ORGANIC PHOSPHATES AND THE REACTION OF N-BUTYL ISOCYANIDE WITH HUMAN HEMOGLOBIN

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Summary: An analysis of the binding of n-butyl isocyanide (BIC) to human hemoglobin requires the consideration of a minimum of two spectrally and functionally distinct components. A variety of evidence indicates very strongly that these components represent the α and β subunits. The presence of diphosphoglycerate (DPG) or inositol hexaphosphate (IHP) not only alters the overall affinity of hemoglobin for BIC but also amplifies the differences between the functional properties of the α and β chains.

It has been reported previously that the isolated hemoglobin subunits differ in their reactivity with BIC (i'_α = 68 $mM^{-1}sec^{-1}$; i_α = 0.40 sec^{-1}; i'_β = 400 $mM^{-1}sec^{-1}$; i_β = 1.35 sec^{-1})* and that these differences although altered are still manifest in a variety of kinetic observations in tetrameric hemoglobin (2,3). Although the effects of DPG and IHP on the oxygen reaction with hemoglobin have been studied extensively (4,5,6,7) it is not known whether or not these molecules exert their effects equally on the properties of the α and β chains. If these phosphates introduce or amplify heterogeneity within the set of heme-binding sites, the apparent cooperativity as measured by the Hill coefficient (n) at 50% saturation will decrease while the overall interaction energy may increase.

Materials and Methods: Stripped human deoxyhemoglobin was prepared according to Gibson (8). BIC was obtained from Aldrich Chemical Co.

* Notation of Ainsworth et. al. (1).

and its specific gravity was determined to be 0.76 at room temperature. IHP was obtained from Sigma Chemical Co., DPG from Calbiochem, and Bis-tris* from Aldrich Chemical Co. Stock solutions of BIC were prepared by adding known small volumes to appropriate deoxygenated buffers. Stopped flow measurements were carried out as described by Gibson (8). Equilibrium measurements were performed basically as described by Anderson et. al. (9) with two modifications. First, the stock ligand solution contained hemoglobin at the same concentration as that present in the initial deoxyhemoglobin solution so that only the total ligand concentration needed to be corrected for dilution effects. Secondly, after each addition of ligand the entire Soret spectrum was recorded on a Cary 14 spectrophotometer, and the final saturation values were calculated from the average of measurements at 410, 430, 429, and 440 nm, wavelengths where the contributions of the α and β chains to the observed difference spectra were judged to be roughly equal (3).

Results and Discussion: It has been suggested that DPG and IHP do not alter the shape of the oxygen equilibrium curve of hemoglobin (5). However, it is apparent from Fig. 1 that these organic phosphates exert a profound influence on the shape and symmetry of the BIC equilibrium curve. While it is possible that the behavior of stripped hemoglobin could be represented by n = 2.0, it is clear that in 0.1 M P_i or DPG solutions "heme-heme" interaction cannot be adequately described by a single Hill coefficient. In the more extreme case of IHP-treated hemoglobin it would appear that the equilibrium curve could be represented by the Hill equation with n = 1.0, but since it has been reported (5) and confirmed in this laboratory that the oxygen equilibrium of IHP-hemoglobin is highly cooperative, it is felt that the curve in Fig. 1 represents the type of "pseudo" non-cooperative situation described in the introduction.

It has been reported (9, 10) that true spectrophotometric isosbestic

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* 2,2-Bis (hydroxymethyl) - 2,2',2"- nitrilotriethanol

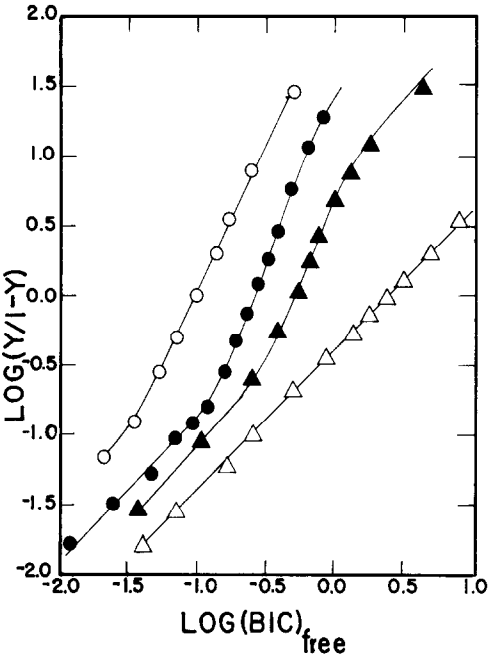

Fig. 1. Hill plots for the equilibrium of BIC with HbA at 22°C. The [BIC] is expressed in millimolar. Conditions: Stripped HbA (o) in 0.05 M Bis-Tris, pH 7.0; Unstripped HbA (●) in 0.1 M P_i, pH 7.0; Stripped HbA (▲) in 0.34 mM DPG and 0.05 M Bis-Tris, pH 7.0; Stripped HbA (△) in 0.4 mM IHP and 0.05 M Bis-Tris, pH 7.0. The concentrations of HbA varied between 6-10 μM heme. All solutions contained a small amount of sodium dithionite (< 0.1%) to ensure anaerobic conditions.

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points exist when hemoglobin is titrated with alkyl isocyanides. However, it is clear from Fig. 2 that none exist when hemoglobin is titrated with BIC and that these deviations from isosbesty are more pronounced when organic phosphates are present. In fact, IHP-hemoglobin possesses a "pseudo" isosbestic point at 420.0 nm for Y = 0 to 20%, indicating that BIC is binding to a single spectrophotometric species in this range of ligand concentrations. This temporary isosbestic point also existed for DPG-treated hemoglobin but over a smaller saturation range (Y=0 to 14%). However, in all cases the final isosbestic point appears to be 421.7±.3 nm, indicating that the organic phosphates are not altering the intrinsic α and β chain difference spectra but rather are affecting the distribution of ligand between the α and β chains.

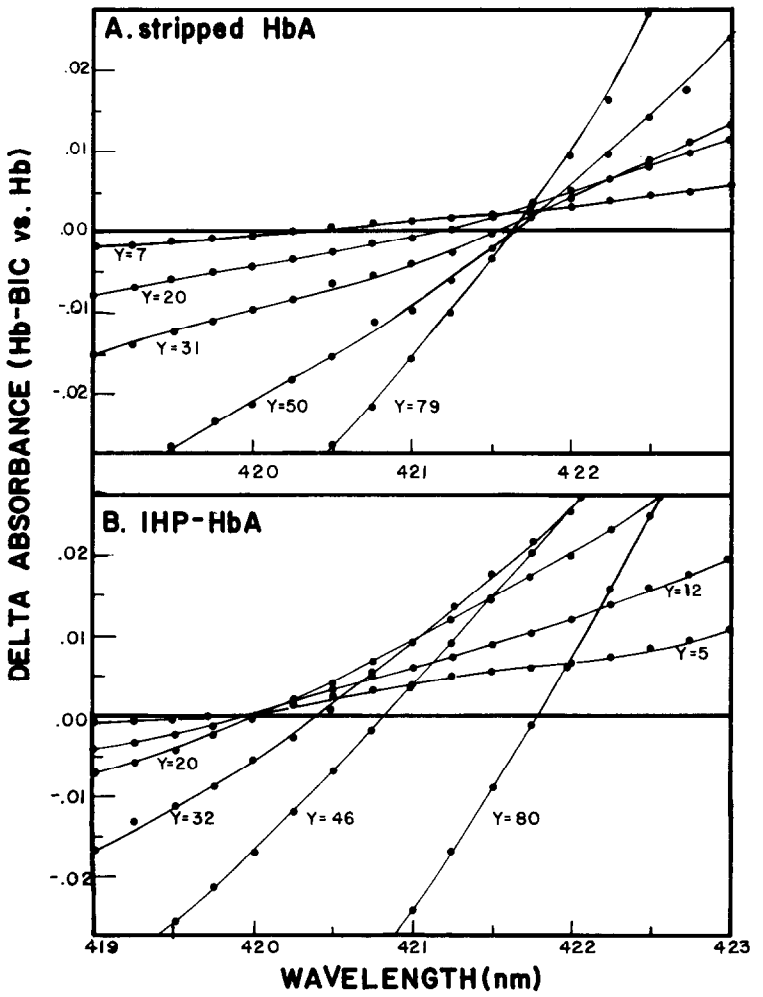

Fig. 2. Difference spectra of HbA-BIC mixtures versus HbA A. Stripped HbA in 0.05 M Bis-Tris, pH 7.0, [Hb] = 6.2 μM.
B. Stripped HbA in 0.4 mM IHP and 0.05 M Bis-Tris, pH 7.0 [Hb] = 8.2 μM. Saturation values were derived from the binding curves in Fig. 1.

The time course for the reaction of BIC with deoxyhemoglobin is wavelength dependent; and under conditions where the reaction is biphasic the absorption changes at 420 nm are slow while those at 424-425 nm are predominantly fast (Fig. 3). When the reaction of BIC with hemoglobin (± organic phosphates) was studied as a function of ligand concentration (see Fig. 4 for a typical experiment), it was found that at BIC concentrations which give rise to small final saturation values the reactions of IHP or DPG-

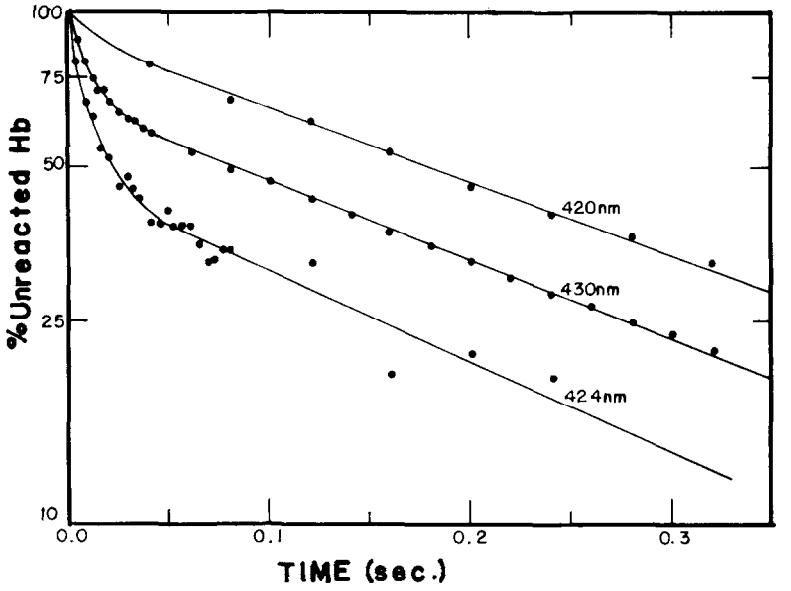

Fig. 3. Time courses for the mixing of unstripped HbA with BIC in 0.1 M P_i, pH 7.0 at 22°C. Conditions: [Hb] = 3.1 μM after mixing; [BIC] = 1.15 mM after mixing; 2 cm light path; 0.5 mm slits. The solid lines represent a fit of the data to $y = w \cdot \exp(-k_1 t) + (1-w) \cdot \exp(-k_2 t)$ where y represents the % Hb remaining and w the fraction of the fast component. It should be noted that only w is wavelength dependent.

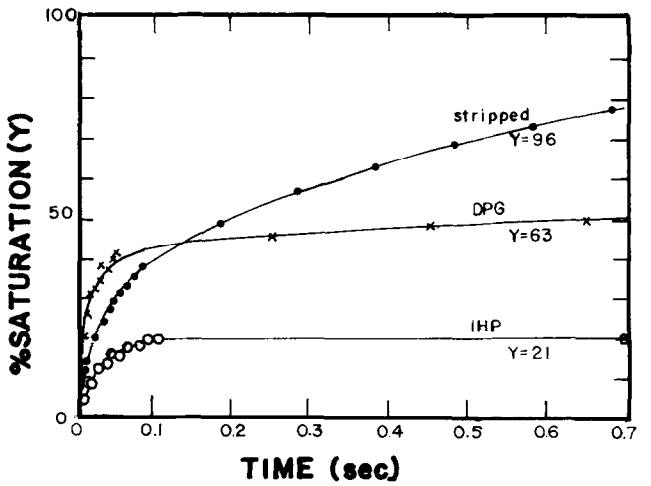

Fig. 4. Time courses for the mixing of stripped HbA with BIC in 0.05 M Bis-Tris, pH 7.0 at 22°C. Conditions: [BIC] for all experiments = 0.58 mM; (•) stripped HbA at [Hb] = 3.2 μm; (x) stripped HbA in 0.7 mM DPG at [Hb] = 50 μM; (o) stripped HbA in 0.4 mM IHP at [Hb] = 3.2 μM. All concentrations are after mixing. The wavelength of observation was 430 nm with 0.5 mm slits.

hemoglobin are virtually homogeneous, fast ($k \sim 30$ sec^{-1}), pseudo first order, and not observable at 420 nm, indicating that under these conditions BIC is binding predominantly to the "fast" spectral species which possesses an isosbestic point at 420 nm. However, in the case of stripped hemoglobin the slow phase did not decrease in amplitude with decreasing BIC concentration over a similar range of final saturation values suggesting concomitant binding to the α and β chains. Since it can be shown by the CO replacement reaction (3) that IHP-hemoglobin at 50% BIC saturation contains roughly three times more liganded β chains than α chains, it would appear that the "fast" component with an isosbestic point at 420 nm corresponds to β chains, a result which is consistent with the relative rates and affinities of the isolated chains.

Conclusions: The results presented here are consistent with the view that organic phosphates exert an unequal influence on the two types of heme-binding sites in human hemoglobin. In the extreme case of IHP-hemoglobin, only the β chains bind ligand up to 20% saturation of the total heme sites with BIC. It is clear that at least ten intermediates are required for an adequate description of the reaction of BIC with stripped hemoglobin because of the nonequivalence of the chains. If one now adds phosphates to such a scheme, the number of intermediates is increased by a factor of two.

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