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THE EFFECT OF pH ON THE RATE OF DISSOCIATION OF THE OXYGENATED BETA CHAIN TETRAMER OF Hb A*

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The effect of pH on the overall assembly of oxyhemoglobin A following mixing of equivalent concentrations of α and β heme subunits has been studied in 0.1 M potassium phosphate buffers at 20°C. The resultant kinetic profiles monitored at 582.5 nm (the maximum of the oxyhemoglobin - oxy chain difference spectrum) were homogeneous and appeared to be first order. The rate of these exponential time courses, reflecting the rate of dissociation of the β chain tetramer, increased from 0.013 min^{-1} at pH 6.4 to 0.30 min^{-1} at pH 8.0 and 1.0 min^{-1} at pH 8.5. Concurrent with this increased rate was a decrease in the overall color yield from the reaction. The absorbance changes, which involve a significant contribution from the β chain tetramer to monomer dissociation step, changed three fold over the pH range studied. The findings indicate that protons enhance the stability of the β chain tetramer.

Processes subsequent to chain synthesis may contribute to the hemoglobin phenotype. There is increasing evidence that the rate of assembly of the hemoglobin tetramer could be an important post-translational determinant of the ultimate distribution of hemoglobin in the erythrocyte (1-3). A precise kinetic assessment of the hemoglobin assembly process *in vitro* should provide a molecular basis for understanding this process *in vivo*.

The mechanism of assembly of the hemoglobin tetramer involves the combination of two dissimilar subunits to form the αβ dimer which then aggregates to form the $\alpha_2\beta_2$ tetramer. The isolated heme subunits themselves are oligomeric (4,5) adding an additional step of complexity to the overall assembly process since dissociation of these oligomeric heme subunits into monomers must occur prior to their combining to form the αβ dimer. Because of the tremendous dif-

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ference in the stability of the nonmonomeric α and β heme subunits, experiments can be carried out at protein concentrations where the amount of α chains is almost entirely monomeric and yet their β chain counterparts exist predominantly as tetramers (5). Such recent studies from our laboratory (6) reveal that the rate of dissociation of the oxygenated β chain tetramers can significantly influence the overall kinetic profile of oxyhemoglobin formation. Here we report the effect of pH on this β chain tetramer dissociation rate.

MATERIALS AND METHODS

Preparation of Isolated Heme Subunits - The subunits of human hemoglobin required in these studies were prepared by appropriate modifications of the method of Bucci and Fronticelli (7) as previously described in detail (6). The ability of these subunits to fully recombine into functioning tetramers was confirmed by electrophoresis and oxygen equilibrium studies.

Spectrophotometric Kinetic Studies - The overall rate of assembly of oxyhemoglobin A can be monitored spectrophotometrically by taking advantage of small spectral differences between intact oxyhemoglobin A and the oxygenated α and β chains (8). Equivalent concentrations (on a heme basis) of oxygenated α and non-α-subunits were mixed in a rectangular tandem-mixing cell and the change in absorbance at 582.5 (±0.2)nm, the spectral maximum, was monitored as a function of time in a Cary 118c recording spectrophotometer equipped with a water jacketed cell holder. Alternatively, the two heme chain solutions were mixed rapidly in a Gibson-Durrum stopped-flow device and the resultant absorbance change (582.5 nm) was monitored by the microcomputer-based OLIS 3820 system (On Line Instruments Systems, Jefferson, GA 30549). All experiments were carried out at 20(±0.1)°C and two independent buffer systems were employed, 0.1 M potassium phosphate buffer (pHs 6-8) and 0.1 M potassium phosphate-borate buffer (pHs 8-9).

RESULTS

We have previously shown (6) that at pH 7 and 20°C in 0.1 M potassium phosphate buffer the resultant time courses following mixing of equivalent concentrations of α and β chains were homogeneous and followed first order kinetics. Under these experimental conditions the dissociation of oxygenated β chain tetramers was the rate limiting step in the overall assembly of Hb A.

In Figure 1 are depicted the rate plots of time courses following mixing of oxygenated heme chains ($[\alpha] \equiv [\beta]$ = 60 uM in heme before mixing) over a wide pH range in 0.1 M potassium phosphate buffer at 20°C. At all pHs shown, straight lines were obtained on these semilogarithmic plots indicative of first order reactions. An increase of more than 20-fold in the overall rate of reaction (k) was seen upon changing the pH from 6.4 to 8.0. The apparent

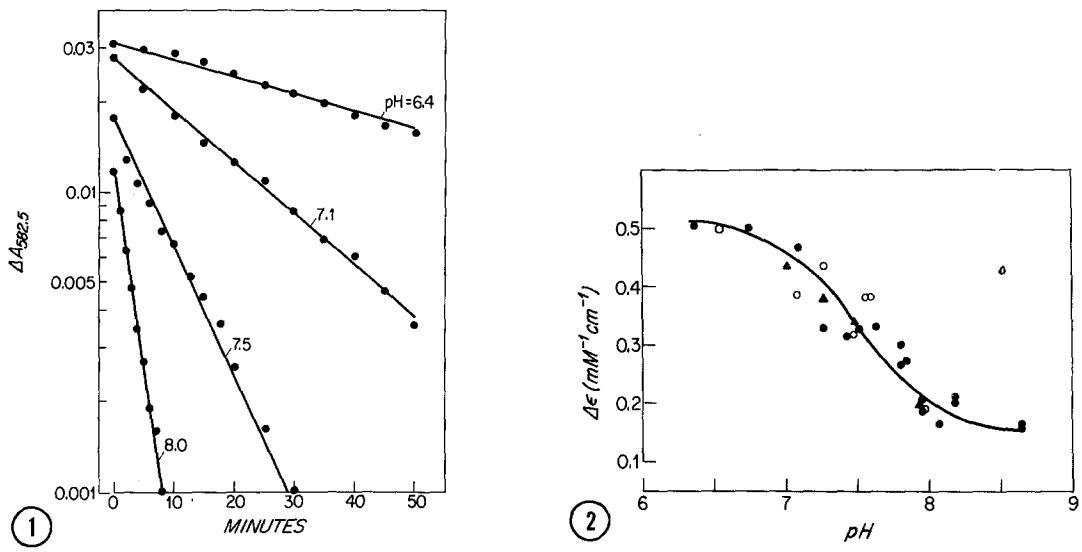

Figure 1: Semilogarithmic plots of time courses following the mixing of oxygenated α and β chains at four distinct pHs. Experimental conditions were: [α]≡[β] = 60 uM in heme prior to mixing; 20(±0.1)°C; 0.1 M potassium phosphate.

Figure 2: The total absorbance change of the reactions as a function of pH. The value of $\Delta\varepsilon$ (mM^{-1} cm^{-1}) at 582.5 nm, the maximum peak of the oxyhemoglobin - oxychain difference spectrum, was determined from the standard equation $\Delta A=\Delta\varepsilon cl$ where ΔA is the change in absorbance of the reaction; c is the concentration of the heme subunits determined in the 576-577 nm range using an ε value of 15.37 mM^{-1} cm^{-1} and l is the optical path length of either the tandem-mix cell (0.83 cm) or the stopped flow cuvette (2 cm). Protein concentrations prior to mixing were (▲), 90 uM; (●), 60 uM; (o), 30 uM in heme.

stability of the oxygenated β chain tetramer, as monitored by its rate of dissociation into monomer, is dependent upon overall pH.

Concurrent with an increase in reaction rate is a decrease in the overall color yield of the reaction as the pH is raised. Although apparent from the plots in Figure 1, this finding is more clearly displayed in Figure 2. This figure is a composite of mixing experiments done at three protein concentrations over an entire pH range from 6 to 9. The value of $\Delta\varepsilon$, the millimolar difference extinction coefficient, changes from a value of 0.5 to 0.15 over this pH range. The overall color changes accompanying the formation of liganded hemoglobin involve a significant contribution from the β tetramer to monomer dissociation step (9). The decreased absorbance seen in the high pH experiments may be a direct consequence of color loss due to the decreased concentration of β chain tetramer available to dissociate.

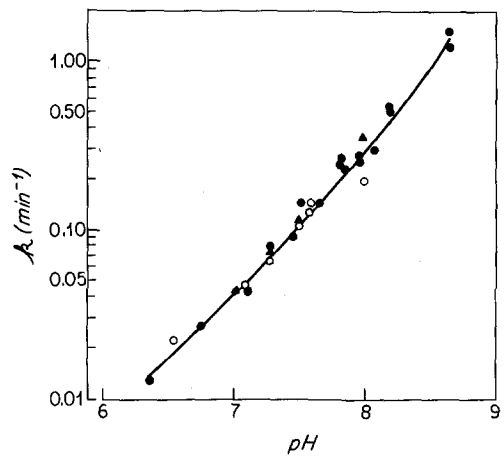

Figure 3: The apparent first order rate constant, $\underline{k}$ (min^{-1}) as a function of pH in a semilogarithmic plot. The protein concentrations are identical to those in Figure 2. The rate constant was either determined from Guggenheim plots of absorbance changes recorded on the Cary 118c spectrophotometer where $\underline{k} = 2.303 (\log A_x - A_{x'}/t_x)$ or from an exponential fitting routine of the microcomputer based OLIS 3820 system. Multiple analysis was carried out on several preparations.

Over a three-fold change in total heme concentration the kinetic profiles of these mixing experiments appear to be first order. Figure 3 reveals the change in rate of reaction as a function of pH, at three protein concentrations. At any given pH from 6.4 to 8.0, the apparent rate constant of the reaction is invariant over the reactant concentration measured; the value of $\underline{k}$ increases from 0.013 min^{-1} at pH 6.4 to 0.30 min^{-1} at pH 8.0. At pH 8.5 this rate constant approaches a value greater than 1.0 min^{-1}; however, we have not yet examined the invariance of this rate with protein concentration. The apparent first order rate of β chain tetramer dissociation and, hence, the overall rate of liganded hemoglobin reassembly is dramatically altered by change in pH.

DISCUSSION

The tetramer-dimer equilibrium of Hb A is linked to ligand, anion and proton binding (10-12). It appears that the self-association property of the β tetramer of Hb A is also affected by these homotrophic and heterotrophic modifiers. Valdes and Ackers (13) observed that the self-association of β chains into tetramers was dependent upon the state of ligation. They measured $K_{4,1}^{deoxy}$ and $K_{4,1}^{oxy}$ equilibrium dissociation constants of 6.6×10^{-15} and 2×10^{-17}

M^3 in heme, respectively, for the β subunits of Hb A (0.1 M Tris/HCl, 0.1 M NaCl, 1 mM Na_2 EDTA, pH 7.4, 21.5°C). Kurtz et al. (14) have recently shown that the presence of inorganic phosphate enhanced the stability of the β chain tetramer (0.05 M N, N'-methylenebisanylamide-Tris, pH 7.3, 20°C, 0.05-0.15 M [Cl^-] total, 0.1-0.15 M Pi). The effect of proton binding on the association-dissociation properties of β chain tetramers has also been reported. Kurtz and Bauer (15) observed that over the entire range of pH from 6 to 9 the unliganded chain tetramer was always more dissociated than the liganded β tetramer (0.05 M bis Tris $\leq$ 7.5 and 0.05 M Tris > pH 7.5; 0.15 M [Cl^-] total and 20°C). Interestingly enough, the pH dependencies of subunit association were complex but identical in the oxygenated and deoxygenated chain tetramer form.

The β tetramer dissociation rate, a kinetic parameter, is apparently also very dependent upon the presence or absence of several of these heterotrophic effectors. In studies on deoxy β chain tetramers McGovern et al. (16) reported a decrease in chain dissociation rates from 15 min^{-1} in the absence of phosphate to 3 min^{-1} in the presence of 1.4 x 10^{-3} M 2,3-diphosphoglycerate and to 0.084 min^{-1} in the presence of 3 x 10^{-5} M inositol hexaphosphate (0.1 M bis Tris, 0.1 M NaCl at pH 7.0 and 20°C). McDonald (6) reported an apparent first order rate constant for the dissociation of oxygenated β chain tetramer to be 0.047 min^{-1} (0.1 M potassium phosphate buffer, pH 7.0 and 20°C). This finding is consistent with the enhanced stability of the liganded tetramer over the unliganded chain tetramer reported by Valdes and Ackers (13). Evidence was also presented (6) that a decrease in phosphate ion concentration correlated well with an overall increase in dissociation rate of the oxygenated β chain tetramer.

Here we report the effect of pH on the β chain tetramer dissociation rate. It appears that the presence of protons greatly stabilizes the β chain tetramer. A dramatic change of nearly 100-fold is observed for the rate of this reaction over the pH spectrum measured. The molecular basis for the apparent enhancement of tetrameric chain structure by proton binding is unknown. The effect of pH may be direct and involve the protonation and/or deprotonation of critical amino

acid residues involved in ββ subunit interactions. On the other hand, the absolute proton ion concentration present could indirectly alter the chain tetramer by affecting the binding of phosphate molecules known to stabilize the β chain tetramer. An evaluation of the effect of protons on the β chain tetramer dissociation reaction in the absence of stabilizing inorganic phosphate ions (currently under investigation in our laboratory) must be completed before the singular and additive properties of these two effectors can be more clearly defined.

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