KINETICS OF ASSOCIATION AND DISSOCIATION PHENOMENA IN HUMAN HEMOGLOBIN STUDIED IN A LASER LIGHT-SCATTERING STOPPED-FLOW DEVICE

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One of the central problems in hemoglobin studies is that of assigning kinetic properties to known aggregated states of the protein. To attack this problem, as well as study the kinetics of association, dissociation, and denaturation, we have constructed a dual-beam stopped-flow device that employs laser (Ar-ion, He-Cd, He-Ne) excitation for kinetic light-scattering measurements. Absorbance and fluorescence changes after rapid pH and concentration jumps can also be measured in the same apparatus. The apparatus has also been used in the flow-flash mode where flash-photolysis measurements at known times after flow permitted a determination of the rate constant for the dissociation of tetrameric hemoglobin. Light-scattering changes as a function of protein concentration after pH jumps can be treated as relaxation phenomena and allow us to determine $K_{4,2}$, the tetramer-dimer equilibrium constant, over the range pH 7–10.9. These determinations are not only orders of magnitude more rapid than those by ultracentrifugation, but are equally or much more precise. We have found that the tetramer to dimer reaction, pH 10.3–11.6, in HbCO is remarkable in that the rate constant increases 100-fold over this interval and reaches a plateau at ca. pH 11.4, well before the onset of denaturation (1). HbCO treated with carboxypeptidase-A, (CPA), which removes βHis(146) and βTyr(145), showed greatly increased rate constants over the interval pH 10.3–11.5 compared to native HbCO, whereas rates for hemoglobin treated with carboxypeptidase-B, which removes αArg(141), were identical. Fig. 1 shows clear evidence of the importance of the β chain COOH-terminus in maintaining the integrity of the tetramer for liganded hemoglobin. The effects of various SH reagents on the tetramer-to-dimer process were studied. Some, such as PMB, greatly increased the rate, whereas *N*-ethyl-maleimide gave a modified hemoglobin (NES) with a decreased dissociation rate (Fig. 1). From the light-scattering amplitudes, both the enzymatically and SH-treated hemoglobins were shown to be more dissociated at pH 7 than native protein. A detailed analysis of the pH kinetic profiles for the native and modified hemoglobins shows that a simple charged-sphere model cannot account for the phenomenon. Various two-state models that assume rapid protonic and conformational equilibria between two forms of the tetramer cannot explain the results. A minimum mechanism for the process must include a pH-dependent conformational change occurring on the same time scale as the tetramer-dimer dissociation. Similar dissociation studies for deoxy-hemoglobin reveal an exceedingly complex problem. First, our kinetic light-scattering studies show that the protein is much more disso-

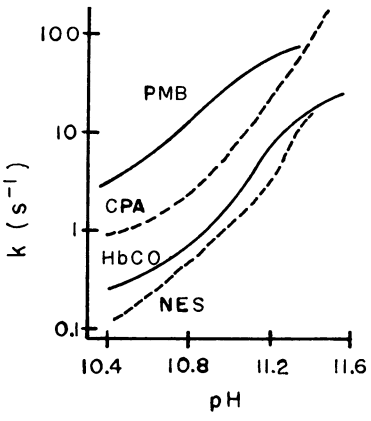

FIGURE 1

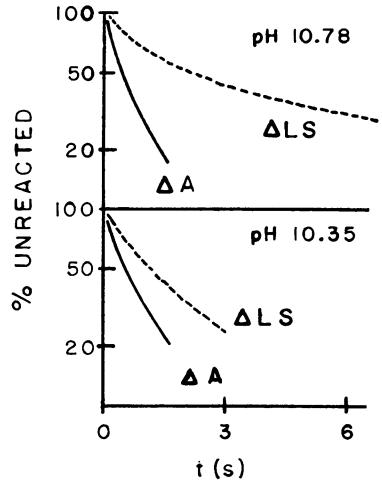

FIGURE 2

ciated at pH values greater than 10 than had been previously assumed. The reaction is at least biphasic in light-scattering, the rate does not increase smoothly with pH, and for some pH values, the rate appears to decrease with increasing total protein concentration. Furthermore, the absorbance changes (ΔA) after a rapid increase in pH precede the light-scattering changes (ΔLS) and display at least triphasic kinetics, with a very rapid change contributing about 50% to the total amplitude (rapid phase not shown in Fig. 2). In the case of HbCO, absorbance changes also occur, but at 287, 408, and 420 nm, they follow rather than precede the light-scattering changes and must therefore correspond to conformational changes in the dimers. Unlike the behavior of HbCO, a description of Hb requires at least five species. Other investigators following only absorbance changes have been entirely misled as to the time-course and the complexity of the process. Fitting the combined absorbance and light-scattering results by various models required the development of minimization procedures that used numerical integration of both the differential equations for the chemical species and the partial differential equations for the rate constants. Eigenvalues and eigenvectors of the relaxation matrix were useful in providing initial parameter values for the minimization routine.

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REFERENCES

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