Folding and Assembly of Hemoglobin Monitored by Electrospray Mass Spectrometry Using an On-line Dialysis System

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The native structure of hemoglobin (Hb) comprises two α - and two β -subunits, each of which carries a heme group. There appear to be no previous studies that report the in vitro folding and assembly of Hb from highly unfolded α - and β -globin in a "one-pot" reaction. One difficulty that has to be overcome for studies of this kind is the tendency of Hb to aggregate during refolding. This work demonstrates that denaturation of Hb in 40% acetonitrile at pH 10.0 is reversible. A dialysis-mediated solvent change to a purely aqueous environment of pH 8.0 results in Hb refolding without any apparent aggregation. Fluorescence, Soret absorption, circular dichroism, and ESI mass spectra of the protein recorded before unfolding and after refolding are almost identical. By employing an externally pressurized dialysis cell that is coupled on-line to an ESI mass spectrometer, changes in heme binding behavior, protein conformation, and quaternary structure can be monitored as a function of time. The process occurs in a stepwise sequential manner, leading from monomeric α - and β -globin to heterodimeric species, which then assemble into tetramers. Overall, this mechanism is consistent with previous studies employing the mixing of folded α - and β -globin. However, some unexpected features are observed, e.g., a heme-deficient β -globin dimer that represents an off-pathway intermediate. Monomeric β -globin is capable of binding heme before forming a complex with an α -subunit. This observation suggests that holo- α -apo- β globin does not represent an obligatory intermediate during Hb assembly, as had been proposed previously. The on-line dialysis/ESI-MS approach developed for this work represents a widely applicable tool for studying the folding and self-assembly of noncovalent biological complexes. (J Am Soc Mass Spectrom 2007, 18, 8–16) © 2007 American Society for Mass Spectrometry

The biologically active state of many proteins comprises several subunits that assemble into a tightly folded quaternary structure. In addition, these complexes often contain functionally essential cofactors. Many of the principles governing the folding of small, monomeric proteins have been uncovered in recent years [1–3]. In contrast, much less is known about the formation of multi-protein assemblies from their unfolded monomeric constituents. Some of these complexes are formed by relatively simple mechanisms, where complete folding of the individual subunits precedes the formation of quaternary contacts [4]. In other cases, however, folding and binding are closely intertwined [5–9]. One difficulty encountered during many in vitro studies on the folding and assembly of protein complexes is the occurrence of aggregation. Another problem is the fact that most spectroscopic techniques cannot readily distinguish intramolecular structural changes from those that are linked to the formation of intermolecular contacts [4].

Direct insights into the structure and dynamics of protein complexes can be obtained by electrospray ionization (ESI) mass spectrometry (MS). The gentle nature of the ESI process allows a wide range of noncovalent assemblies to be transferred into the gas-phase, such that their composition can be determined from their mass [10–13]. In addition, the ESI charge state distribution provides a highly sensitive probe for changes in the overall protein conformation. Unfolded solution-phase proteins generally produce higher charge states during positive ion ESI than tightly folded structures [14–16]. As a result, ESI-MS is rapidly being adopted as a standard method in structural biology [17].

Hemoglobins (Hb) from higher organisms are among the most thoroughly studied multi-protein complexes. The quaternary structure of Hb comprises two pairs of heme containing α - and β -subunits in a tetrahedral arrangement. The noncovalent bonds stabilizing this complex encompass nonpolar and van der Waals interactions, hydrogen bonds, and salt bridges. Each α chain impinges upon the two β chains along two different surfaces. The noncovalent contacts connecting the $\alpha 1\beta 1$ and $\alpha 2\beta 2$ subunits are more extensive than those found at the $\alpha 1\beta 2$ and $\alpha 2\beta 1$ interfaces [18, 19]. The cooperative binding of oxygen to Hb is a particu-

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larly important area of research. Although the basic principles underlying the oxy (R) to deoxy (T) transition have been elucidated several decades ago [20], new details regarding the mechanism of these structural switching events continue to emerge [21–24].

Hb also serves as an important model system for studying protein assembly. The canonical heterotetrameric structure is in equilibrium with $\alpha\beta$ dimers as well as with α - and β -monomers [25]. Adding to this complexity is the fact that, in principle, every subunit can exist in its heme-bound form (holo, i.e., α^h and β^h) or in its heme-free form (apo, i.e., α^a and β^a) [26]. Removal of heme causes the subunits to adopt extensively disordered structures [27]. Previous studies on the binding equilibria and assembly of Hb mostly employed UV-Vis spectral changes in the Soret region of the heme absorption spectrum. Occasionally, this was complemented by CD and fluorescence spectroscopic measurements [27, 28]. Other studies were based on measurements of haptoglobin binding [29], auto-oxidation kinetics [30], radioactive labeling [31], as well as stopped-flow pH measurements [32]. The *in vitro* assembly of Hb has most commonly been triggered by mixing initially separated α^h and β^h subunits under native solvent conditions. Before mixing, the isolated subunits form noncovalently bound $(\alpha^h)_2$ and $(\beta^h)_4$ complexes, respectively. Dissociation of these species allows the formation of $\alpha\beta$ dimers to occur, which can then form heterotetrameric Hb [33–38]. Electrostatic interactions play a key role in guiding these assembly processes [39–41]. The exact heme-binding state of the dimeric $\alpha\beta$ intermediate(s) ($\alpha^h\beta^h$, $\alpha^h\beta^a$, $\alpha^a\beta^h$, or $\alpha^a\beta^a$) is still a matter of debate [28, 42–44], and apparently there is more than one viable assembly pathway leading from the isolated subunits to native Hb [45].

The *in vivo* mechanism of Hb formation is thought to partly resemble that inferred from *in vitro* studies [46]. It is important to note, however, that during Hb biosynthesis the monomeric subunits undergo co-translational folding and heme binding [47, 48]. Another interesting aspect is the recent discovery of a chaperone in red blood cell precursors. This chaperone, termed AHSP (α -hemoglobin-stabilizing protein), specifically binds to nascent monomeric α^h , thereby preventing the formation of cytotoxic α -globin aggregates [46, 49–54].

To our knowledge, there exist no previous studies that report the *in vitro* folding and assembly of Hb from extensively denatured α - and β -subunits in a “one-pot” reaction. Such a process would have to involve (1) the folding of individual subunits, (2) subunit binding to heme, and (3) subunit assembly to form tetrameric Hb (obviously, the three steps do not necessarily have to occur in this particular order). Previous attempts to carry out related experiments were frustrated by very low heterotetramers yields due to extensive aggregation and precipitation [55]. In fact, aggregation is a common problem even for reconstitution schemes employing folded α - and β -subunits [45]. This could imply that Hb folding and self-assembly necessarily requires a

chaperone system, and possibly other components of the cellular machinery. However, the experiments described in the current study demonstrate that this is not the case. We employ a dialysis system that is coupled on-line to an ESI mass spectrometer, such that conformational changes, as well as the formation of heme-protein and protein-protein contacts can be monitored directly. Under carefully controlled solution conditions the gradual removal of denaturants from a Hb solution causes the protein to refold and assemble without any apparent aggregation.

Experimental

Materials

Bovine ferri-Hb (Sigma, St. Louis, MO) was dialyzed against 10 mM ammonium acetate (Fluka, Buchs, Switzerland). All stock Hb solutions were made to 1 mM (as tetramer) and were centrifuged to remove small amounts of insoluble debris. Post-dialysis samples were flash frozen in liquid nitrogen as 100 μ L aliquots and stored at -80°C . All Hb solutions contained 10 mM ammonium acetate. Reagent grade ammonium hydroxide and glass distilled acetonitrile (ACN) were obtained from Caledon Laboratories, Georgetown, ON. Consistent with previous reports, the subunit masses of the protein were found to be 15,053 Da and 15,954 Da for α^a and β^a , respectively [56]. The heme group accounts for an additional 616 Da for each subunit. Both β -subunits have a reduced Cys residue in position 93 [21]. ESI mass spectra recorded under denaturing acidic conditions confirmed that no intermolecular disulfide bridging took place under any of the conditions used in this work (data not shown). In contrast to physiologically active ferro (Fe^{2+}) Hb, the ferri (Fe^{3+}) form of the protein used here (“metHb”) is not capable of reversible oxygen binding. $\text{Fe}^{2+} \rightarrow \text{Fe}^{3+}$ auto-oxidation occurs readily upon exposure of the protein to air. The X-ray crystal structure of ferri-Hb corresponds to that of ferro-Hb in its R conformation [19].

On-line Dialysis

To monitor the refolding and assembly of Hb from its denatured constituent parts, an on-line dialysis setup was developed. Hb (50 μ M as tetramer) was denatured by first raising the pH from 6.8 to 10.0 (ammonium hydroxide), subsequently ACN was added to a final concentration of 40% (vol/vol). After exposing the protein to these conditions for ca. 10 min, 2.8 mL of the Hb solution were transferred into a slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) with a nominal 7 kDa molecular weight cut-off. Refolding commenced after placing this cassette in an air tight screw-cap container filled with 2.8 L of dilute ammonium hydroxide (pH 7.90). Equilibration of the protein solution with the dialysis buffer resulted in pH 8.0 and less than 0.04% ACN. Refolding was monitored as a function of dialysis

Figure 1. pH and acetonitrile (ACN) concentration changes during on-line dialysis. (a) pH as a function of time; the inset shows the same data after conversion to hydroxide ion concentration. (b) Refractive index as a function of time, reflecting the drop in ACN concentration within the solution during dialysis. The dotted line indicates the refractive index of water ($n = 1.333$). Inset: calibration curve used to determine the ACN concentration. Filled circles represent experimental data, solid lines are spline curves.

time by pressurizing the headspace within the container with nitrogen, causing the protein solution to exit the dialysis cassette via a metal capillary, that was directly connected to the ESI source of the mass spectrometer via PEEK tubing. A nitrogen pressure of 3 psi resulted in an analyte flow rate of $30 \mu\text{L min}^{-1}$, corresponding to a flow delay of ca. 2 min between the dialysis cassette and the outlet of the ESI source. Time-dependent changes of pH and ACN concentrations within the dialysis cell were monitored with an AB15 pH meter (Fisher Scientific, Nepean, ON) and Atago refractometer (HSR-500, Atago, Tokyo, Japan), respectively, (Figure 1). The total protein loss upon refolding was found to be ca. 25% by UV-Vis spectroscopy. We attribute this loss to protein leakage through the dialysis membrane, despite the nominal 7 kDa cut-off. In addition, protein adsorption to the membrane may play a role.

ESI-MS

Online dialysis was monitored with an LCT time-of-flight mass spectrometer (Waters/Micromass, Manchester, UK) employing a commercial Z-spray source operating in positive ion mode. All MS parameters were optimized to give the best Hb tetramer signal

at pH 8.0. A capillary voltage of 3.5 kV, extraction cone voltage of 10 V, and sample cone voltage of 60 V were found to be optimal. Desolvation and source temperatures (30 and 80 °C, respectively) were kept low to minimize tetramer dissociation. The mass spectrometer was operated at elevated pressure in the first pumping stage to facilitate the detection of noncovalent complexes [57–59]. This was accomplished by throttling the source turbo pump by means of a speedy valve to a pressure reading of 10 mbar. Hb refolding was monitored as a function of dialysis time, as explained above, with a scan time of 2 s for 40 min. Spectra were grouped and averaged every 2 min from 0 to 10 min, then every 5 min from 10 to 20 min and, finally, every 10 min from 20 to 40 min. Normalized peak intensity traces for all species present were generated from these nine spectra. The mass spectrometer was calibrated with CsI. All data were acquired and analyzed using MassLynx software provided by the instrument manufacturer. Baseline subtraction and data smoothing was performed before analysis.

Optical Spectroscopy

Fluorescence emission spectra were measured on a PTI fluorimeter (Mississauga, ON, Canada) using an excitation wavelength of 280 nm. All solutions analyzed contained 5 μM Hb (as tetramer). Absorption measurements were carried out on a Cary-300 Varian UV-Vis spectrometer (Palo Alto, CA) using a protein concentration of 2.5 μM . Circular dichroism (CD) spectra were collected on a Jasco J-810 spectropolarimeter (Easton, MD) by employing a 0.1 cm path length cuvette and 5 μM Hb.

Results and Discussion

Reversible and Irreversible Denaturation of Hemoglobin

Griffith and Kaltashov recently conducted a detailed study on the disassembly and unfolding of Hb by monitoring ESI mass spectra at increasing acid concentrations [60]. Ionic signals corresponding to the native Hb tetramer were most intense around pH 8.0. Acidification was found to disrupt the tetramer, initially leading to the formation of $\alpha^h\beta^h$ and $\alpha^h\beta^a$ dimers which dominate the spectrum at pH 5. These dimeric structures become unstable below pH 4, forming highly unfolded monomeric apo-subunits around pH 3. This stepwise process resembles the denaturation mechanism observed in kinetic ESI-MS experiments, where unfolding was triggered by a sudden pH jump [61].

Unfolding studies can provide direct information on the refolding mechanism of a protein only if the experiments are carried out under reversible conditions. Unfortunately, the acidic pH environment employed previously [60, 61] results in irreversible denaturation of Hb. For the current work it was attempted to refold

Figure 2. Photographs of microtubes after centrifugation of hemoglobin solutions at 10,000 rpm for 10 min. (a) Acid-denatured protein in 10% acetic acid (no pellet); (b) initially acid-denatured protein after exchange into aqueous solution at pH 8.0. Note the pellet of precipitated protein (indicated by the arrow); (c) denatured protein at pH 10.0 in water/ACN (60:40 vol/vol) (no pellet); (d) initially base/ACN denatured protein, refolded by exchange into aqueous solution at pH 8.0 (no pellet).

the acid-denatured protein in different ways, e.g., by slowly titrating it back to pH 8.0, or by carrying out a single-step pH change. Extensive protein aggregation was observed in all of these experiments (Figure 2a and b). ESI-MS analysis of the supernatant obtained after centrifugation did not reveal the presence of any refolded protein. In contrast, we found hemoglobin denaturation at pH 10.0 in 40% (vol/vol) ACN to be reversible. A dialysis-mediated change in conditions back to a purely aqueous solution of pH 8.0 does not result in any detectable aggregation (Figure 2c and d). It seems likely that one contributing factor for the different behavior observed for the two denaturing conditions is the fact that heme itself tends to precipitate in acidic solution due to its very low solubility. Under these conditions, refolding may be limited by the lack of freely available heme, which is required for both globin chains to fold into a compact conformation [27]. As a result, the polypeptide chains can become trapped in

conformations that are prone to aggregation. At basic pH this problem does not occur due to the much higher heme solubility [62,63]. The data discussed below show that refolded Hb after exposure to pH 10.0/40% ACN closely resembles the native protein before denaturation.

Characterization of Denatured Hb by ESI-MS

Figure 3a depicts a typical ESI mass spectrum of Hb obtained at pH 8.0, before exposing the protein to denaturing conditions. Consistent with previous work [60,61,64], this spectrum reveals the presence of α^h and β^a monomers, $\alpha^h\beta^a$ and $\alpha^h\beta^h$ dimers, and Hb heterotetramers. These gas-phase species reflect the presence of the corresponding solution-phase quaternary structures and are consistent with the known binding equilibria of the protein [64]. Acidification to pH 2.0 leads to disruption of all protein-protein interactions (Figure 3b). ESI-MS data obtained under these conditions exclusively show α^a and β^a ions which, based on their charge state distributions, represent highly unfolded solution-phase structures [60,16,65]. The spectrum of reversibly denatured Hb (pH 10.0/40% ACN) is somewhat more complex (Figure 3c). It exhibits a bimodal α^a charge state distribution. The most intense charge states around 14+ are attributed to apo- α -globin that is unfolded to a similar degree as under acidic conditions, whereas α^a ions around 8+ indicate the presence of apo- α -globin in a more compact solution-phase conformation. A similar bimodal distribution at lower intensity is observed for α^h ions, representing monomeric α -globin that maintains contact with its heme-cofactor, resembling the behavior observed for myoglobin under similar solvent conditions [66]. Both holo- and apo- β -globin appear in charge states 7+ up to at least 14+. In addition, the spectrum shows dimers of the composition $\beta^a\beta^h$.

As an interesting side aspect, it is noted that the solution charge states of apo- α -globin at pH 2.0 and 10.0 are ca. 25+ and 4–, respectively. Yet, the corresponding ESI charge state distributions in Figure 3b and c both exhibit a maximum around 14+. This observation is consistent with the notion that the protein charge states observed in ESI-MS are *not* determined by the charges of the protein in solution, rather the ESI charge states are determined by the solution-phase protein conformation [67].

Characterization of Denatured and Refolded Hb by Optical Spectroscopy

The Trp fluorescence intensity of native Hb at pH 8.0 is relatively low. A dramatically increased emission level is observed upon exposure of the protein to pH 2.0, and an even higher intensity is seen at pH 10.0 in 40% ACN (Figure 4a). In native hemoglobin the fluorescence of α Trp14, β Trp15, β Trp37 is quenched by Förster reso-

Figure 3. ESI mass spectra of hemoglobin recorded under different solvent conditions. (a) “Native”, aqueous solution, pH 8.0; (b) acid-unfolded, aqueous solution, pH 2.0; (c) unfolded protein at pH 10.0 in water/ACN (60:40 vol/vol). Notation: α^h and α^a represent holo- and apo- α -globin, respectively; β -globin is labeled analogously. (c) inset: expansion of the region around m/z 2000, revealing the presence of both α - and β -subunits in their apo and holo forms. β_{ox}^a represents an $(M + 32)$ species. The expanded spectrum also shows signals corresponding to $\beta^a\beta^h$ ions carrying Na^+ and K^+ adducts.

nance energy-transfer to the nonfluorescent hemes [68–70]. Protein unfolding and disruption of the heme/protein interactions leads to increased heme-Trp distances, thereby reducing the energy-transfer efficiency and increasing the Trp emission intensity [71]. Control experiments on *N*-acetyl tryptophan amide indicate that the lower emission intensity seen at pH 2.0 is due to solvent-chromophore interactions, not to a lesser degree of unfolding when compared to pH 10.0/40% ACN (data not shown).

Changes in the heme environment can be probed by monitoring the Soret region of the heme absorption spectrum. Hemoglobin at pH 8.0 exhibits a narrow spectral band with a maximum at 406 nm (Figure 4b). Denaturation at pH 10.0/40% ACN leads to lowering of the absorption intensity, broadening of the spectrum, and a slight blue shift. Even more pronounced changes are observed upon exposing the protein to pH 2.0. The protein absorption spectra recorded under both acidic and basic conditions resemble those of isolated heme in the corresponding solvents (data not shown).

Far-UV CD spectroscopy provides information on secondary structure [17,72]. Hb^o at pH 8.0 exhibits with

Figure 4. Characterization of native, denatured, and refolded hemoglobin by optical spectroscopy. (a) Fluorescence emission, (b) Soret absorption, and (c) circular dichroism spectra. The various solvent conditions used are indicated in (a).

two pronounced minima around 208 and 222 nm, which reflect the protein's largely helical conformation. Upon exposing Hb to pH 10.0/40% ACN the intensity of the CD signal at 222 nm is diminished by ca. 50%, which suggests that the protein loses about half of its native helicity under these conditions. An even more pronounced loss of helicity (ca. 75%) is observed for the acid-denatured protein (Figure 4c).

The optical data reported here are consistent with the ESI-MS spectra of Figure 3, confirming that Hb at pH 10.0/40% ACN adopts a significantly unfolded structure. The native heme-protein interactions are largely disrupted and the heme groups are mostly solvent-accessible. Yet, the persistence of weak residual heme-protein interactions under these non-native conditions is quite likely [66, 73], which is supported by the observation of low intensity heme-bound species by ESI-MS (Figure 3c). Also, "free" heme would get lost during dialysis, thus precluding refolding of the protein. CD spectroscopy (Figure 4c) shows that Hb at pH 2.0 is more extensively unfolded than the protein in its reversibly denatured form, a result that is in line with the charge state distributions displayed in Figure 3b and c. The most important aspect of Figure 4 is that the fluorescence, absorption, and CD spectra obtained after dialysis-mediated refolding of the base-denatured protein are indistinguishable from those of native Hb (the dotted spectra almost perfectly match the dash-dotted ones in all three panels of Figure 4).

Hemoglobin Refolding Monitored by ESI-MS

The dialysis setup developed for this work allows the folding and reassembly of Hb to be monitored in an on-line fashion by ESI-MS. Mass spectra were recorded while the solvent inside the dialysis chamber was gradually changed from pH 10.0/40% ACN to a purely aqueous environment of pH 8.0. The spectrum measured after a dialysis time of one minute (Figure 5a) is similar to that obtained under the denaturing conditions of Figure 3c. After 3 min, the relative intensities of highly charged α^a ions are greatly diminished, and α^h 8+ has become the most intense peak (Figure 5b). This spectral change reflects the transition of unfolded apo- α -globin to a more compact holo- α -globin conformation. Heme-bound heterodimers are the dominant species for a dialysis time of 9 min (Figure 5c). The spectrum recorded after 35 min shows tetramers, heme-bound and heme-deficient dimers, α^h ions in low charge states, and a small contribution from β^a ions in high charge states (Figure 5d). This spectrum resembles that recorded for native Hb (Figure 3a), confirming that the quaternary structure after refolding is similar to that before denaturation. The slightly higher tetramer intensities and lower α^h intensities, however, indicate the presence of minor structural differences, despite the fact that the corresponding optical spectra are indistinguishable.

For a better visualization of the spectral changes taking place during refolding, the peak intensities of

Figure 5. ESI mass spectra recorded during hemoglobin refolding in an on-line dialysis cell. (a) $t = 1$ min; (b) $t = 3$ min; (c) $t = 9$ min; (d) $t = 35$ min.

gas-phase ions corresponding to individual solution-phase species were added and normalized, resulting in the traces depicted in Figure 6. Within the first 10 min, the signals of α^a , β^a , and β^h decrease to levels close to zero (Figure 6a, b, and d). Following an initial increase, the time profile of α^h in charge states 9+ to 23+ exhibits a drop and then levels off at an intensity around 0.1. The lower charge states of α^h increase to an intensity level of 0.2 within ca. 15 min (Figure 6c). The $\beta^a\beta^h$ dimer is most strongly populated around 5 min, later on it completely disappears from the spectrum. Figure 6f–h displays the traces of three species that were not populated at the onset of the reaction. A sigmoidal rise to 0.17 is seen for $\alpha^h\beta^a$ (Figure 6f), whereas the trace for $\alpha^h\beta^h$ exhibits its highest intensity around ten minutes

Figure 6. Normalized intensity-time profiles extracted from ESI mass spectra recorded during hemoglobin folding and assembly; (a)–(d), monomeric species; (e)–(g), dimeric species, (h) hemoglobin tetramer. Data in panels (a) and (c)–(h) represent intensity sums for all charge states corresponding to a specific heme binding state or quaternary structure. Different time profiles for high and low charge states were only observed in the case of α^h ; those data are displayed separately (c). Data for native hemoglobin (based on the spectrum in Figure 3a) are included for comparison in each panel.

before dropping to a level close to 0.2 (Figure 6g). Following an initial lag phase, tetrameric Hb exhibits a slow and steady increase up to a maximum of 0.25 towards the end of the experimental time window (Figure 6h).

As noted in previous studies [61, 64], a certain fraction of β -globin carries a +32 Da modification (signals labeled β_{ox} in Figure 3c inset, Figure 6b). This effect is attributed to partial methionine oxidation, a notion that is consistent with the known chemical behavior of Met residues [74] and with peptide mapping studies on β -globin [75]. Interestingly, this modification does not preclude β -globin from participating in Hb assembly; MS/MS experiments reveal the presence of modified β -globin in $\alpha^h\beta^a$, $\alpha^h\beta^h$, and $(\alpha^h\beta^h)_2$ (data not shown). This observation is consistent with previous biochemical data [75], and with the fact that

none of the three β Met residues is directly involved in heme binding or protein-protein interactions [21].

The time profiles in Figure 6 allow an overall mechanism for the refolding and assembly of Hb to be proposed (Figure 7). The data in Figure 6a and c suggest that folded holo- α -globin is formed in a stepwise-sequential manner from largely unfolded apo-protein, via unfolded holo- α -globin as reaction intermediate. The situation is less clear for monomeric β -globin, as both its apo and holo forms disappear from the spectrum in parallel (Figure 6b and d). Possibly $\alpha^h\beta^h$ is formed by binding of α^h to β^h , and $\alpha^h\beta^a$ by binding of α^h to β^a . Based on the characteristic rise and drop of the profile in Figure 6g, $\alpha^h\beta^h$ appears to be a direct precursor of the $(\alpha^h\beta^h)_2$ heterotetramer. The subunit constituents of the heterodimer likely interact through their $\alpha 1\beta 1$ interface [18, 19]. The question whether $\alpha^h\beta^a$ is another direct precursor of tetrameric hemoglobin or an off-pathway complex cannot be decided with certainty from the data presented here. Certainly, $\beta^a\beta^h$ is an off-pathway species, as all of the contacts between the two subunits are non-native and have to be disrupted in order for $\alpha^h\beta^h$ or $(\alpha^h\beta^h)_2$ to form.

In general terms, the mechanism proposed in Figure 7 suggests a hierarchical sequence of events, where a certain degree of folding at the monomer level precedes the assembly of heterodimers, ultimately resulting in the formation of heterotetramers. Hierarchical folding schemes of this type have previously been deduced for other multi-protein complexes using a variety of different experimental approaches [76]. For the conditions used in this study, both α - and β -globin are capable of binding heme in their monomeric forms. This behavior is different from the mechanism inferred from acid-induced unfolding experiments, which suggested that heme binding to β -globin can take place only after the formation of a $\alpha^h\beta^a$ heterodimer [60]. Thus, the data presented here do not provide support for the idea that $\alpha^h\beta^a$ is an obligatory intermediate en route towards $(\alpha^h\beta^h)_2$ [43]. As noted above, however, likely the exact sequence of events during Hb folding and assembly in vitro can be modulated by external conditions such as solvent, pH, presence of chaperones, etc. [45].

Figure 7. Proposed mechanism of hemoglobin assembly for the conditions used in this study. Further information is given in the text.

Conclusions

Exposure of Hb to pH 10.0/40% ACN induces the breakdown of the protein's quaternary structure and significant unfolding. The native heme-protein interactions become largely disrupted. In contrast to the more commonly employed acidic unfolding conditions, denaturation of Hb in pH 10.0/40% ACN is highly reversible. Optical spectroscopy as well as ESI-MS show that the properties of refolded hemoglobin are very similar to those of the protein before unfolding. This demonstrates that the spontaneous self-assembly of Hb from highly unfolded subunits can take place in the absence of cellular chaperones. Experiments employing an on-line dialysis cell allow Hb refolding to be monitored by ESI-MS in a time-dependent fashion. The capability of ESI-MS to distinguish coexisting protein conformations, ligand binding states, and quaternary structures provides detailed insights into the mechanism of this coupled folding/binding process. The on-line dialysis approach developed for this work could also be useful for monitoring the assembly of other protein complexes. The experiments described here were carried out on ferri-hemoglobin. In future work, we will attempt to reconstitute the protein in its physiologically active ferro (Fe^{2+}) form. Those studies will require a careful control of the redox conditions. Subsequent O_2 -binding tests will provide a stringent test for the nativeness of the refolded ferro-Hb.

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