intermediates at low pH. The dramatic changes in the flounder hemoglobin heme crevice, however, did not lead to a significant liberation of heme or iron from the protein, as only about 4.8% of released iron was found for hemoglobin at pH 2.5, which was not statistically higher than the 2.7% liberated iron found at pH 7 (**Table 1**). This suggests that the heme was not released but rather displaced in the protein on acid unfolding, staying closely associated with the heme pocket. This is not unexpected, as the heme is highly hydrophobic and would not partition into water but would stay associated with a more hydrophobic, partially unfolded protein. These changes in the heme pocket at low pH were further accompanied by changes in the protein's ability to bind to oxygen as the two peaks between 500 and 600 nm were lost and the heme group in the protein had become oxidized to methemoglobin at low pH (**Figure 3**). This loss in oxygen-binding capability and oxidation of the protein was irreversible according to refolding studies (data not shown). Unfolding of the distal heme pocket at low pH, which most likely led to the irreversible loss of oxygen binding and oxidation, may have contributed to the changes in the heme pocket, as oxidation and ligand binding have a large bearing on the proximal histidine–

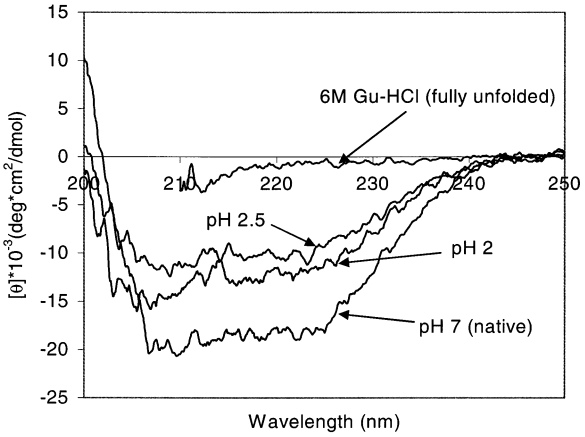

Figure 4. Far-UV circular dichroism spectra of flounder hemoglobin at pH 7 (native), pH 2, pH 2.5, and 6 M Gu-HCl (fully unfolded). Protein concentration was 8.33 μM, and circular dichroism spectra were recorded after 1 h at 5 °C.

heme interactions (*33*, *36*). The loss in helical structure at low pH (**Figure 4**), concurrent with the loss of proximal and distal histidine interactions with the heme, strongly suggests local perturbations in the helixes making up the proximal and distal side of the heme pocket, which is expected to significantly expose the heme group to the solvent with unknown functional consequences. Furthermore, it has been reported that the heme group consumes more than 97% of the tryptophan excitation energy (*27*), and on acid unfolding this quenching is substantially decreased (*37*). The increase in tryptophan fluorescence at low pH previously discussed (**Figure 1**), therefore, indicates not only unfolding of the protein but also concurrent exposure of the heme group to solvent.

Evidence for a Molten Globular Conformation at Low pH. It is interesting to note that below pH 2.5, where the protein was maximally acid-unfolded, fluorescence intensity decreased (**Figure 1**), presumably because tryptophan residues had less access to the surrounding solvent. This may denote a refolding behavior of the acid-unfolded protein. The refolding below pH 2.5 was verified by far-UV CD experiments, where the protein had higher secondary structure at pH 1.5 and 2 compared to that at pH 2.5. When the tryptophan fluorescence emission maximum is looked at, it can be seen that, in the absence of salt at pH 2.5, the emission wavelength was significantly red shifted to 348.5 nm, compared to native hemoglobin at pH 7, which had a maximum of 338 nm (**Figure 1** inset), indicating that the environment around the tryptophan residues became more polar at low pH, presumably as the residues became exposed to solvent on unfolding. The emission wavelength at low pH, however, was not as red shifted as that of fully unfolded hemoglobin in 6 M Gu-HCl (354 nm), and the fully unfolded proteins also exhibited substantially higher tryptophan fluorescence intensity than the protein at pH 3 (data not shown), which strongly indicates that the acid-unfolded protein was only partly unfolded. To verify this, the far-UV CD spectra of hemoglobin were recorded at pH 2 and 2.5 (**Figure 4**), which showed that hemoglobin still retained a large amount of secondary structure at acid pH. From the CD spectra, it can be seen that at pH 2, the protein had more secondary structure than at pH 2.5, 69% vs 55% (from data values at 222 nm). This is in excellent agreement with the fluorescence studies, which showed that at pH 2, tryptophan fluorescence was less than that at pH 2.5 (**Figure 1**). This provides strong evidence that the protein (a) is only partially unfolded at low pH and (b) gains a more folded conformation as more HCl is titrated below pH 2.5. These data

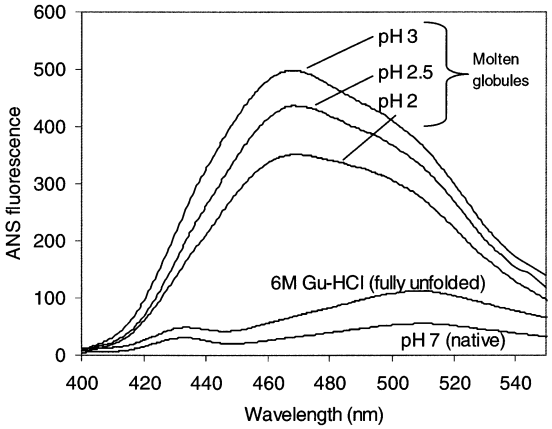

Figure 5. Exposure of hydrophobic clusters of flounder hemoglobin at pH 7 (native), pH 2 (molten globule), and 6 M Gu-HCl (fully unfolded) as assessed by fluorescence emission spectra of ANS bound to hemoglobin. Protein concentration was 0.4 μM, and fluorescence was recorded after 1 h at 5 °C. Excitation wavelength was 380 nm.

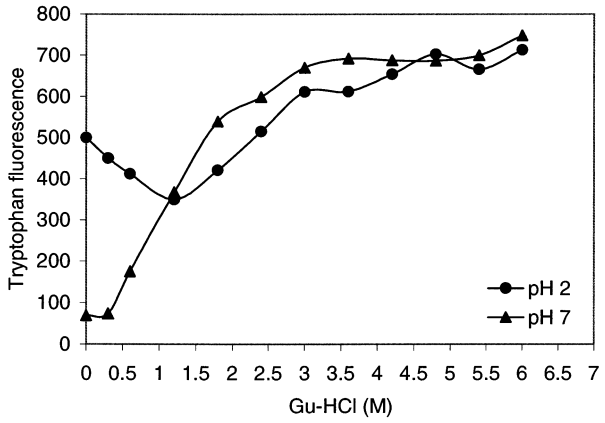

Figure 6. Gu-HCl-induced unfolding of native hemoglobin (pH 7) and molten globular hemoglobin (pH 2) as determined from the protein's intrinsic maximum tryptophan fluorescence intensity. Protein concentration was 0.4 μM, and fluorescence was recorded after 30 min at 5 °C. Excitation wavelength was 280 nm, and emission spectra were recorded from 300 to 450 nm.

are consistent with those often seen in transitions of native structures to molten globular states (*38*, *39*). To verify that this was the case, the protein was studied for loss of tertiary structure (near-UV CD) and exposure of hydrophobic residues (ANS binding). At pH 2 and 2.5, flounder hemoglobin had near-UV CD spectra similar to that of a fully unfolded protein in 6 M Gu-HCl (data not shown), indicating that acid pH leads to a complete loss in tertiary interactions, regardless of the amount of secondary structure retained at low pH. The ability of the protein to bind the hydrophobic dye ANS was, however, significantly increased at low pH, whereas native (pH 7) and fully unfolded (6 M Gu-HCl) protein exhibited very low binding (**Figure 5**). The increase in fluorescence intensity and wavelength shift from ~510 to ~470 nm on ANS addition to hemoglobin at pH 2 is an indication that clusters of otherwise buried hydrophobic groups become exposed upon acid unfolding (*40*). In its native state these groups would be buried and inaccessible for ANS, and in the fully unfolded state the protein could be in an extended configuration with no ordered hydrophobic clusters for ANS to bind to. The lower ANS binding of hemoglobin at pH 2 is a further indication that the protein was less unfolded than at pH 2.5 and 3. To get even more assurance on the conformational state of hemoglobin at pH 2, it was subjected to serial Gu-HCl-induced unfolding and compared to native hemoglobin at pH 7 (**Figure 6**). Results confirm that the molten globule at pH 2 does have considerable structure, as increasing concentrations did induce further unfolding of its structure. If the protein were fully unfolded at acid pH, there would have been no additional fluorescence increase as Gu-HCl concentration was increased. An interesting observation is that low concentrations of Gu-HCl actually induced more structure to the protein at pH 2, possibly due to addition of Cl^- from the Gu-HCl. This is consistent with the data of Hagihara et al. (*41*) on cytochrome *c*. In its native state, however, the hemoglobin cooperatively unfolded, with a midpoint of unfolding at ~1.2 M Gu-HCl.

These results taken together, i.e., considerable retention of secondary structure, loss of tertiary structure, partially exposed tryptophan residues, and exposure of hydrophobic clusters as indicated by increased ANS binding, provide strong evidence that flounder hemoglobin takes on a molten globular state at acid pH (*9*, *10*, *13*, *22*, *42*). At low pH, the protein unfolds by electrostatic repulsion between positively charged residues. However, the repulsion is not strong enough to overcome all the hydrophobic forces holding the protein together, and for that reason it is only partially unfolded. It has been previously reported that the presence of anions is critical in determining the extent of unfolding at acid pH (*8*, *10*). The reason the protein exhibited a refolding behavior below pH 2.5 is therefore likely via the action of the increased HCl concentration, which would contribute more Cl^- anions to an already maximally protonated protein. The Cl^- anions will therefore act to lower the electrostatic repulsion that promotes acid unfolding, most probably via direct electrostatic binding of Cl^- to the positively charged sites of the proteins or screening of the repulsive charges. This would result in the manifestation of the intrinsic hydrophobic interactions of the protein, which favor partial refolding (*8*, *10*, *43*), as is seen with flounder hemoglobin below pH 2.5.

Effect on Pro-oxidative Activity. Hemoglobin is known for its potential to catalyze various oxidative and peroxidative reactions, which may lead to toxic conditions in vivo and to undesirable reactions in muscle-based food products (*15*, *44*). This is a particularly important issue for a recently developed acid-aided process aimed at producing functional protein isolated from fish muscle, as the highly acidic conditions could modify the oxidative properties of hemoglobin in the raw material (*3*). Very little is known about how highly acidic conditions affect hemoglobin's participation in lipid oxidation. It was therefore of much interest to investigate if the conversion of hemoglobin to a molten globular state would influence its pro-oxidative activity. Hemoglobin was added to a linoleic acid emulsion, and oxidation development was followed by monitoring the formation of conjugated dienes (CDs). The concentration in the assay was comparable to hemoglobin and heme concentrations of 5.94 and 0.22 mg, respectively, per 100 g of fish muscle (wet weight). This quantity is typical of the hemoglobin and heme content in white muscle species such as flounder (*45*−*47*). At pH 7, hemoglobin-catalyzed lipid oxidation was complete after 10 min, with an oxidation midpoint (50% of CD formation) of 6.2 min (**Figure 7**). When hemoglobin was added to the emulsion at acid pH (pH 2−3), oxidation developed substantially faster and was complete after ca. 3−6 min, depending on the specific low pH. The fully acid-unfolded hemoglobin at pH 2.5 exhibited the most pro-oxidative activity (oxidation midpoint at 2 min), with hemoglobin at pH 3 having

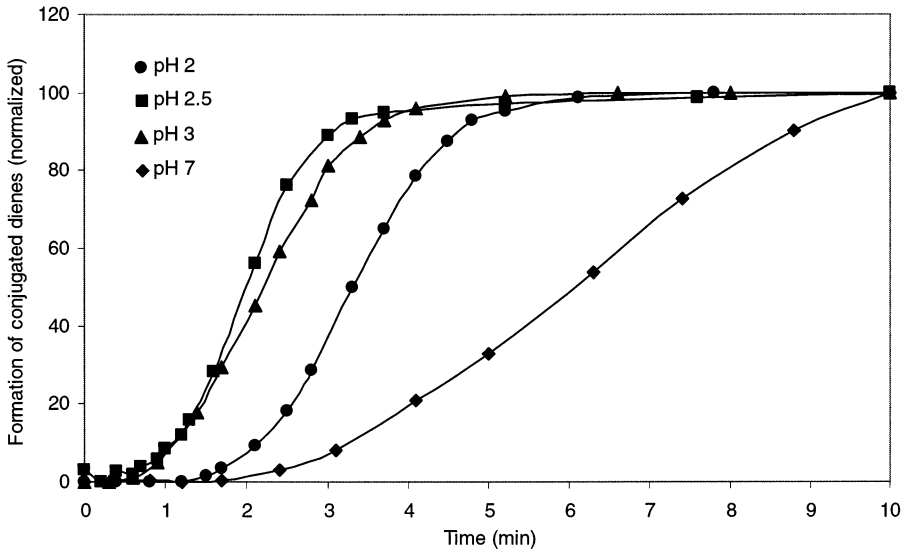

Figure 7. Effect of pH on the pro-oxidative activity of flounder hemoglobin on linoleic acid. Hemoglobin (0.9 μM) was added to a linoleic acid emulsion after 1 h at 5 °C, and formation of conjugated dienes followed at 234 nm at 5. The data were normalized such that 100 represents maximum absorbance at 234 nm, i.e., formation of conjugated dienes, and 0 refers to no absorbance. Temperature was maintained at 5 °C during the assay.

slightly less activity (midpoint of oxidation at 2.2 min). Interestingly, hemoglobin at pH 2, which had partially refolded and thus had more structure than hemoglobin at pH 2.5 and 3, did exhibit significantly less pro-oxidative activity (midpoint of oxidation at 3.3 min). These results show that the molten globular form of hemoglobin at acid pH leads to a significant increase in the pro-oxidative activity of the protein, and that different partially unfolded structures translate to different activities, most notably that pro-oxidative activity is lowered for the acid-induced refolded proteins. Similar results were obtained with trout hemoglobin previously (*3*). The greatly enhanced activity could be attributed to a more open heme crevice at low pH, which would facilitate the access of fatty acids to the crevice and thus the participation of the heme in the catalysis of oxidation. A study using mutated hemoglobin with a more open heme crevice showed that it had increased pro-oxidative activity (*48*). It is known that proteins in their molten globular state can more readily interact with and penetrate into lipid bilayers due to their hydrophobic and flexible nature (*13*, *49*). Hemoglobin has been found to be capable of binding to membranes (*50*) and can partially enter phospholipid liposomes (*51*). Flounder hemoglobin in its molten globular state at acid pH may therefore interact more effectively with linoleic acid compared to when it is in its native state at pH 7, thus exerting its action more effectively as a pro-oxidant. Furthermore, the loss of contact between the heme group and the proximal histidine, along with its greater exposure, is expected to make it less strongly associated with hemoglobin and could make the heme more readily partition into a nonpolar phase, such as the linoleic acid emulsion, where the free heme could more effectively oxidize the fatty acids. Destabilization of the structure of hemoglobin, e.g., induced by pH, has been shown to make the protein more readily give up its heme (*33*, *52*). The reason the protein at pH 2 has less activity than the protein at pH 2.5 and 3 is therefore likely due to a less exposed heme group, in accord with more quenched tryptophan fluorescence (**Figure 1**). The increased secondary structure and also less exposed hydrophobic surface (from ANS binding data), and the presumably decreased flexibility at pH 2 compared to the more unfolded protein at pH 2.5 and 3, could also make hemoglobin less prone to interact with the linoleic acid phase.

CONCLUSION

These results give important insight into what could occur with hemoglobin's structure and pro-oxidative activity under highly acidic conditions, e.g., in a low-pH environment, or when the hemoglobin comes into contact with acidic membrane interfaces. The results show that there is a connection between the conformation of hemoglobin and its pro-oxidative activity, such that more unfolding of the heme crevice and the structure overall leads to higher pro-oxidative activities. A refolding behavior of the protein at pH 2 was also linked to a decrease in the protein's pro-oxidative activity. The results show that the choice of different low pHs in acid extraction of fish muscle proteins may lead to different extents of hemoglobin-mediated lipid oxidation. The next step is to study in more detail the mechanism of pro-oxidation under highly acidic conditions, as it is important to develop appropriate antioxidative strategies to minimize hemoglobin-induced oxidation in fish muscle-based products experiencing very acidic pH values.

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