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AN INVESTIGATION OF HUMAN OXYHEMOGLOBIN BETA TETRAMER DISSOCIATION USING HAPTOGLOBIN BINDING*

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Haptoglobin was used as a macromolecular probe to investigate the formation of human oxyhemoglobin beta chain dimers from tetramers in 0.1 M potassium phosphate buffer, 20° C at pH 7 and pH 8. Monitoring of spectral changes upon mixing haptoglobin with beta heme chains (2.5 and 5 micromolar) revealed an overall decrease in absorbance accompanied by a shift of the Soret spectral peak from 415 to 417 nm. The magnitude of the absorbance decrease was proportional to the beta concentration; the time courses consistently yielded greater color at pH 8 than at pH 7. At pH 8, two exponential phases of 0.47 min^{-1} and 0.084 min^{-1} were seen whose rates remained invariant with concentration. In contrast, only one exponential process was evident at pH 7, yielding a first order rate constant of 0.21 min^{-1}. We have spectrophotometrically followed the beta chain tetramer to dimer dissociation reaction, thus providing information about the contribution of this step to hemoglobin assembly.

Hemoglobin is a model system for understanding protein-protein interactions (1). Previous investigations of human oxyhemoglobins have used visible spectroscopy to monitor subunit assembly *in vitro* (2,3). Native subunit alpha and beta chains can be isolated which recombine to produce fully functional hemoglobin tetramers via an alpha-beta dimer intermediate (4-6). Over the protein concentration range previously studied, alpha chains are monomeric, while beta subunits are self-associated (7,8). Indeed, the beta tetramer dissociation can be the rate-limiting step in the reconstitution of hemoglobin (2).

The uncoupling of beta tetramers has been fitted to a tetramer-monomer equilibrium based on gel permeation studies (7) and absorbance changes accompanying dissociation (8). No attempt has been made to assess the role of an intermediate dimer species in this overall process, even though evidence for the existence of the beta chain dimer comes from sedimentation studies (9) and

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more recently from ligand binding studies (10). Furthermore, Atassi's group has demonstrated that beta chains combine with haptoglobin (11, 12), which is known to bind a dimer species of hemoglobin (13-15). By following Soret spectral changes upon mixing of human oxyhemoglobin beta chains with haptoglobin, we have succeeded in defining the beta tetramer to dimer reaction, a probable intermediate step in hemoglobin assembly *in vitro*.

MATERIALS AND METHODS

Preparation of reagent proteins - Isolated beta heme chains were purified from human adult hemoglobin by modifications (5,6) of the method of Bucci and Fronticelli (4). Prior to use in these experiments, the beta chains were stripped of phosphate ions (2) and subsequently buffered to the appropriate pH in 0.1 M potassium phosphate. To minimize stability problems, a fresh micromolar dilution was prepared for each Cary run and for each series of stopped flow traces. Concentration on a heme basis was verified spectrophotometrically at 414 nm (ϵ = 131 $mM^{-1}cm^{-1}$). The human haptoglobin was phenotype 1:1 and > 98% pure (Sigma, St. Louis, MO). Its concentration was maintained at a stoichoimetric excess (0.2 mg/ml before mixing) in 0.1 M potassium phosphate buffer at the desired pH. Multiple preparations of haptoglobin and beta chains were employed.

Static and kinetic spectrophotometric measurements - A Cary 2200 recording spectrophotometer maintained at 20° C was used to make all successive spectroscopic measurements. The mixing experiments were performed in rectangular tandem mixing cuvettes. The sample cell held equal volumes of beta and haptoglobin solutions, whereas the reference cell contained equal volumes of beta solution and buffer. For some trials, successive scans from 380-450 nm at 1 nm/sec were repeated after mixing at intervals sufficient to provide resolution of individual traces. In addition, the change in absorbance as a function of time was followed at 415 nm in either the Cary 2200 or a Kinetic Instruments stopped-flow apparatus integrated by the 4120AT microcomputer-based system (On-Line Instrument Systems, Inc., Jefferson, GA). A minimum of four traces was collected and analyzed for each experimental condition.

Data reduction - All spectral data were corrected to 1 cm path length cell and for small variations in beta chain concentration (< 10%). Data from the successive spectral measurements and absorbance profiles were analyzed using the Guggenheim approximation. Stopped-flow data were fitted directly by the routines in the 4120AT software to obtain first order rate constants.

RESULTS AND DISCUSSION

Since the alpha monomer binds essentially irreversibly to the beta monomer (16,17), our previous studies of subunit assembly utilizing alpha chains as the probe allowed investigation of the rate limiting beta chain tetramer dissociation step (2,3). However, in the presence of alpha chains, the pseudo steady-state approximation with respect to the beta dimer was necessary since the relative absorbance contributions of the tetramer to dimer and the dimer to monomer steps were unknown. The present approach addresses the formation of the beta dimer *in vitro*.

Human oxyhemoglobin beta chains were mixed with human haptoglobin, phenotype 1:1, and the change in absorbance as a function of wavelength and time was monitored on a Cary 2200 recording spectrophotometer. A typical experiment employing the spectral overlay technique is shown in Figure 1 for beta chains at 5 micromolar in heme prior to mixing, 0.1 M potassium phosphate buffer, pH 8 and 20° C. By spectrally monitoring the region from 380 to 450 nm, it was evident that a small but definite Soret absorption peak shift from 415 to 417 nm occurs during the course of the reaction. Such a red shift has been previously reported in 0.1 M Tris, 0.1 M NaCl, 1 mM Na_2EDTA, pH 7.4 and 10° C for beta chain concentration difference spectra and has been assigned to a tetramer dissociation step (8). Presumably, since haptoglobin binding allows the tetramer to dimer reaction to be isolated, our studies suggest that this dissociation step contributes to the overall spectral shift.

The haptoglobin binding reaction resulted in an overall decrease in absorbance of 0.071 at 415 nm, consistent with a beta chain tetramer to dimer dissociation (8). If this reaction is indeed analogous to that of hemoglobin (14), a homogeneous first order dissociation profile should be seen. Successive spectral scans allowed initial inspection of kinetic parameters; however,

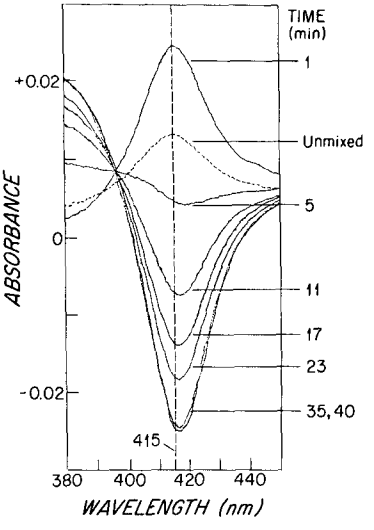

Figure 1: Successive spectra recorded on a Cary 2200 spectrophometer after mixing haptoglobin with oxygenated beta chains in 0.1 M potassium phosphate buffer, pH 8 and 20° C. Kinetics were analyzed at 415 nm. Beta chain concentration on a heme basis prior to mixing was 5 micromolar. Only experimental data demonstrating isosbesty at 395 (±1) nm were used in the present studies. Lack of a clean isosbestic point was inevitably due to methemoglobin formation of beta chains, as revealed by absolute spectra taken immediately following the mixing experiment.

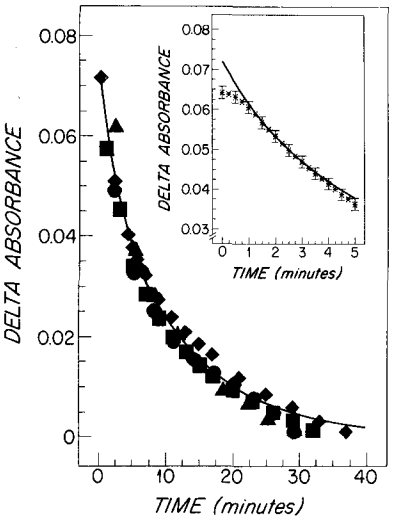

Figure 2: Overall time course for the reaction of beta heme chains with haptoglobin. The figure displays the results of successive scan experiments and absorbance profiles together with the fitted two exponential rate plot. The inset superimposes the same fitted curve with stopped flow data points obtained as the average of four individual traces; average points rather than individual data from each experimental run are shown in the interest of clarity. Conditions are the same as Figure 1. (An initial deviation from the fitted curve is seen and was not further analyzed in the present study.)

to better define the overall profile, the reaction was further monitored on either a Cary 2200 spectrophotometer or a Kinetic Instruments stopped-flow apparatus (Figure 2). Surprisingly, resultant time courses could be fitted to a two exponential process yielding first order rate constants of 0.47 and 0.084 min^{-1} for the fast and slow phases, respectively (See Table I). When the concentration of beta chains was halved to 2.5 micromolar in heme prior to mixing, the fast phase alone contributed the majority of the color change and yielded a rate of

Table I
Absorbance changes and rate constants for the reaction of oxyhemoglobin beta chains with haptoglobin. Results determined as described in text in 0.1 M potassium phosphate buffer and 20° C.

pH	[BETA] before mixing	TOTAL DELTA ABSORBANCE	k_1 min^{-1}	DELTA ABSORBANCE RXN#1	k_2 min^{-1}	DELTA ABSORBANCE RXN#2
8	2.5 uM	0.031	0.49 (±.021)	0.031	*	*
8	5.0	0.071	0.47 (±.035)	0.017	0.084 (±.0033)	0.054
7	2.5	0.016	0.24 (±.098)	0.016		
7	5.0	0.035	0.21 (±.020)	0.035		

* Slow phase contributed less than 10% of overall delta absorbance and was not further evaluated.

tetramer dissociation identical to that seen at 5 micromolar. The slow phase was only marginally detectable at 2.5 micromolar. The unexpected result of a two phase reaction may be explained by recently reported evidence for heterogeneity in the beta tetramer population (10, 18). More probably, this heterogeneity can be accounted for by differential phosphate binding to beta tetramers. Crystallographic evidence from Arnone's laboratory (19,20) demonstrates the existence of two phosphate binding sites on the beta chain tetramer. In addition, our work (2,3,21) as well as that of McGovern *et. al* (22) indicates a strong effect of phosphate on beta chain tetramer dissociation kinetics.

Further studies were conducted in a more physiologically relevant system, 0.1 M potassium phosphate buffer at pH 7 and 20° C at identical protein concentrations. The Soret spectral shift seen at pH 8 was also apparent at pH 7, strongly suggesting that beta chain binding to haptoglobin at both pH's involves a similar mechanism. The results of the spectral and kinetic analyses are shown in Figure 3 and Table I. The color change of the reaction was, again, directly proportional to initial heme concentration. However, the magnitude of the absorbance change at pH 7 was consistently less than that at pH 8. In addition, the overall time course could be fitted to a single exponential curve yielding a rate of 0.21 min^{-1}. This slower rate of beta tetramer to dimer

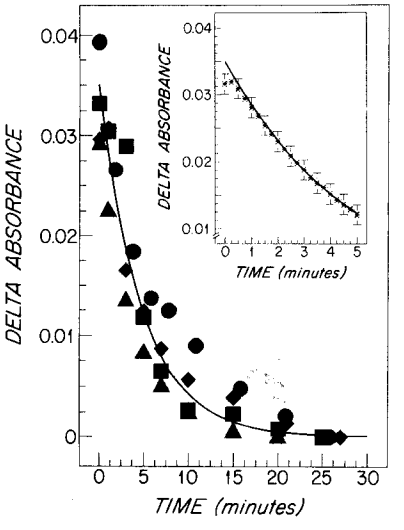

Figure 3: Overall time course for the reaction of beta heme chains with haptoglobin, similar to Figure 2 with conditions of: beta chain concentration of 5 micromolar in heme prior to mixing, 0.1 M potassium phosphate, pH 7 and 20° C. (The initial deviation from the fitted curve is again observed, but not evaluated.)

dissociation at pH 7 is consistent with the proposed stabilization of the beta tetramer by protons (21). Overall beta tetramer to monomer dissociation has been shown spectrophotometrically to be rate limiting in hemoglobin reconstitution (2,3,21-25). We have isolated the tetramer to dimer reaction of oxygenated beta chains and have demonstrated that this step contributes to the Soret absorbance change. Furthermore, the first order rates measured were in the range of those defined as rate limiting in assembly.

The studies reported here are exciting because this method potentially allows monitoring of the tetramer-dimer dissociation in isolated human beta chains by a spectrophotometric technique traditionally used to analyze this dissociation in hemoglobin (26-28). Our findings should encourage further investigations aimed at analyzing the intriguing phenomenon of beta chain self-association, a key reaction in human hemoglobin assembly.

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