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Changes in Trout Hemoglobin Conformations and Solubility after Exposure to Acid and Alkali pH

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The effect of different acid and alkali treatments followed by pH readjustment on solubility and conformation of trout hemoglobins was investigated. At low pH (1.5−3.5) hemoglobin was unfolded at faster rates as the pH was lowered. Inclusion of 500 mM NaCl at low pH significantly increased the rate of unfolding. At alkaline pH (10−12) the conformation of hemoglobin was much less affected than at acid pH, and the presence of salt had little additional effect. When hemoglobin solutions were adjusted to neutrality at different stages of unfolding, the recovery of native structure on refolding was proportional to the extent of unfolding prior to pH readjustment: the more unfolded the protein, the less was the recovery of native structure. The presence of salt led to a smaller recovery of native structure. The more improperly unfolded the hemoglobin was (and hydrophobic), the lower was its solubility. Results suggest that the presence of NaCl (25−500 mM) may not only interfere with the refolding process but also enhance the hydrophobic interactions of improperly refolded hemoglobin, possibly due to charge screening. These results show that proper control of unfolding and refolding time and ionic strength in processes using highly acidic or alkaline conditions can minimize loss of hemoglobin solubility.

KEYWORDS: Hemoglobin; trout; pH; ionic strength; conformation; unfolding; refolding; solubility; aggregation

INTRODUCTION

Hemoglobin is the main protein of red blood cells and is found in high amounts in dark muscle of fish due to its large oxygen demand (*1*), but it is also the predominant heme protein found in fish white muscle (*2*). There is evidence that the presence of blood, and thus hemoglobin, in fish muscle leads to significant color and lipid oxidation problems (*2*−*4*). The adverse effect of hemoglobin on color occurs when its reduced state oxy- (Hb-$Fe^{2+}-O_2$) or deoxyhemoglobin (Hb-Fe^{2+}) oxidizes to the brown met-hemoglobin (Hb-Fe^{3+}) form. Both the reduced and oxidized forms of hemoglobin are reported to be pro-oxidative, but primarily under acidic conditions (*2*, *5*). The oxidized met form of hemoglobin can be transformed (e.g., via interaction with H_2O_2) into a higher oxidation state species, perferryl hemoglobin (•Hb-Fe^{4+}═O), which rapidly reduces to ferrylhemoglobin (Hb-Fe^{4+}═O), both of which are thought to play an important role in initiating lipid oxidation (*5*−*7*). The formation of these species is accelerated as pH is reduced (*5*). Many fish hemoglobins are far more sensitive to a drop in pH than their mammalian counterparts due to differences in their structure, most notably the interactions of their subunits (*8*−*10*). The enhancement of autoxidation on pH reduction is thought to be due to increased dissociation of hemoglobin subunits (*11*) as well as a loss of oxygen, leaving the heme more exposed and having less oxidative stability (*12*). On the other hand, when the pH is increased to even high alkaline pH values, the heme group is stabilized, resulting in greatly increased oxidative stability (*12*−*14*). Decreasing the pH to extremely low values leads to the exposure of the heme as the heme cavity and the global structure of the protein unfold, which greatly increases the rate of heme autoxidation (*14*). For more background information on fish hemoglobin and reactions of heme proteins, readers are referred to the excellent reviews by Riggs (*8*) and Baron and Andersen (*5*), respectively.

The influence of extreme pH values on the structure and function of hemoglobin is of much importance for a new process aimed at extracting and precipitating fish muscle proteins from underutilized fish species and byproducts (*1*, *15*). This process involves subjecting homogenized fish tissue to very high or low pH values to solubilize the muscle proteins, followed by a high-speed centrifugation separating the proteins from other constituents of muscle (bones, scales, connective tissue, neutral lipids, and membrane phospholipids). The proteins are then recovered by isoelectric precipitation (pH 5.5). An important

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step in the process is the removal of membrane phospholipids, which are highly susceptible to oxidation. Hemoglobin coprecipitated with the muscle protein isolate could oxidize any lipid remaining and may result in adverse color changes in the final protein isolate. For this reason it was of interest to investigate how different low- and high-pH treatments that might be used in preparing protein isolates might affect hemoglobin's solubility at pH 5.5, where the muscle proteins are precipitated. In addition, we were interested in studying what molecular changes in hemoglobin may account for changes in its solubility characteristics. This work will enable us to understand on a molecular level what affects the solubility of acid- and alkali-treated hemoglobin and provide suggestions of how coprecipitation can be minimized during processes requiring exposure of a mixture of muscle proteins and hemoglobin to high or low pH.

MATERIALS AND METHODS

Preparation of Trout Hemolysate/Hemoglobin. Rainbow trout (*Onchyrynchus mykiss*) was purchased from Mohawk Trout hatchery (Sunderland, MA) and kept live in a recirculating water bath (5 °C). Blood was drawn from the caudal vein of trout anesthetized in aminobenzoic acid, as described by Rowley (*16*). Hemolysate was prepared from the blood according to the method of Fyhn et al. (*17*) as modified by Richards and Hultin (*18*), where blood is washed and lysed to release hemoglobin. For all studies the hemoglobin levels were quantified spectrophotometrically as described by Hudzik (*19*).

Low- and High-pH Treatments of Hemoglobin. Hemolysates were added to distilled deionized H_2O (ddH_2O) containing no NaCl or 500 mM NaCl, to give a hemoglobin concentration of ~20 μM as determined by using the method of Hudzik (*19*). The pH of the hemoglobin solution was lowered to 3.5, 3, 2.5, 2, or 1.5 or increased to 10, 10.5, 11, 11.5, or 12 by HCl or NaOH titration, respectively. Hemoglobin solutions were held at these pH values for (a) 10 s or (b) 20 min to evaluate how the extent of pH-induced unfolding would affect solubility. The solutions were then readjusted to pH 5.5 by delivering a predetermined volume of HCl or NaOH into the solutions; the pH was checked and adjusted if necessary. To maintain the same protein concentration in all samples, water was added to those samples that required less HCl or NaOH addition on unfolding and pH readjustment. The effect of ionic strength (0−500 mM NaCl) was further assessed on samples subjected to pH 3 and 10.5 for 20 min and then readjusted to pH 5.5. The role of ionic strength was then further evaluated by subjecting hemoglobin to pH 1.5−3.5 in 20 and 50 mM NaCl for 20 min followed by adjustment to pH 5.5. The role of unfolding time was studied in more detail by unfolding hemoglobin at pH 2.5 in no salt and at pH 3.5 in 500 mM NaCl for 10, 30, and 90 s and 5, 10, 20, and 60 min, followed by pH readjustment. The role of different rates of unfolding from pH 2.5 and 3 in the absence of NaCl and from pH 3 in the presence of 50 mM NaCl was also studied. For these studies samples were acidified and then pH readjusted back in 10 s and 2, 30, 60, 100, and 180 min, with NaOH being titrated gradually to the samples (linear increase in pH with time).

Solubility of Hemoglobin after pH Treatments. The pH-adjusted hemoglobin solutions were tested for hemoglobin solubility by centrifuging them at 2200*g* for 10 min in a Sorvall RT6000 refrigerated centrifuge at 4 °C to sediment-aggregated hemoglobin. Supernatant containing soluble hemoglobin was collected and its protein content determined according to the method of Lowry et al. (*20*). Protein concentration in the supernatant after centrifugation was divided by the protein concentration of the protein solution before centrifugation and solubility reported as percent solubility. Controls were untreated hemoglobin at pH 5.5 and 7.

Effect of Low- and High-pH Treatments on Hemoglobin Conformation. *Unfolding Kinetics of Hemoglobin at Low and High pH.* Unfolding of hemoglobin (0.8 μM) with time at pH 1.5−3.5 and 10−12 (in $ddH_2O \pm 500$ mM NaCl) was studied by following changes in heme peak absorbance (408 nm) with time at 5 °C by scanning in the visible range from 600 to 350 nm in a Hitachi-3110 double-beam spectrophotometer (Hitachi Instruments, Inc., San Jose, CA) at 120 nm/min. Mixing time was ~2 s.

Conformation of Hemoglobin and Recovery of Native Structure upon pH Readjustment. Hemoglobin was added to acid (pH 1.5−3.5) and alkaline (pH 10−12) solutions and kept at these pH values for 10 s or 20 min. Refolding of hemoglobin was initiated by adding an aliquot of the low/high-pH sample to 4 volumes of 10 mM potassium phosphate buffer, giving a final pH of 7, and allowing the protein to refold for 1 h. This time was found to be sufficient because more time did not result in more refolding according to circular dichroism (CD) and visible spectra. The final hemoglobin concentration after refolding was ~0.8 μM. Hemoglobin kept at pH 2.5 and 3.5 for different times, as outlined in the previous section, was also refolded at pH 7 to examine the reversibility of unfolding. Recovery of native structure was also evaluated for hemoglobin unfolded at pH 3 for 20 min in 0−500 mM NaCl. For the time-dependent unfolding studies a hemoglobin solution was subjected to the same treatment as in the solubility study, but at much lower concentrations to avoid aggregation to determine native structure recovered. Evaluation of protein refolding was done by acquiring hemoglobin's visible spectra from 350 to 600 nm (as outlined above) and measuring hemoglobin tryptophan fluorescence emission. Measurements for tryptophan fluorescence used 280 nm as the excitation wavelength and emission was followed from 300 to 400 nm using a Hitachi F-2000 fluorescence spectrophotometer (Hitachi Instruments, Inc.). The extent of protein refolding after low- and high-pH unfolding was calculated on the basis of the change in the absorbance of the heme peak (Soret peak) for the refolded proteins versus the native proteins according to a two-state unfolding mechanism

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

where f represents the observed absorbance at 408 nm under the given conditions, and f_U and f_N are the corresponding values in the unfolded (absorbance at 408 nm in 6 M Gu-HCl) and native state (absorbance at 408 nm at pH 7), respectively.

Selected treatments (pH 2, 2.5, and 3.5 after 10 s and 20 min of unfolding followed by refolding to pH 7) were furthermore subjected to CD analysis to evaluate the effect on secondary structure. Changes in secondary structure were monitored by scanning hemoglobin solutions in a quartz cell from 260 to 190 nm using a JASCO J-715 spectropolarimeter (Jasco, Inc.) and a cell width of 2 mm. Resolution was set at 0.2 nm, bandwidth was 1 nm, sensitivity was 20 mdeg, response was 1 s, and scanning speed was 20 mdeg/min. Five spectra were acquired for each sample, and results are averaged in one spectrum. The ellipticity was calculated from the CD spectra in Microsoft Excel 2000 (Microsoft, Bellevue, WA) using the formula

$$[\theta] = [\theta]_{obs}(MRW)/10cl$$

where $[\theta]$ represents the mean residue molar ellipticity ($deg \cdot cm^2 \cdot dmol^{-1}$), $[\theta]_{obs}$ the degree ellepticity, c the protein concentration (g/mL), and l the quartz cell path length (cm).

RESULTS

To understand how different pH, time, and ionic strength combinations differed in their effect on the solubility of hemoglobin, the extent of unfolding with time was studied and the effect on hemoglobin conformation on pH readjustment (refolding) evaluated. Rate of unfolding as evaluated by the drop in heme peak absorbance (**Figure 1**) depended greatly on both the pH of the medium and the presence of 500 mM NaCl. There was an increase in unfolding rate as pH was lowered, with both pH 3.5 and 3 unfolding much more slowly than pH 1.5−2.5 in the absence of salt (**Figure 1a**). At pH 2 and 1.5 unfolding was complete within seconds, whereas it took >30 min for the protein at pH 3 and 3.5 to reach a stable absorbance value. In the presence of 500 mM NaCl unfolding proceeded much more quickly than in its absence (**Figure 1b**). At pH values from 1.5

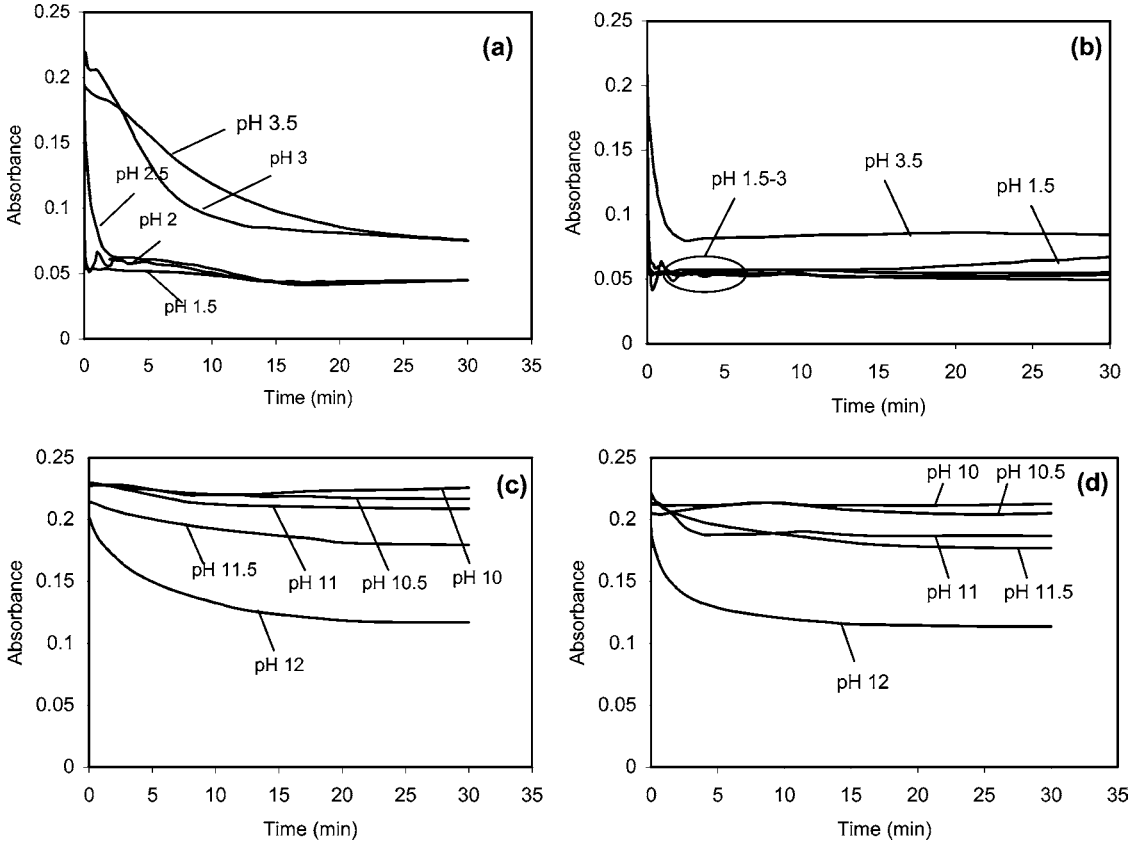

Figure 1. Changes in trout hemoglobin conformation with time as assessed by drop in heme group absorbance at 408 nm: (**a**) pH 1.5–3.5 in no NaCl; (**b**) pH 1.5–3.5 in 500 mM NaCl; (**c**) pH 10–12 in no NaCl; (**d**) pH 10–12 in 500 mM NaCl.

to 3 hemoglobin was almost instantaneously unfolded (within seconds), whereas it took hemoglobin at pH 3.5 ~2 min to reach a stable value.

At alkaline pH much more subtle changes in heme absorbance (**Figure 1c**,**d**) were observed, indicating that conformation was affected much less than at acid pH values. In the lower alkaline pH range (pH 10−11) the heme peak absorbance (and tryptophan fluorescence; data not shown) changed only slightly with time (**Figure 1c**). At pH 11.5−12 heme peak absorbance dropped significantly with time, more at pH 12 than at pH 11.5. The presence of salt aggravated this effect only slightly for pH 11.5 and 12 but did lead to a greater decrease in absorbance for pH 11 in salt compared to no salt (**Figure 1d**). It was therefore apparent that the rate and extent of unfolding greatly depended on how low (or high) the pH was and if salt was present in the solution. The question now became how these different extents of unfolding at different pH values and ionic strengths would affect the refolding of the hemoglobin molecule as samples were readjusted to neutrality.

Proper refolding of the protein from acid conditions to neutrality was affected by (a) how low the pH was, (b) how long the protein was kept at the low pH, and (c) the ionic strength of the mixture (**Figures 2** and **3**). For example, unfolding hemoglobin at pH 2.5 for 10 s and then refolding it resulted in only ~10−15% change in its visible spectra, tryptophan fluorescence intensity (**Figure 2**), and secondary structure (**Figure 3**). However, leaving the protein to unfold for 20 min at the same pH clearly resulted in a substantially improperly refolded protein from all conformational indicators. On the other hand, subjecting hemoglobin to pH 3.5 without 500 mM NaCl led to a protein structure with mostly native conformation on pH readjustment. Therefore, the lower the pH was and the longer the time at that pH, the less properly the protein was refolded. In the presence of salt proper refolding became even more difficult to achieve. After 20 min of unfolding in the presence of 500 mM NaCl, all low pH values yielded species less refolded than their counterparts in the absence of salt, except for samples at pH 3.5 for 10 s (**Figure 2b**,**e**,**f** and **3b**). It was also evident from the dissappearance of the two peaks between 550 and 700 nm (**Figures 2** and **4**) that oxygen binding was greatly affected by the extent of refolding and thus more in the presence of salt than in its absence.

As alkaline conditions clearly had less effect on hemoglobin conformation, pH readjustment led to only moderate changes in the tryptophan fluorescence or heme absorbance for hemoglobin subjected to pH 10−11.5 even after a 20 min unfolding period (**Figure 4**). The presence of salt also had much less effect at alkaline pH than at low pH (**Figure 4**). Hemoglobin conformation at pH 12 was, however, somewhat affected and as a result did not fully refold according to an increased tryptophan fluorescence and suppressed heme and oxygen peaks. It was interesting to note, however, that the partially refolded alkali-treated hemoglobin did not have a shifted heme peak absorbance like that of the acid-treated hemoglobin.

The solubility of the trout hemoglobin samples after various acid and alkali treatments was studied at pH 5.5. From **Figure 5a** it is evident that in the absence of salt the lower the unfolding pH and the longer the unfolding time (20 min vs 10 s), the larger the drop in hemoglobin solubility at pH 5.5. Adjusting the hemoglobin solution to pH 3.5 for 10 s or 20 min led to a solubility similar to that of the pH 5.5 control, whereas pH 3.0 resulted in only a slight loss of solubility compared to the control. Lowering the pH further led to more loss in solubility on pH readjustment, with the longer unfolding time more negatively affecting solubility compared to the short unfolding

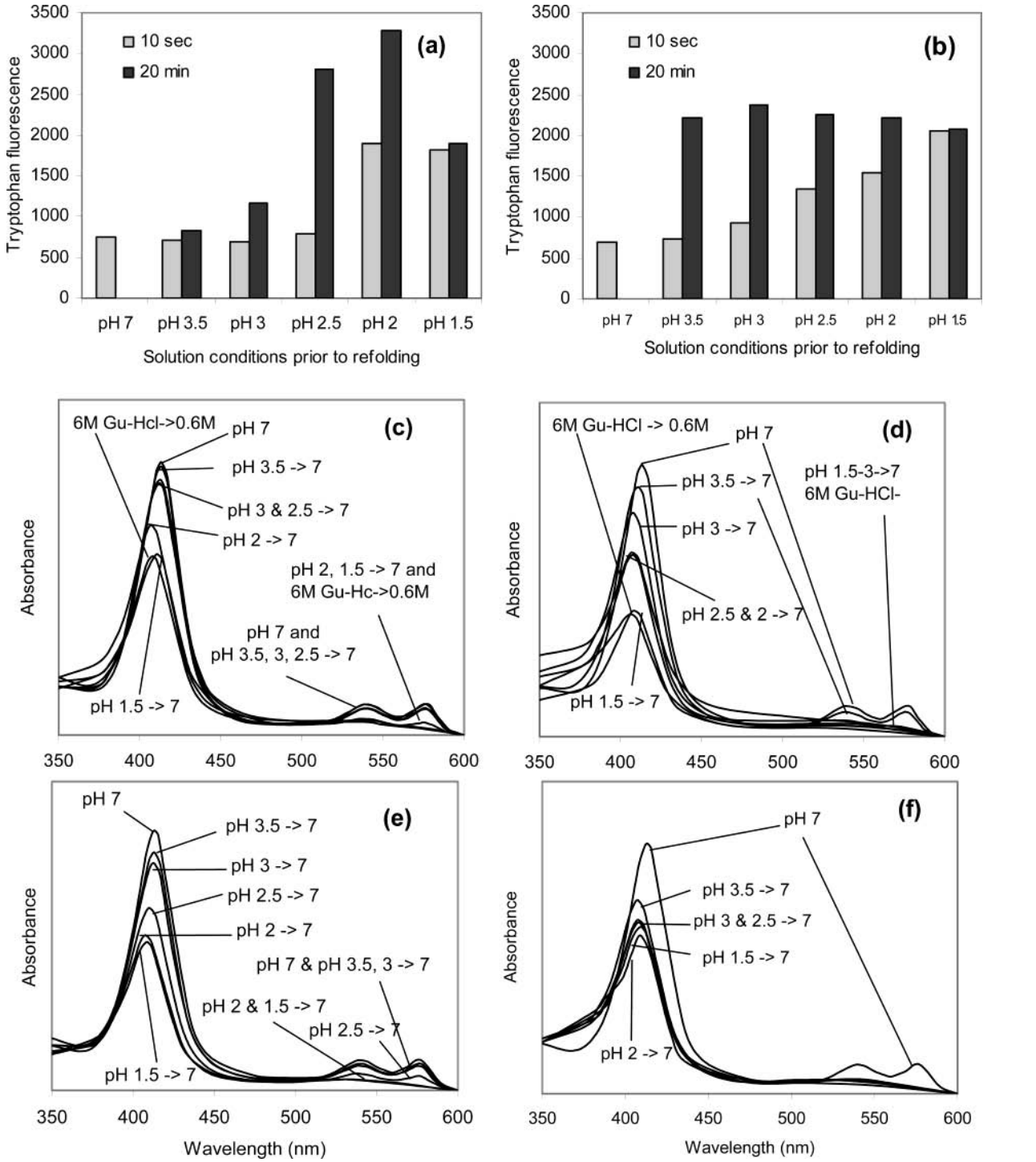

Figure 2. Effect of refolding from acid pH (1.5–3.5) on trout hemoglobin conformation. Tryptophan fluorescence intensity of refolded hemoglobin was measured in the absence (**a**) or presence (**b**) of 500 mM NaCl; visible spectra of refolded protein were taken after 10 s (**c**) or 20 min (**d**) at low pH in no NaCl; visible spectra of refolded protein were taken after 10 s (**e**) or 20 min (**f**) at low pH in 500 mM NaCl. For comparison, hemoglobin was unfolded in strong denaturant (6 M Gu-HCl) and refolded after 10 s and 20 min. The fluorescence values for these hemoglobins were 3180 after 10 s of treatment and 6538 after 20 min of treatment.

time. The two unfolding times did not, however, appear to significantly differ for pH 2.

When hemoglobin was acidified in the presence of salt (**Figure 5b**), a different story emerged compared to that in the absence of salt. Although short unfolding at pH 3.5 and 3 in salt had little negative effect, extended unfolding (20 min) at all low pH values tested drastically reduced the solubility of hemoglobin when acidified to pH 5.5. Only 10 s of unfolding at and below pH 2.5 significantly reduced the solubility of hemoglobin at pH 5.5, yielding results similar to those obtained at 20 min of unfolding.

The effect of alkaline treatment on solubility at pH 5.5 was also studied (**Figure 5c**,**d**). Subjecting hemoglobin to pH 10−11.5 for 10 s or 20 min, in salt or no salt, had little effect on hemoglobin's solubility when adjusted to pH 5.5, compared to the control at pH 5.5. Only at pH 12 was there a significant drop in solubility. Solubility at pH 12 was almost the same for the two holding periods.

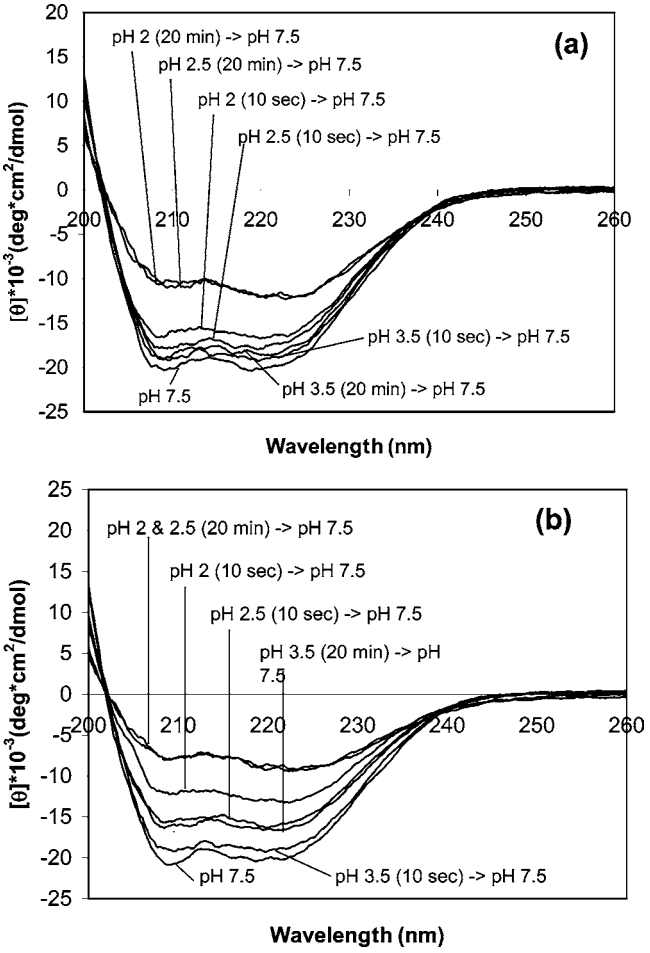

Figure 3. Recovery of secondary structure on refolding hemoglobin from pH 2, 2.5, and 3.5 after 10 s or 20 min in the absence (**a**) or presence (**b**) of 500 mM NaCl compared to native hemoglobin.

The sensitivity of hemoglobin to unfolding time was further investigated by keeping the protein at pH 2.5 in no salt and at pH 3.5 in 500 mM NaCl for various times as indicated in **Figure 6**. It is evident that increased unfolding time did result in more loss in solubility at pH 5.5, more dramatically for the protein at high versus low ionic strength (**Figure 6**). The extent of refolding upon pH readjustment was calculated and plotted in **Figure 6** and shows that the drop in solubility was accompanied, or caused by, less refolding. There was an excellent correlation with the level of refolding and solubility in the absence of salt, but in the presence of salt solubility dropped more that the drop in percent native heme peak, suggesting a direct role of the salt in the solubility loss.

As diluted homogenized muscle, which is the starting material in the acid and alkaline process, could have different ionic strengths depending on the dilution and the final use of the material, the salt sensitivity of pH-readjusted trout hemoglobin was further investigated by (a) unfolding hemoglobin at pH 3 for 20 min in 0−500 mM NaCl (**Figure 7a**) and (b) unfolding hemoglobin at pH 1.5−3.5 for 20 min in 20 and 50 mM NaCl (**Figure 7b**) followed by readjustment to pH 5.5. From **Figure 7a** it is clear that even the presence of relatively low amounts of salt (e.g., 20 mM) led to significantly reduced solubility at pH 5.5 and that solubility was gradually reduced as ionic strength increased. Furthermore, it can be seen from **Figure 7b** that in the presence of 20 and 50 mM NaCl, the lower the unfolding pH, the lower the solubility at pH 5.5, with the higher salt concentrations reducing solubility more than the lower salt concentration, except at pH 1.5. In terms of hemoglobin conformation the results show that increased NaCl concentration did lead to a less refolded protein, as assessed by heme peak absorbance (**Figure 7a**). However, in salt the level of refolding did not show the same correlation with solubility as hemoglobin did in the absence of salt, signifying that the ionic strength dependent loss of solubility of the refolded protein is caused by more factors than just different levels of refolding.

One idea to potentially manipulate the solubility behavior of hemoglobin on refolding was to determine if unfolded hemoglobin could be trapped into different intermediate states by different rates of refolding. This was tested by acidifying hemoglobin in no salt or in the presence of 50 mM NaCl (to see if ionic strength had any effect) and then readjusting the pH gradually to pH 5.5 in 2, 30, 60, 100, and 180 min, with the pH increase with time being linear. Results show that increasing pH-readjustment time (i.e., increasingly slower pH increase) was accompanied by a greater loss in solubility at pH 5.5 for samples with no added salt (**Figure 8a**). The drop in solubility was more for hemoglobin readjusted from pH 2.5 versus pH 3. In the presence of salt the same decrease in solubility was seen with increase in pH-readjustment time (**Figure 8b**); however, the drop in solubility was considerably greater in comparison with the results in the absence of salt. Extent of refolding was less as more time was taken to readjust hemoglobin from acidic conditions (**Figures 8**).

DISCUSSION

The solubility of proteins is controlled by a sensitive balance between repulsive electrostatic forces and attractive hydrophobic forces, which depend on the protein surface residues and solution conditions. A recent process builds upon the fact that muscle proteins solubilize at highly acid or alkaline pH values, enabling one to selectively extract them from other constituents of muscle. The proteins are then precipitated at their isoelectric point, pH ∼5.5 (*1*). Many raw materials used for this process are high in heme proteins, such as hemoglobin, and their presence in the protein isolate is highly undesirable as they could lead to major color and oxidation problems (*14*). At pH 5.5 the majority of trout hemoglobin is expected to remain in solution. Hemoglobins in fish are variable both within and between species. Trout has both cationic and anionic hemoglobin components. The primary component of trout hemoglobin (IV) is anionic (*8*), and because native hemoglobin was largely soluble at pH 5.5, its isoelectric point is suggested to be higher than that of myofibrillar proteins. However, when hemoglobin is subjected to unfolding pH values, it may readily coprecipitate with the improperly refolded and hydrophobic muscle proteins at pH 5.5. In a commercial operation, processing times, pH values, and ionic strengths can vary. For that reason it was of much interest and importance to systematically study and understand how unfolding of hemoglobin at different low- and high-pH values would affect its solubility at pH 5.5, at which the protein isolate is precipitated.

It has been demonstrated that hemoglobin is denatured to different extents as it experiences very low or high pH values (*14*). It is known that refolding a denatured protein from a low or high pH is sometimes accompanied by transient association of partly folded intermediates (*21*−*25*). This propensity to associate or aggregate is moreover believed to be a general characteristic that many non-native proteins share (*21*, *25*). In the refolding pathway the protein may become trapped in an energy well, making the protein unable to attain its native form (*26*). The formation of aggregates would therefore be the result of nonspecific interactions between exposed hydrophobic areas

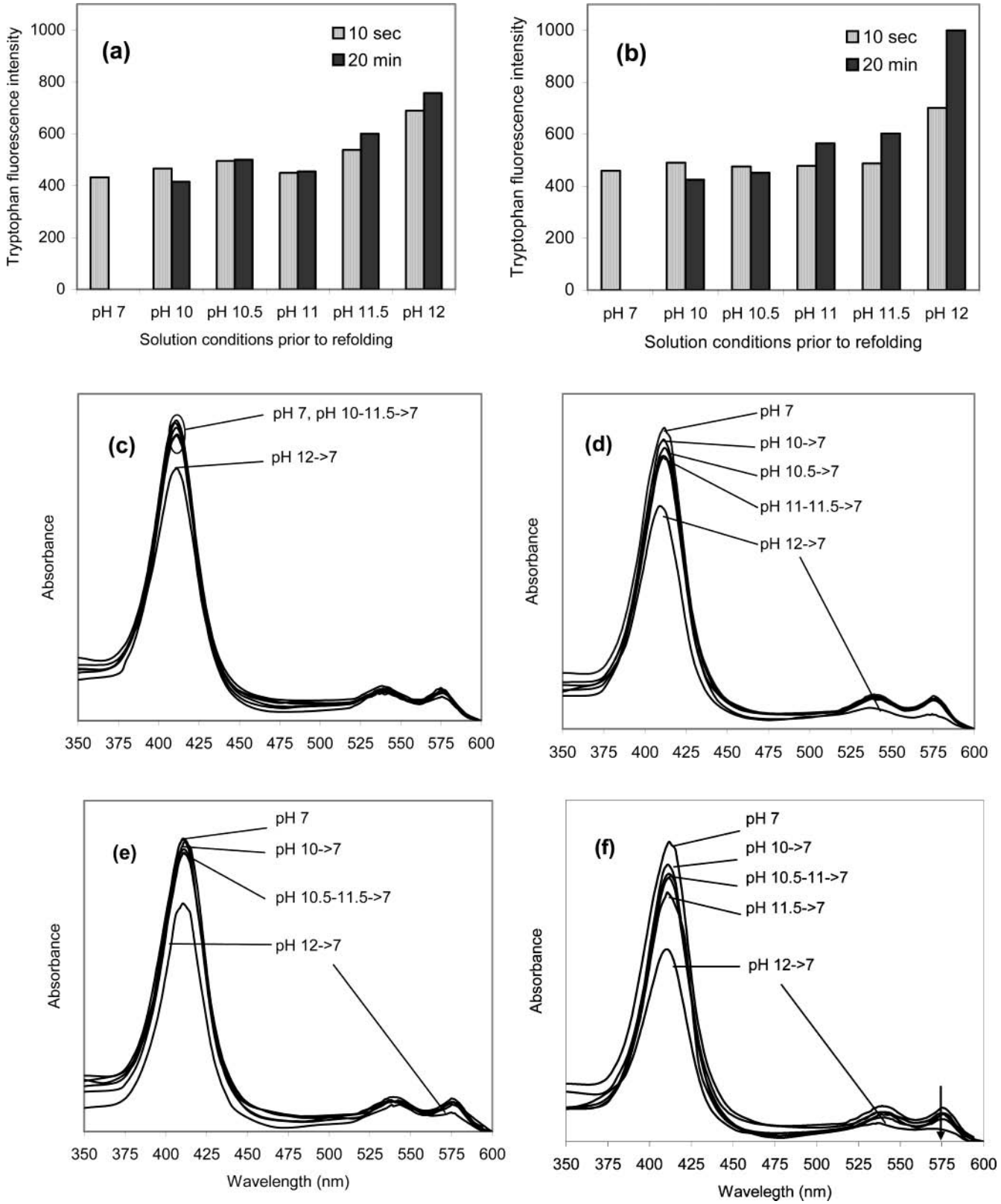

Figure 4. Effect of refolding from alkaline pH (10–12) on trout hemoglobin conformation. Tryptophan fluorescence intensity of refolded hemoglobin was measured in the absence (**a**) or presence (**b**) of 500 mM NaCl; visible spectra of refolded protein were taken after 10 s (**c**) or 20 min (**d**) at alkaline pH in no NaCl; visible spectra of refolded protein were taken after 10 s (**e**) or 20 min (**f**) at alkaline pH in 500 mM NaCl.

in the intermediates in the folding pathway (*27*). The more improperly the protein is refolded, the more hydrophobic sites are expected to be exposed and in turn the higher the tendency of the protein to aggregate. To test this, hemoglobin was subjected to several different low and high pH values for different durations of time. It was clear from the results with hemoglobin that the more unfolded the protein was (as a result of an increasingly lower or higher pH and increased unfolding time), the more difficult it became to refold the protein back to its native state on pH readjustment, as assessed by heme

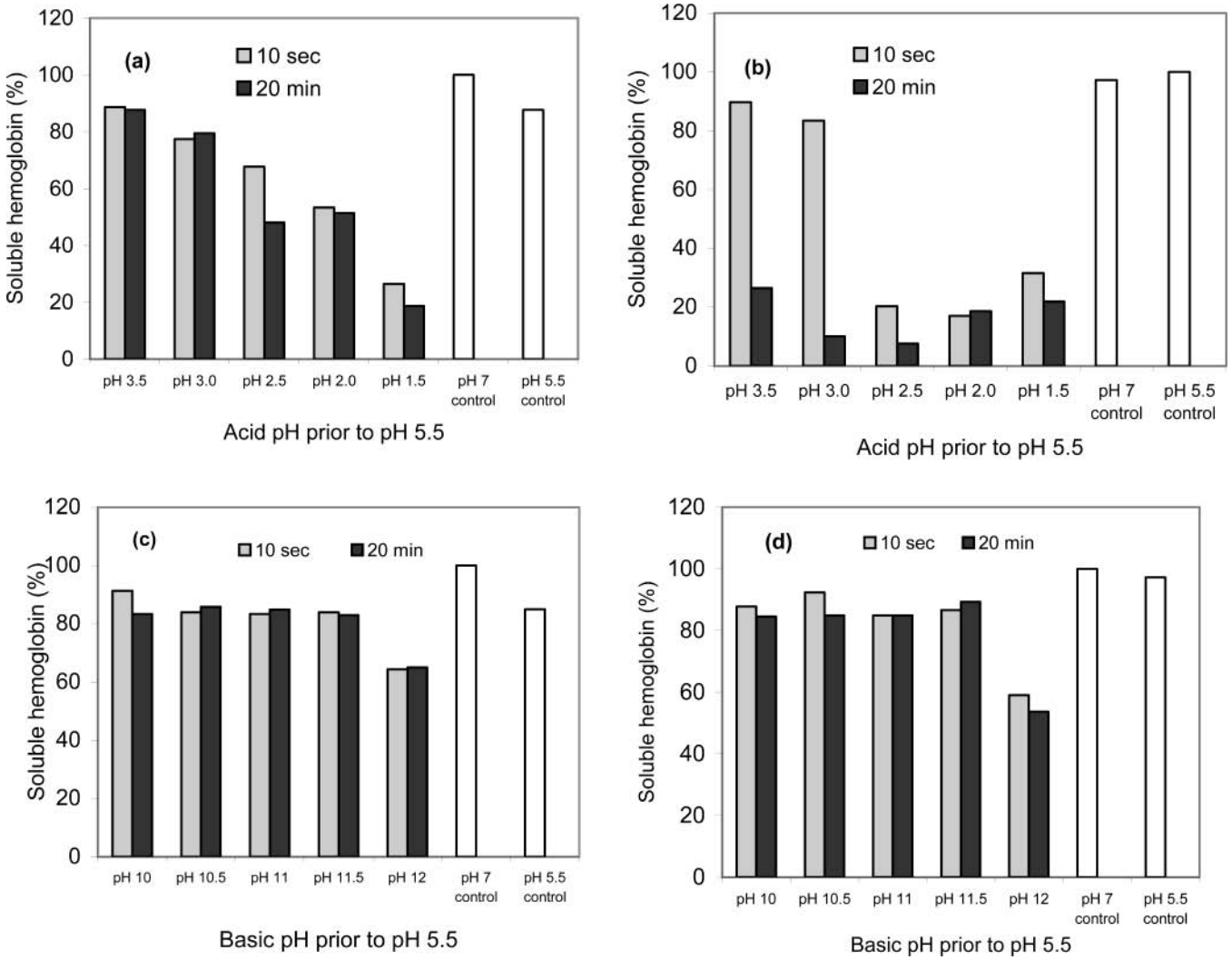

Figure 5. Effect of acid and alkaline pH on the solubility of trout hemoglobin readjusted to pH 5.5: (**a**) pH 1.5–3.5 in the absence of added NaCl; (**b**) pH 1.5–3.5 in the presence of 500 mM NaCl; (**c**) pH 10–12 in the absence of added NaCl; (**d**) pH 10–12 in the presence of 500 mM NaCl. Controls were hemoglobin at pH 7 and 5.5 without any pH treatment.

environment, tryptophan fluorescence, and secondary structure (**Figures 2**, **3**, and **6**). These data are consistent with previous studies showing that a longer duration at low pH for both hemoglobin and myoglobin leads to less refolded proteins (*28*−*30*). A similar finding was observed for alkali-unfolded pepsinogen (*31*). The longer the refolding time of hemoglobin from acid pH, the more improperly it refolded, more so for pH 2.5 than for pH 3 (**Figure 7**). This is in agreement with the results of Shen and Hermans (*30*), who found that myoglobin needed a longer time to refold to a stable conformational state when unfolded at low pH (3.4) versus moderately low pH (4.16) (*30*). The important finding in the current study is that the less hemoglobin was refolded, the lower its solubility at pH 5.5 (**Figures 5**−**8**). The more improperly refolded the protein was after pH readjustment, the more exposed hydrophobic groups would be on the protein surface. An increase in tryptophan fluorescence, as seen here with the pH-readjusted protein, has been found to correlate well with increase in hydrophobicity (*14*). The conformational data (**Figure 2** and **3**) reveal that it is very likely that an increase in hydrophobicity and concurrent loss in solubility stem from the environment around the heme group in hemoglobin, which would be left exposed in improperly refolded hemoglobin. This would also explain the loss in oxygen binding because a proper conformation of the heme environment is required for oxygen binding. Our data are consistent with the data from Shen and Hermans (*32*), who suggested that the presence of heme interferes with the refolding of acid-unfolded myoglobin. Free heme is known to be extremely prone to aggregate in solution (*33*), so its increased exposure not surprisingly would be expected to lead to decreased solubility. Recent studies indicate that low pH may irreversibly convert hemoglobin to a denatured low-spin compound, called a hemichrome (*5*, *34*, *35*). This form has been associated with unfolding intermediates (i.e., molten globules) of hemoglobin (*35*). This species has its heme group exposed and a narrowed heme pocket and as a result polymerizes with other hemicromes and precipitates, that is, loses solubility (*34*, *35*). It is possible that this species is formed at low pH in the current study and may in part be responsible for the reduction in solubility.

Why longer unfolding time and lower pH led to less refolding can be understood from looking at the time-dependent unfolding of the protein (**Figure 1**). The unfolding rate increased considerably as the pH was lowered from 3.5 to 1.5. This $[H^+]$ dependency on unfolding has been previously noted for sperm whale apomyoglobin and has been linked to the more rapid protonation of histidine residues as the pH becomes lower, many of which are confined to the environment around the heme group (*32*). For example, it was easier to refold the protein from pH

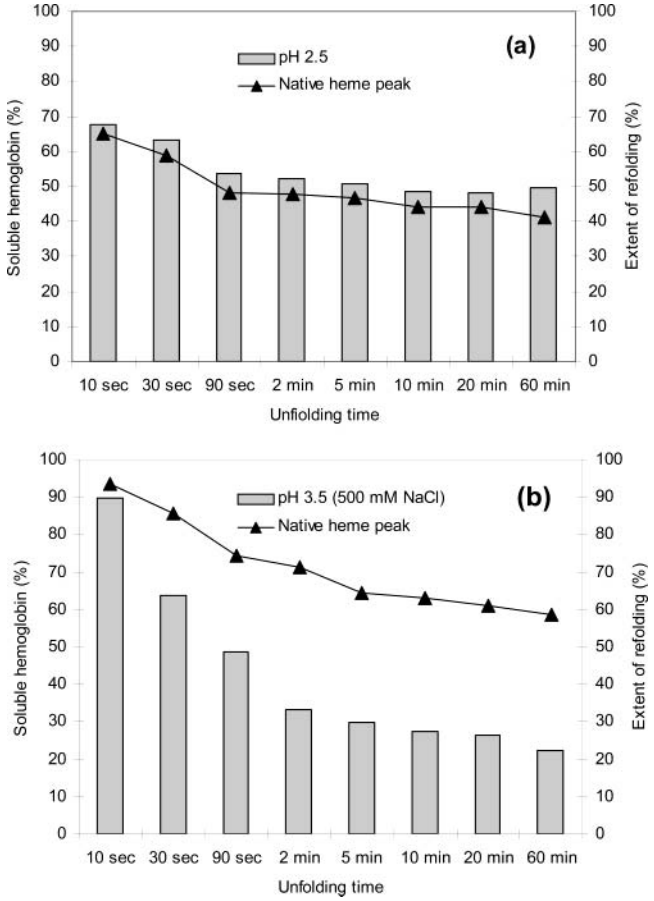

Figure 6. Effect of unfolding time on hemoglobin solubility at pH 5.5 and extent of refolding: (**a**) hemoglobin unfolded at pH 2.5 in no salt; (**b**) hemoglobin unfolded at pH 3.5 in 500 mM NaCl. Extent of refolding was calculated on the basis of the absorbance value at 408 nm after refolding, with the absorbance value at pH 7 representing a fully folded protein (100%) and the absorbance in 6 M Gu-HCl representing fully unfolded (0%). Solubility of acid-treated hemoglobins was calculated as a percentage of fully soluble untreated hemoglobin at pH 5.5 (hemoglobin was ~90% soluble at pH 5.5 in comparison to pH 7).

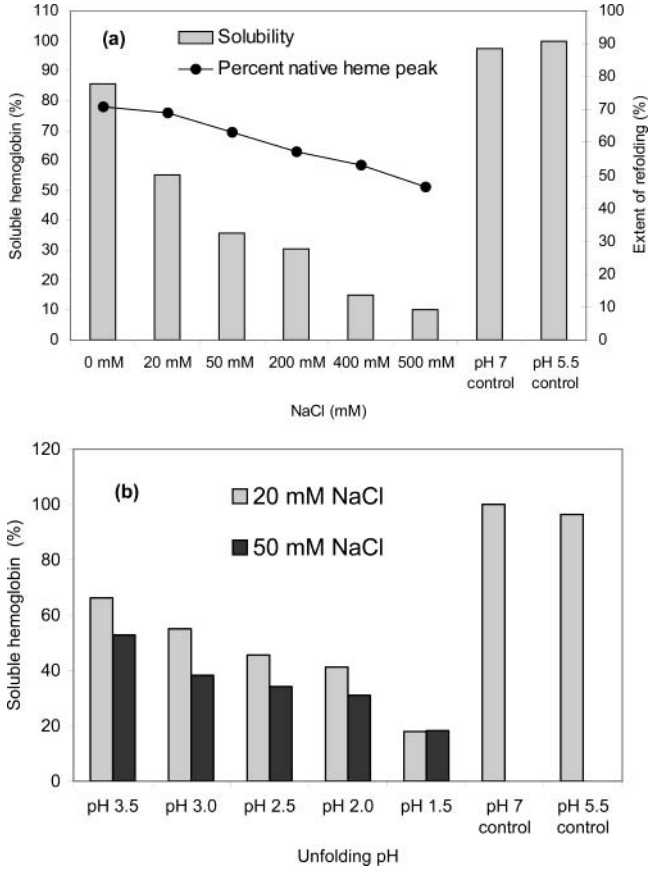

Figure 7. Role of ionic strength in solubility behavior of hemoglobin refolded from acid pH to pH 5.5: (**a**) solubility and recovery of native heme peak of hemoglobin at pH 5.5 after 20 min of unfolding at pH 3 in the presence of 0–500 mM NaCl; (**b**) influence of 20 and 50 mM NaCl on solubility of hemoglobin refolded after 20 min at pH 1.5–3.5. Controls were hemoglobin held for 20 min at pH 7 and 5.5.

2.5 after 10 s compared to pH 1.5 after 10 s because the former had unfolded less in that time period. The same applies in general for the longer unfolding times; for example, it was easier to refold the protein from pH 3 versus pH 2.5 after 20 min because the latter had unfolded to a greater extent than the former in that time. If irreversible hemichromes are formed at low pH, a longer unfolding time would allow for a larger formation of these and thus a lower amount of refolded proteins would be expected after pH readjustment. Possibly the pH-readjusted samples contain a mixture of irreversibly unfolded protein (e.g., hemichromes), partially refolded proteins (i.e., molten globules), and fully refolded proteins. The more irreversible unfolded and partially refolded proteins there would be in the samples after pH readjustment, the lower would be the expected solubility because both forms are known to have low solubility (*26*). Hydrophobic interactions may be more easily formed with misfolded proteins. At pH 5.5, partially refolded and unfolded hemoglobin, in contrast to native hemoglobin, would have a propensity to aggregate largely dependent on how exposed its hydrophobic groups are, as results did in fact indicate.

The lack of proper refolding on pH readjustment is also likely caused by the presence of increasingly more ions as the pH is lowered. These results show that the pH-readjusted hemoglobins were more sensitive to ionic strength than native hemoglobin, a result similarly observed for many partially unfolded/refolded globular proteins including the molten globular state (*36*−*38*). For example, at pH 1.5 substantially more HCl has been added compared to at pH 2.5, which means that on refolding NaCl concentration will be higher (due to more addition of HCl to reach pH 1.5 and more NaOH required to reach pH 5.5 compared to pH 2.5). There are many possible mechanisms through which the presence of ions may interfere in the refolding of the protein. It has, for example, been demonstrated that as the pH is lowered, the proteins form progressively more stable, but yet hydrophobic, molten globular forms due to increasing levels of Cl^- ions (*14*, *38*). The more stable these molten globules are, the more they may have resisted refolding to the native state on pH readjustment, thus leading to less refolded proteins. In addition to this, increased ionic strength would also screen electrostatic repulsive interactions, allowing enhanced protein association via hydrophobic interactions (*21*, *39*, *40*). That the solubility of hemoglobin unfolded at pH 1.5 was almost as low after 20 min compared to 10 s of unfolding further strengthens this ionic dependency theory. If solubility was solely governed by the extent of refolding, the protein should have a higher solubility after 10 s of unfolding at low pH compared to 20 min of unfolding because refolding was higher in the former as assessed by UV−vis (**Figure 2**). This was not the case. The drop in solubility is therefore a combination of a faster rate of unfolding and an increased ionic strength after refolding when the sample is held at increasingly lower pH values. The increase

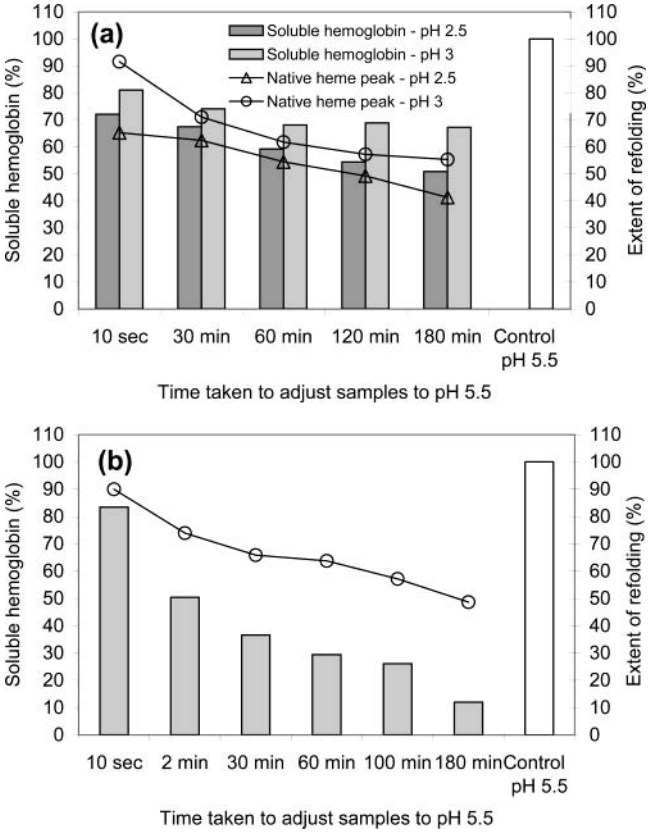

Figure 8. Effect of time used to refold hemoglobin from acid pH conditions on solubility and recovery of native heme peak at pH 5.5: (**a**) hemoglobin was refolded from pH 2.5 and 3; (**b**) hemoglobin was refolded from pH 3 in the presence of 50 mM NaCl. Control was hemoglobin at pH 5.5 held for 20 min (there was minimal change in solubility with holding time at pH 5.5). Native heme peak was calculated as described in **Figure 2**.

in ionic strength does, however, not appear to become important until very low pH (below pH 2). At pH values typically used in the acid and alkali process (pH from 2.5 to 3.5; pH from 10.5 to11), increased unfolding time leading to less refolding seems to be the governing factor in causing the subsequently reduced solubility.

Although improper refolding could explain most of the loss in solubility for hemoglobin in the absence of salt (except for highly acidic pH values), it cannot do so for hemoglobin unfolded and refolded in the presence of increasing concentrations of NaCl. This is clearly demonstrated by the results in 500 mM NaCl (**Figures 2**, **3**, **5**, and **6**) and in increasing ionic strength at pH 3 (**Figure 7**), which show that an increased level of salt led to less solubility. This is interesting because hemoglobin is actually known to have increased solubility as NaCl ionic stength is increased (*41*). This highlights the importance of improper refolding in the presence of salt. The dramatic loss in solubility after acid unfolding in the presence of salt corresponded in all cases to less refolding compared to results in the absence of salt. At the same pH and time of unfolding the proteins in salt had refolded less and had lower solubility than those in no salt. This suggests that hemoglobin resisted refolding after being subjected to salt at low pH. Looking at the time-dependent unfolding in 500 mM NaCl (**Figure 1c**,**d**), it can be seen that in salt the proteins unfolded much more quickly than in its absence, which in part could explain why they were more difficult to refold. This ionic strength-dependent rate of unfolding has been previously demonstrated for myoglobin, which was found to unfold much more quickly at 200 mM NaCl (*42*) compared to the same protein in no salt (*43*, *44*), in agreement with our findings in this paper. It is well-known that high ionic strength destabilizes the native state of myoglobin at low pH, thus leading to more rapid unfolding compared to low ionic strength (*43*−*48*). This is because in high ionic strength the pK_a values of the histidines in myoglobin (and hemoglobin) are shifted up and more positively charged imidazole side groups are therefore expected in salt versus no salt at a given low pH (*48*). Furthermore, the acid-unfolded hemoglobins in the presence of salt were likely far more difficult to refold to their native state than their counterparts in the absence of salt due to interfering action of the high amount of NaCl ions present during refolding, as previously discussed. This is strongly indicated by the generally larger lack of heme environment refolding, larger blue shift of heme peak on refolding, and more incomplete oxygen binding capacity on refolding (due to improper refolding of heme environment) of hemoglobin in the presence versus the absence of NaCl. However, the much larger loss in solubility than the accompanying loss in structure at pH 5.5 in the presence of increasing levels of NaCl suggests that charge screening may play a more important role compared to its effect on the extent of refolding.

Although acid pH had a striking and rather complicated effect on the solubility of hemoglobin after pH readjustment, the same treatments at alkaline pH values up to 11.5 had little effect (**Figure 5**). This is not surprising as alkaline conditions (pH 10−11.5) have a only a small effect on the conformation of hemoglobin, as illustrated by the time-dependent unfolding (**Figure 1c**,**d**). As these pH values had little effect on conformation, the proteins were mostly in their native state on pH readjustment (**Figure 4**), which explains why the solubility was little affected. The solubility was, however, negatively affected for samples subjected to pH 12, as this pH did partially unfold the protein (**Figure 1c**,**d**) and only partial refolding was achived on pH readjustment (**Figure 4**). Exposure to pH 12 likely exposed more hydrophobic regions on the hemoglobin compared to proteins subjected to pH 10−11.5, therefore causing increased aggregation. What is interesting to note is that an unfolding time of 20 min in the presence of salt at pH 12 led to a less refolded species compared to that in the absence of salt, but the solubility was the same for both species, at ∼60%. These results were in stark contrast to corresponding results at acid pH. As a comparison, hemoglobin refolded from pH 2.5 after 20 min refolded to a similar extent (as measured by heme peak absorbance) as hemoglobin at pH 12 did, but it was <10% soluble. A clue for this difference may lie in the positioning of the heme peak of the refolded protein. Unfolding in salt at acid pH led to a refolded species with a significantly blue-shifted, suppressed heme peak, whereas unfolding at pH 12 led to a similarly suppressed peak but at the same wavelength as the native peak at pH 7. The spectra suggest that the bond between the heme group and the globin (between histidine and the heme iron) is not disrupted by alkali treatment (in contrast to acid treatment), thus leaving the heme groups and heme crevice less exposed and thus having lower tendency than the acid-unfolded hemoglobin to aggregate due to lower surface hydrophobicity (*14*, *45*, *46*). It is known that hemi/hemochrome structures of hemoglobin can form at high pH (*5*). However, these are reversible on pH readjustment, in contrast to the structures formed at low pH. These results therefore suggest that alkali-induced unfolding has a fundamentally different effect on hemoglobin conformation and native structure recovery than

acid-induced unfolding, which leads to a less detrimental effect on solubility.

CONCLUSION

This study showed that the solubility of hemoglobin after acid and alkali treatment is highly influenced by the conformation of the protein. The more misfolded the protein is, the lower is its solubility at pH 5.5, suggesting that change in conformation is a driving force for loss in solubility. However, the data also suggest that the presence of salts may play an important role in solubility loss, likely via direct interference in the refolding of hemoglobin and also in enhancing hydrophobic interactions of the refolded protein. These results show that to be able to keep hemoglobin soluble at pH 5.5 and minimize its coprecipitation with muscle proteins recovered in the acid and alkali fish protein recovery process, it is essential to (a) not reduce the pH too low, (b) not hold hemoglobin too long at low pH, (c) readjust the pH from low pH quickly, and (d) keep ionic strength as low as possible. From the data presented it appears that the use of the alkali process will lead to substantially fewer problems with hemoglobin and could therefore be useful for species high in heme proteins.

ABBREVIATIONS USED

Hb-Fe^{2+}-O_2, reduced state oxyhemoglobin; Hb-Fe^{2+}, reduced state deoxyhemoglobin; Hb-Fe^{3+}, met-hemoglobin; •Hb-Fe^{4+}═O, perferryl hemoglobin; Hb-Fe^{4+}═O, ferrylhemoglobin; ddH_2O, distilled deionized water; ANS, 8-anilino-1-naphthalenesulfonic acid.

LITERATURE CITED

(1) Hultin, H. O.; Kelleher, S. D. Surimi processing from dark muscle fish. In *Surimi and Surimi Seafood*; Park, J. W., Ed.; Dekker: New York, 2000; pp 59−77.

(2) Richards, M. P.; Hultin, H. O. Contributions of blood and blood components to lipid oxidation in fish muscle. *J. Agric. Food Chem.* **2002**, *50*, 555−564.

(3) Connell, J. J. *Control of Fish Quality*; Whitefriars Press: London, U.K., 1975.

(4) Wheaton, F. W.; Lawson, T. B. *Processing Aquatic Food Products*; Wiley: New York, 1985.

(5) Baron, C. P.; Andersen, H. J. Myoglobin-induced lipid oxidation. A review. *J. Agric. Food Chem.* **2002**, *50*, 3887−3897.

(6) Halliwell, B.; Gutteridge, J. M. C. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.* **1986**, *246*, 501−514.

(7) Alayash, A. I. Hemoglobin-based blood substitutes: oxygen carriers, pressor agents, or oxidants? *Nat. Biotechnol.* **1999**, *17*, 545−549.

(8) Riggs, A. Properties of fish hemoglobins. In *Fish Physiology*; Hoar, W. S., Randall, D. J., Eds.; Academic Press: New York, 1970; pp 209−252.

(9) Perutz, M. F.; Brunori, M. Stereochemistry of cooperative effects in fish and amphibian haemoglobins. *Nature* **1982**, *299*, 421−426.21. De Young, A.; Kwiatkowski, L. D.; Noble, R. W. Fish hemoglobins. *Methods Enzymol.* **1994**, *231*, 124−150.

(10) Mylvaganam, S. E.; Bonaventura, C.; Bonaventura, J.; Getzoff, E. Structural basis for the Root effect in haemoglobin. *Nat. Struct. Biol.* **1996**, *3*, 275−283.

(11) Manning, J. M.; Dumoulin, A.; Li, X.; Manning, L. R. Normal and abnormal protein subunit interactions in hemoglobins. *J. Biol. Chem.* **1998**, *273*, 19359−19362.

(12) Shikama, K. The molecular mechansim of autoxidation for myoglobin and hemoglobin: A venerable puzzle. *Chem. Rev.* **1998**, *98*, 1357−1373.

(13) Hargrove, M. S.; Olson, J. S. The stability of holomyoglobin is determined by heme affinity. *Biochemistry* **1996**, *35*, 11310−11318.

(14) Kristinsson, H. G. Conformational and functional changes of hemoglobin and myosin induced by pH: Functional role in fish quality. Ph.D. dissertation, University of Massachusetts at Amherst, 2002.

(15) Hultin, H. O.; Kelleher, S. D.; Feng, Y.; Kristinsson, H. G.; Richards, M. P.; Undeland, I. A.; Ke, S. High Efficiency Alkaline Protein Extraction, U.S. Patent Appl. 60/230,397, 2000.

(16) Rowley, A. F. Collection, separation and identification of fish leucocytes. In *Techniques in Fish Immunology*; Stolen, T. C. F. J. S., Anderson, D. P., Robertson, B. S., van Muiswinkel, W. B., Eds.; SOS Publications: NJ, 1990; pp 113−135.

(17) Fyhn, U. E.; Fyhn, H. J.; Davis, B. J.; Powers, D. A.; Fink, W. L.; Garlick, R. L. Hemoglobin heterogeneity in amazonian fishes. *Comp. Biochem. Biophys.* **1979**, *62A*, 39−66.

(18) Richards, M. P.; Hultin, H. O. Effect of pH on lipid oxidation using trout hemolysate as a catalyst: a possible role for deoxyhemoglobin. *J. Agric. Food Chem.* **2000**, *48*, 3141−3147.

(19) Hudzik, E. Comparison of haem iron content estimation according to Hornsey's method with aqueous extracts absorption at 410 nm. *Sci. Aliment.* **1990**, *10*, 209−213.

(20) Lowry, O. H.; Rosenbrough, N. J.; Farr, L. A.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

(21) Tanford, C. Protein denaturation. *Adv. Protein Chem.* **1968**, *23*, 121−282. Fink, A. L.; Calciano, L. J.; Goto, Y.; Kurotsu, T.; Palleros, D. R. Classification of acid denaturation of proteins: Intermediates and unfolded states. *Biochemistry* **1994**, *33*, 12504−12511.

(22) De Young, A.; Kwiatkowski, L. D.; Noble, R. W. Fish hemoglobins. *Methods Enzymol.* **1994**, *231*, 124−150.

(23) Ptitsyn, O. B. Molten globule and protein folding. *Adv. Protein Chem.* **1995**, *47*, 83−229.

(24) Uversky, V. N.; Segel, D. J.; Doniach, S.; Fink, A. L. Association-induced folding of globular proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 5480−5483.

(25) Kuznetsova, I. M.; Biktashev, A. G.; Khaitlina, S. Y.; Vassilenko, K. S.; Turoverov, K. K.; Uversky, V. N. Effect of self-association on the structural organization of partially folded proteins: inactivated actin. *Biophys. J.* **1999**, *77*, 2788−2800.

(26) Kumar, S.; Ma, B.; Tsai, C.-J.; Sinha, N.; Nussinov, R. Folding and binding cascades: Dynamic landscapes and population shifts. *Protein Sci.* **2000**, *9*, 10−19.

(27) Price, N. C. Conformational issues in the characterization of proteins. *Biotechnol. Appl. Biochem.* **2000**, *31*, 29−40.

(28) Geddes, R.; Steinhardt, J. Factors affecting the release by dilute acid of hidden prototropic groups in carbonylhemoglobin. *J. Biol. Chem.* **1968**, *243*, 6056−6063.

(29) Allis, J. W.; Steinhardt, J. Acid denaturation of carbonylhemoglobin. Protein unfolding without heme detachment. *Biochemistry* **1970**, *9*, 2286−2293.

(30) Shen, L. L.; Hermans, J. Kinetics of conformation change of sperm-whale myoglobin. I. Folding and unfolding of metmyoglobin following pH jump. *Biochemistry* **1972**, *11*, 1836−1841.

(31) McPhie, P. Swine pepsinogen folding intermediates are highly structured, motile molecules. *Biochemistry* **1982**, *21*, 5509−5515.

(32) Shen, L. L.; Hermans, J. Kinetics of conformation change of sperm-whale myoglobin. III. Folding and unfolding of apomyoglobin and the suggested overall mechanism. *Biochemistry* **1972**, *11*, 1845−1849.

(33) Kobald, M.; Luck, T.; Holley, W.; Klostermeyer, H. Decolorization of slaughterhouse thick blood for the recovery of a colourless protein preparation and an iron-containing heme fraction. In *Food Proteins: Structure and Functionality*; Schwenke, K. D., Mothes, R., Eds.; VCH: Weinheim, Germany, 1991; pp 103−105.

(34) Sugawara, Y.; Kadono, E.; Suzuki, A.; Yukuta, Y.; Shibasaki, Y.; Nishimura, N.; Kameyama, Y.; Hirota, M.; Ishida, C.; Higuchi, N.; Haramoto, K.; Sakai, Y.; Soda, H. Hemichrome formation observed in human haemoglobin A under various buffer conditions. *Acta Physiol. Scand.* **2003**, *179*, 49−59.
(35) Robinson, V. L.; Smith, B. J.; Arnone, A. A pH-dependent aquomet-to-hemichrome transition in crystalline horse methemoglobin. *Biochemistry* **2003**, *42*, 10113−10125.
(36) Ptitsyn, O. B. Protein folding: hypotheses and experiments. *J. Protein Chem.* **1987**, *6*, 273−293.
(37) Fink, A. L.; Calciano, L. J.; Goto, Y.; Kurotsu, T.; Palleros, D. R. Classification of acid denaturation of proteins: Intermediates and unfolded states. *Biochemistry* **1994**, *33*, 12504−12511.
(38) Goto, Y.; Fink, A. L. Acid-induced folding of heme proteins. *Methods Enzymol.* **1994**, *232*, 3−15.
(39) Kristinsson, H. G. Acid-Induced Unfolding of Flounder Hemoglobin: An Evidence for a Molten Globular State with Enhanced Pro-oxidative Activity. *J. Agric. Food Chem.* **2002**, *50*, 7669−7676.
(40) Harwalkar, V. R.; Kalab, M. Thermal denaturation and aggregation of beta-lactoglobulin at pH 2.5. Effect of ionic strength and protein concentration. *Milchwissenschaft* **1985**, *40*, 31−34.
(41) Cohn, E. J.; Edsall, J. T. *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*; Reinhold Publishing: New York, 1943.
(42) Phillips, L. G.; Whitehead, D. M.; Kinsella, J. *Structure−Function Properties of Food Proteins*; Academic Press: San Diego, CA, 1994.
(43) Shen, L. L.; Hermans, J. Kinetics of conformation change of sperm-whale myoglobin. II. Characterization of the rapidly and slowly formed denatured species (D and D*). *Biochemistry* **1972**, *11*, 1842−1844.
(44) Konermann, L.; Rosell, F. I.; Mauk, A. G.; Douglas, D. J. Acid-induced denaturation of myoglobin studies by time-resolved electrospray ionization mass spectrometry. *Biochemistry* **1997**, *36*, 6448−6454.
(45) Sage, J. T.; Morikis, D.; Champion, P. M. Spectroscopic studies of myoglobin at low pH: Heme structure and ligation. *Biochemistry* **1991**, *30*, 1227−1237.
(46) Tang, Q.; Kalsbeck, W. A.; Bocian, D. F. Acid-induced transformations of myoglobin. II. Effect of ionic strength on the free energy and formation rate of the 426-nm absorbing deoxyheme intermediate. *Biospectroscopy* **1997**, *3*, 17−29.
(47) Tang, Q.; Kalsbeck, W. A.; Olson, J. S.; Bocian, D. F. Disruption of the heme iron-proximal histidine bond requires unfolding of deoxymyoglobin. *Biochemistry* **1998**, *37*, 7047−7056.
(48) Friend, S. H.; Gurd, F. R. N. Electrostatic stabilization in myoglobin. pH dependence of summed elestrostatic contributions. *Biochemistry* **1979**, *18*, 4612−4619.

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