Introduction. It has been recently recognized that structural plasticity plays an important role in the function (assembly, signaling, recognition, etc) of quite a number of protein systems. Tetrameric mammalian hemoglobins (Hb) (composed of four subunits: two α and two β chains) exhibit such structural plasticity in a very surprisingly asymmetric manner. Although the structure and dynamics of the native (tetrameric) Hb are well understood, the mechanism of the protein assembly remain largely unknown. A working knowledge of this mechanism is needed in order to combat disorders like thalassemia and in the preparation of hemoglobin solutions for blood transfusion. In this work we study the in vitro assembly of Hb by monitoring the assembly/dissociation equilibria under a variety of conditions. It highlights the utility of ESI MS for studies of both structural and dynamic aspects of protein interaction in highly heterogenous systems (in this case 7 different species), allowing us to monitor both the composition and conformation of protein complexes in a species-specific fashion.

Methods. All mass spectra were acquired on a JMS-700 MStation (JEOL, Tokyo, Japan) two-sector mass spectrometer equipped with a standard ESI source. All circular dichroism spectroscopy experiments were performed with a JASCO J-715 CD spectropolarimeter (Jasco Corporation, Tokyo, Japan). All sample solutions were prepared by the dilution of a stock solution into 10 mM ammonium acetate solution whose pH (in the range 3 through 10) was adjusted to the desired level with NH₄OH or CH₃CO₂H. All sample solutions were equilibrated at room temperature (24 °C) for 1 hour prior to analysis.

Results. Hemoglobin concentration in vivo is about 5 mM where the protein exists in its tetrameric form. By decreasing the concentration in solution, a reversible dissociation of the complex is induced. A typical mass spectrum of bovine Hb at near-physiological pH and at the concentration used in the experiment shows the presence of multiple species corresponding to monomers, dimers, and a tetramer, with the latter one being the most abundant in the spectrum (Figure 1). By acquiring mass spectra under a variety of solution conditions the equilibria between the species can be shifted (Figure 2). For the most part, these equilibria are as expected from vast amount of literature available. In addition to the abundant ionic signal corresponding to the Hb dimer (αβ*), the mass spectrum also shows the existence of a dimeric species lacking one heme (αβ*). This species has previously been suggested to be an important player in the Hb association/dissociation pathway. No ionic signal corresponding to either a putative apo-dimeric form of Hb (αβ) or homo-dimers (α₂, α₂*, β₂, β₂*) can be detected in the spectra. Out of four possible monomeric species (α, α*, β, β*), only the holo-α and apo-β chains are present in the spectra unless the solution pH is lowered to 4.1 and below, at which point the protein becomes destabilized and the apo-monomeric species predominate. In addition to lacking the heme groups, β chains also exhibit a significantly higher charge density and a broader charge-state distribution, as compared to the α* ionic species. Charge state distributions of globin monomer conformations have been processed using a chemometric procedure previously employed with myoglobin (Figure 3), a structurally similar protein with the same evolutionary origin. It indicates the presence of four conformers for each globin: N, the most compact state; I, an intermediate state (“pH 4 intermediate”); E, extended conformation; and U, unfolded state (random coil). Regardless of solution condition, the β chain mostly populates the I and E states. Considering the high sequence homology and near-identical structure between the α and β chains, this conformational asymmetry is very surprising. It is possible that this intrinsic lack of structure enables the precise control of the assembly process and that such asymmetry was developed under the evolutionary pressure to minimize globin aggregation in the crowded cytosolic environment in erythrocytes. The oligomeric Hb species ([αβ*]₂, αβ*), and αβ) always maintain highly structured compact conformations as suggested by low protein charge density and narrow charge state distributions. The data show the assembly mechanism to be as follows: α* N + β₀ ⇄ (αβ)ₙ + heme ⇄ (αβ*)ₙ ⇄ ([αβ*]₂)ₙ (Figure 4). The percentage of natively folded protein and the fraction of heme-bound globin chains have been estimated.
at each pH by processing the mass spectra. These results are in good correlation with the results from circular dichroism spectroscopy.

Figure 1: ESI mass spectrum of bovine hemoglobin, pH 8

Figure 2: ESI mass spectra of bovine hemoglobin, pH 3-5 and 8

Figure 3: Charge state deconvolution of myoglobin, α-globin and β-globin contributions to ESI mass spectra at pH 4 and 8

Figure 4: Hemoglobin assembly mechanism concluded

\[ \alpha^* + \beta \iff \alpha^*\beta \iff \alpha^*\beta^* \iff (\alpha^*\beta^*)_2 \]