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Characterization of the pH dependence of hemoglobin binding to band 3 Evidence for a pH-dependent conformational change within the hemoglobin-band 3 complex

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Abstract

The pH dependence of hemoglobin binding to inside-out red cell membrane vesicles was studied using 90° light scattering (Salhany, J.M. et al., Biochemistry 19 (1980) 1447–1454). Hyperbolic binding curves were observed for high-affinity hemoglobin binding to the cytoplasmic domain of band 3 (CDB3) within the intact transporter. Analysis of these saturation curves yielded the apparent K_d and the maximum light scattering signal change (ΔLS_{max}). The apparent K_d for hemoglobin binding did not change substantially between pH 5.5 and 7.0, while at pH 8, there is no binding. In contrast, ΔLS_{max} decreased by about 20-fold between pH 5.5 and 7.0, with an apparent pK of 6.5. These results suggest that hemoglobin binds to CDB3 with high affinity at both neutral and acid pH, a suggestion that was confirmed using a centrifugation method. Thus, the pK for the light scattering signal change is significantly lower than the pK for the actual binding process. We show that the change in ΔLS_{max} is not related to a change in band 3 binding capacity, which remains constant as a function of pH. We also show that hemoglobin binding to non-band 3 sites contributes less than 10% to ΔLS_{max} under our specific experimental conditions. On the basis of these and previous fluorescence quenching results in the literature, we propose a new model for hemoglobin binding to band 3, where raising the pH from 6 and 7 causes the CDB3–hemoglobin complex to undergo a conformational change leading to the movement of the bound hemoglobin away from the surface of the bilayer. The possible implication of this new mechanistic interpretation is discussed briefly. © 1998 Elsevier Science B.V.

Keywords: Membrane protein; Protein–protein interaction; Band 3; Hemoglobin; Light scattering; Fluorescence

Abbreviations: CDB3, cytoplasmic domain of band 3; RET, resonance energy transfer; AS, 12-(9-anthroyl)stearic acid; GAPDH, glyceraldehyhde-3-P-dehydrogenase; PBS, 5 mM sodium phosphate, 150 mM NaCl, pH 8.0; 5P(8), 5 mM sodium phosphate pH 8.0; IOVs, inside-out membrane vesicles

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1. Introduction

Hemoglobin binds to CDB3 [1–10], which is a major cytoskeletal and cytosolic protein binding domain attached to the intracellular-facing aspect of band 3, the erythrocyte anion exchanger. In the original hemoglobin binding studies [1,2], fluorescence RET was used to monitor the interaction. The fluorescent fatty acid AS was incorporated into the membrane, and quenching by hemoglobin was measured. Quenching involved donor (AS)–acceptor (hemoglobin) RET in that: (a) trivial reabsorption could be ruled out, and (b) no significant change in AS fluorescence was observed consequent to binding of GAPDH to the hemoglobin binding site on band 3 [2]. The hemoglobin binding process was shown to be highly sensitive to pH. Fluorescence quenching was apparently eliminated by raising the pH from 6 to 7, with an apparent pK of about 6.3 [1]. Light scattering studies of hemoglobin binding also described a similarly low pK [6].

The interpretation of the loss in fluorescence quenching with elevated pH was that dissociation of the hemoglobin/band 3 complex made the distance between donor and acceptor molecules too large to produce RET quenching [1]. However, all of the pH-dependent studies cited above were performed at only one hemoglobin concentration, making such mechanistic interpretations open to question. Given the limited data available, it is possible that the hemoglobin/band 3 complex does not dissociate over this pH range, but rather that a conformational change occurs in the complex leading to movement of hemoglobin away from the membrane surface as the pH is raised. Such a conformational change could result in a significantly diminished fluorescence RET for the membrane-bound complex at pH 7.

To investigate this latter possibility, it is necessary to construct entire saturation curves at several pH values, and determine if the pH dependence of both the maximal signal amplitude and the K_d change together or not. The results presented in this paper show that the amplitude of the light scattering change has a pK of 6.5, but that the pK for the K_d is significantly higher (> 7.0), indicating the presence of substantially tighter binding at pH 7.0 than was suggested in the earlier studies. We have reinterpreted the pH dependence of fluorescence quenching [1] to indicate that a conformational change occurs within the hemoglobin–CDB3 complex, which results in a movement of hemoglobin away from the inner surface of the red cell membrane.

2. Materials and methods

2.1. Materials

Blood was supplied by the Omaha Chapter of The American Red Cross. Chymotrypsin was from Sigma, and other chemicals were of reagent grade, and were used without further purification.

2.2. Preparation of IOVs

IOVs were prepared by the method of Steck and Kant [11]. Red blood cells were washed in PBS and made into white ghosts by lysis and washing in 5P(8). These ghosts were salt stripped in 200 mM NaCl + 5P(8), and then washed 4 times in 5P(8). They were then suspended 1:40 in 0.5P(8), incubated on ice for at least 30 min, and then centrifuged for 30 min at $30{,}000 \times g$. IOVs were brought to the original volume of the ghosts in 0.5P(8) and passed 8 times through a 1-ml syringe fitted with a 27-gauge needle. To remove CDB3, IOVs were mixed 1:1 with 200 μg/ml chymotrypsin and incubated for 45 min at 37°C in a water bath. The IOVs were then washed 2 times, and brought to the original volume in a graduated tube.

2.3. Preparation of hemoglobin

Hemoglobin was prepared essentially as described previously [12]. Washed red blood cells were lysed 1:3 in 5P(8) and centrifuged 20 min at $30{,}000 \times g$. Hemoglobin was aspirated with a Pasteur pipet and recentrifuged. It was then passed over a 1×30 cm, G25 column equilibrated in 5P(7), and then dialyzed overnight, 1/100 against a 5-mM sodium phosphate buffer at a pH that would bring it close to the desired pH for a given experimental condition. After dialysis, the sample was titrated to the required pH with 5 mM phosphate tribase or acid. After determining the concentration of hemoglobin present, stock solutions were prepared using the appropriate buffers. For a wide

range of concentrations, up to three different stock solutions were prepared by serial dilution of 1/10. The concentrations of these were checked and portions pipetted to make a series of 1 ml samples at twice the final concentration desired.

2.4. Hemoglobin binding studies using light scattering

One ml of IOVs was washed 2 times in the appropriate buffer, and brought to 77 ml, or 1.3% hematocrit (~0.24 μM band 3) in the same buffer. One ml of this stock was then added to each 1 ml hemoglobin sample prepared as described above. Ninety-degree light scattering measurements were made in a 650-40 Perkin-Elmer fluorescence spectrophotometer with both slits set at 2 nm, and with the excitation and emission wavelengths set at 700 nm. The excitation and emission path lengths were 4 mm and 10 mm, respectively.

2.5. Hemoglobin binding studies using centrifugation

Hemoglobin and IOVs were prepared in the same way as for the light scattering experiments, except that the IOVs were suspended to 32% hematocrit. 0.75 ml of this stock were mixed with 0.75 ml of various hemoglobin concentrations in Centricon retentate cups, and within 10 min, they were centrifuged in the Beckman Model J2-21 Centrifuge for 15 min at 5000 rpm. Supernatants were aspirated and recentrifuged, and the second supernatant was read at 415 nm in a 4 mm × 10 mm path length cuvette.

3. Results

The 90° light scattering change associated with hemoglobin binding to unsealed ghosts or IOVs has been characterized previously [4]. When the light scattering signal change was compared with centrifugation measurements of hemoglobin binding under stoichiometric binding conditions ([band 3] > [hemoglobin]), the signal change was found to be linearly related to the fraction of bound hemoglobin. This observation, that a given signal change is directly proportional to the stoichiometric binding of a ligand, establishes the validity of the light scattering method as an empirical assay for measuring the hemoglobin binding process. In the previous work, we showed that there are two classes of binding sites [4], a high-affinity, chymotrypsin-sensitive class of sites, involving hemoglobin binding to band 3, and a low-affinity, chymotrypsin-insensitive class of sites, involving hemoglobin binding to glycophorin A and to lipid [5]. The two classes of sites differed in affinity by more than 40-fold [4]. Thus, by studying binding in dilute membrane suspensions, only the high-affinity class of hemoglobin binding sites will contribute significantly to the observed signal change [4].

Construction of complete saturation curves for the high-affinity component in hemoglobin binding shows that the maximal light scattering change is significantly reduced at pH 7 compared to pH 6 (Fig. 1A). Yet, double reciprocal plots of the data indicate that the apparent K_d for binding does not change significantly (*x*-axis intercept in Fig. 1B). We constructed similar saturation curves at numerous incremental pH values between pH 5.5 and 7.5, and quantitatively

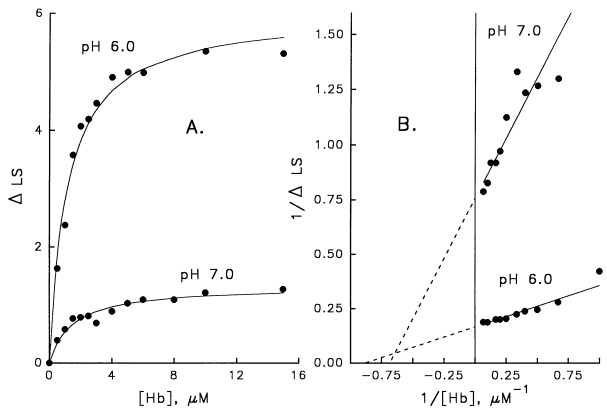

Fig. 1. Ninety-degree light scattering measurements of hemoglobin binding to IOVs at pH 6 and 7. Hemoglobin was added to IOVs ([band 3] = 0.12 μM) at the final concentrations indicated. Measurements were made in 5 mM sodium phosphate buffer at 25°C, with both excitation and emission wavelengths set at 700 nm. The change in light scattering signal (ΔLS) showed saturation behavior for the high-affinity binding component (A). Double reciprocal plots (B) were linear, indicating the involvement of one class of binding sites over this hemoglobin concentration range. The lines through the data in both A and B represent fits to Eq. (1) of the text with constants: pH 6.0: $\Delta LS_{max} = 6.0 \pm 0.2$; $K_d = 1.1 \pm 0.1$ μM and pH 7.0: $\Delta LS_{max} = 1.3 \pm 0.07$ $K_d = 1.5 \pm 0.3$ μM.

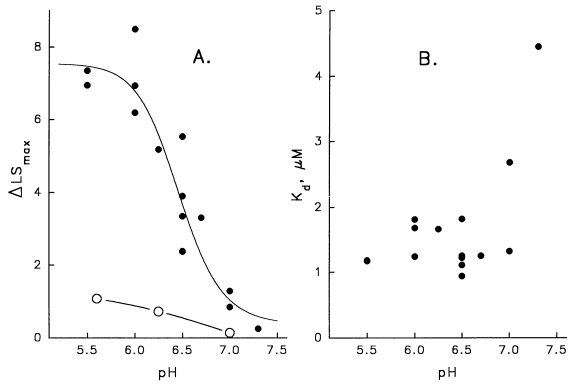

Fig. 2. Plot of the maximum light scattering signal (ΔLS_{max}) (A), and the apparent K_d (B) vs. pH. Saturation curves like those in Fig. 1 were determined at each pH, and values of the two parameters calculated from the fits. The ΔLS_{max} values titrated with a pK of 6.5 ± 0.1. In contrast, the K_d values showed little significant change between pH 5.5 and 7, and tended to increase in value above 7. The open circles are ΔLS values for hemoglobin binding to chymotrypsin-treated IOVs measured over the same hemoglobin concentration range that leads to saturation of the high-affinity hemoglobin binding site in control IOVs.

determined the values of ΔLS_{max} and K_d by fitting a simple hyperbolic function to the data:

$$\Delta LS = \{\Delta LS_{max}(Hb)\}/\{K_d + (Hb)\} \quad (1)$$

The dependence of these two parameters on pH is shown in Fig. 2. The ΔLS_{max} parameter for control IOVs (Fig. 2A, closed circles) follows a well-defined titration with a pK of 6.5. In contrast, the K_d value does not change significantly between pH 5.5 and 7, after which, there is a tendency for the K_d value to increase (Fig. 2B). It was not possible to determine the K_d values at higher pH, since the light scattering signal becomes too small for accurate measurement. However, other evidence suggests that hemoglobin does not bind to the membrane at pH 8 [3].

While light scattering and centrifugation measurements gave identical results in our previous studies at pH 6.0 under stoichiometric binding conditions [4], it seemed important to have independent evidence indicating that hemoglobin binds to band 3 at pH 7.0. Furthermore, it was important to show that hemoglobin binding to band 3 is the dominant process producing the light scattering change under so-called affinity conditions ([band 3] < [hemoglobin]), and that binding to non-specific sites makes a minimal contribution to the light scattering signal over the hemoglobin concentration range used to saturate the high-affinity binding site [4].

We prepared matching suspensions of control and chymotrypsin-treated IOVs at high band 3 concentration (3.3 μM). Chymotrypsin treatment completely cleaves all copies of band 3 as determined by gel electrophoresis studies (data not shown). Fig. 3 shows the absorbance of hemoglobin (415 nm) in the supernate after centrifugation of initial hemoglobin/IOV mixtures, plotted vs. total hemoglobin added. Binding is indicated in control (curves labeled 1), by the lag in the appearance of free hemoglobin in the supernate. That these binding sites at pH 7 are exclusively protein binding sites, was conclusively demonstrated by the fact that chymotrypsin pretreatment completely eliminated the lag at pH 7. Furthermore, the linearity of the plot is indicative of no hemoglobin binding to chymotrypsin-treated IOVs at pH 7.

At pH 6, it is evident that the binding capacity of the IOVs increases by about twofold in control (compare x-axis intercept values for the curves labeled 1 in Fig. 3, at each pH). Chymotrypsin treatment causes a 50% reduction in binding capacity at pH 6, but binding to non-protease sensitive sites is still significant under these stoichiometric binding conditions. In terms of band 3 stoichiometry, the stoichiometry of the chymotrypsin-sensitive binding at pH 7, is ap-

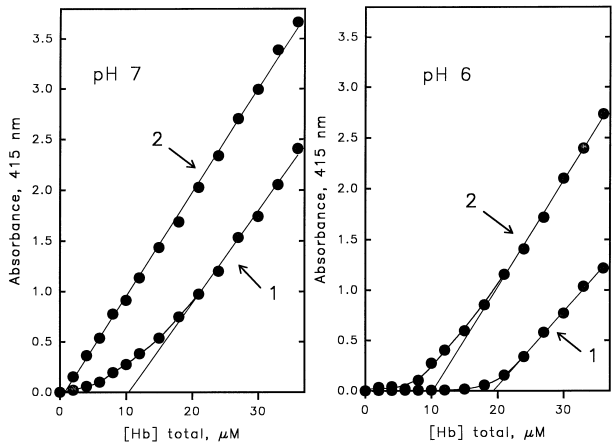

Fig. 3. Plot (vs. total added hemoglobin) of the 415-nm absorbance of hemoglobin present in the supernate after centrifugation of mixtures of control or chymotrypsin-treated IOVs at pH 7 or pH 6. IOVs ([band 3] = 3.3 μM) were equilibrated in the presence of various concentrations of hemoglobin, and then centrifuged as described in the text. Note that absorbance values above 1 were calculated based on readings from diluted supernate samples.

proximately equal to one hemoglobin tetramer per band 3 subunit (i.e., 10 μM heme/4 = 2.5 μM tetramer compared to $\sim$3.3 μM band 3). The protease sensitive sites at pH 6 have almost exactly the same stoichiometry, as indicated by the fact that chymotrypsin treatment reduces the hemoglobin binding capacity by approximately one hemoglobin tetramer per mole of band 3 subunit at this pH. It is known that hemoglobin binds to a band 3 subunit with a stoichiometry of one hemoglobin tetramer per band 3 subunit [7,13].

It is also important to note that although hemoglobin binding to protease insensitive sites increases upon lowering the pH, this increased binding capacity does not contribute significantly to the light scattering signal change over a hemoglobin concentration range, where the high-affinity binding site is saturated. This is illustrated by open circles shown in Fig. 2A, where ΔLS for chymotrypsin-treated IOVs was found to be only about 10% that of control.

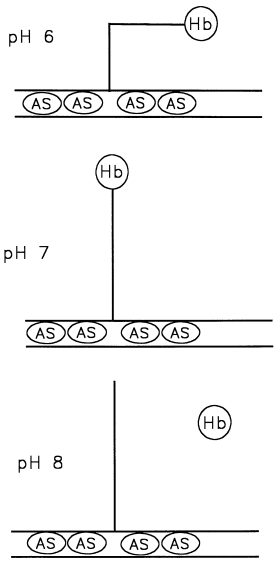

Fig. 4. Schematic model to explain data on the pH dependence of hemoglobin binding to membrane-bound band 3. Hb = hemoglobin and AS = 12-(9-anthroyl)stearic acid. See text for additional details.

4. Discussion

The results of this paper show that the light scattering signal produced with hemoglobin binding to CDB3 titrates with a lower pK than was found for the associated binding constant (K_d) (Figs. 1 and 2). The change in the maximal light scattering signal intensity could be related to one of two processes: (a) an increase in hemoglobin binding capacity of CDB3 at lower pH, or (b) a change in the light scattering properties of the hemoglobin-bound system as a function of pH, without a change in the hemoglobin binding capacity of CDB3. The results in Fig. 3 show that the hemoglobin binding capacity of CDB3 does not change upon lowering the pH from 7 to 6. The hemoglobin binding capacity at pH 6, which is lost by treatment with chymotrypsin, is exactly the same as the number of hemoglobin binding sites lost by proteolysis at pH 7. The only difference between the two pH values is the twofold increase in hemoglobin binding capacity to the protease insensitive sites, which we showed previously as low-affinity binding sites [4], involving hemoglobin binding to glycophorin A and, to a lesser extent, to bilayer lipids [5]. Importantly, binding to these latter sites contributes only about 10% to the light scattering signal under affinity binding conditions (Fig. 2A), because of the very low affinity of these sites, and thus, because they are minimally occupied when binding is studied under affinity conditions.

The pK for the titration of the light scattering signal associated with hemoglobin binding to band 3 is virtually identical to the pK reported earlier for hemoglobin quenching of AS fluorescence [1]. Yet, if hemoglobin is bound to band 3 at pH 7, why does not hemoglobin quench the fluorescence of AS located within the lipid bilayer? The schematic diagram in Fig. 4 illustrates a simple model that may offer an answer to this question. The donor, AS, is assumed to be uniformly placed in the lipid bilayer [1]. Thus, concerns about donor orientation from RET theory can be ignored. Furthermore, there is considerable evidence that membrane-bound protein/CDB3 complexes rotate rapidly with respect to the plane of the membrane [14,15]. This would also eliminate concerns about acceptor orientation. Finally, the absence of any significant change in AS fluorescence when GAPDH binds to the hemoglobin binding site on CDB3 [2], suggests strongly, that hemoglobin quenching of AS fluorescence is indeed due to RET, and is not related to some non-specific effect associated with protein binding to the membrane. With

these considerations in mind, we have arrived at the potentially important conclusion that loss of hemoglobin-induced quenching of AS fluorescence at pH 7.0 in the experiments by Shaklai et al. [1] arises from a conformational change in the membrane-bound hemoglobin/CDB3 complex, which results in hemoglobin residing further away from the membrane surface at pH 7.0, than it does at pH 6 (Fig. 4).

The idea is not new, that as the pH rises, CDB3 can undergo a conformational change which increases its Stokes' radius [16]. The isolated CDB3 dimer was shown to have two pH-dependent conformational transitions, one with a pK of 7.2, and one with a pK of 9.2 [16]. Unfortunately, it is not possible to compare directly, our pK data, or that observed by Shaklai et al. [1] with the pK for the conformational transitions of isolated CDB3 owing: (a) to differences in solution conditions; (b) to the fact that hemoglobin is bound to CDB3 in our case, and (c) to the fact that we study membrane-bound CDB3, not isolated CDB3. Nevertheless, the work with isolated CDB3(16) shows that a conformational elongation of the type needed to support the model in Fig. 4 is possible for CDB3. Such an elongation is thought to occur at a pH-regulated flexible hinge region near the center of CDB3 (residues 175–190), which is a proline-rich region [16]. We have shown previously [10] that hemoglobin binding to its site on the N-terminus of CDB3 can affect the conformation of CDB3 in the vicinity of cysteines 201 and 317, which are located in a pocket of cysteines C-terminal to the flexible hinge region. Thus, a pH-dependent 'bending-type' conformational change in the CDB3 dimer (Fig. 4) of the type needed to move the CDB3–hemoglobin complex's various distances from the bilayer is a reasonable hypothesis.

Thevenin et al. [17] have presented experimental evidence for membrane-bound band 3, which suggests that cys 201 lies 39 Å from the bilayer, near the band 3 vertical axis of symmetry. Their evidence suggested further that the subdomain between the hinge and the membrane is not extended, but that the subdomain N-terminal to the hinge can change conformation with respect to the membrane surface in a manner analogous to that suggested in Fig. 4. If cys 201 is about 40 Å from the bilayer, it is not difficult to see how subtle movements of the N-terminal subdomain/hemoglobin complex could move hemoglobin from a distance that would quench AS fluorescence to a distance that would not.

We believe that the model in Fig. 4 may be of some relevance when attempting to define mechanisms involved in the hemoglobin–band 3 interaction in pathological or senescent red cells. For example, there is clear evidence that in sickle red blood cells, the binding of denatured hemoglobin to band 3 promotes aggregation with ankyrin, glycophorin and autologous IgG [18]. Formation of such an aggregate may signal the sequestration of sickle red cells in the reticuloendothelial system. Since there is evidence for hemoglobin binding to CDB3 at physiological pH and salt concentration [9], it is possible that the 'pH 6 conformation' of the hemoglobin–CDB3 complex (Fig. 4) more readily supports aggregation of band 3. Indeed, precipitation of isolated CDB3/hemichrome mixtures [19] has a pK that is similar to that seen for ΔLS_{max} (Fig. 2A), and for the hemoglobin/AS fluorescence quenching process [1]. Thus, the pK for such aggregation may be linked to the proposed pH 7 to pH 6 CDB3–hemoglobin conformational change. We have preliminary gel filtration evidence using isolated intact band 3, which suggests that hemoglobin binding may promote band 3 aggregation of the isolated transporter protein in non-ionic detergent solutions (or it binds tightly to the aggregated transporter) (unpublished observations). Others have published evidence that hemoglobin forms a complex with aggregated or precipitated isolated band 3 [13,20]. Indeed, hemoglobin seems to promote aggregation of isolated CDB3 [8]. There is considerable evidence that attachment of CDB3 to the integral domain is required for isolated band 3 to aggregate [21–23].

In summary, our results show that the hemoglobin binds to membrane-associated band 3 with a higher pK than is found for the associated light scattering signal. This result caused us to reinterpret the pH-dependent loss of hemoglobin-induced fluorescence quenching [1], and the pH-dependent diminution in the light scattering signal intensity [6] seen previously. We now suggest that a pH-dependent conformational change occurs within the hemoglobin–CDB3 complex that allows for movement of hemoglobin away from the plane of the membrane bilayer. This conformational change may be a key event in certain red cell membrane pathophysiologi-

cal processes, where abnormal hemoglobin–band 3 interactions have been implicated.

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